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Iontophoretic release of acetylcholine, noradrenaline, 5-hydroxytryptamine and D-lysergic acid diethylamide from micropipettes

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Summary

1. The *in vitro* iontophoretic release of tritium-labelled acetylcholine and 5-hydroxytryptamine from large and small micropipettes and noradrenaline and D-lysergic acid diethylamide from small micropipettes was determined by liquid scintillation counting.

2. The release was directly proportional to the electrical charge passed in the range normally used in the iontophoretic study of these compounds. The transport numbers obtained for the large micropipettes were approximately double those with the small micropipettes. A very low transport number was found for D-lysergic acid diethylamide.

3. The spontaneous leakage was small and did not vary appreciably with time.

4. The iontophoretic release of acetylcholine *in vitro* agreed with the *in vitro* measurements.

5. The brain-stem tissue concentration of D-lysergic acid diethylamide after intravenous injection into intact and decerebrate cats was determined.

Introduction

The technique of micro-iontophoresis for applying putative synaptic transmitters, and centrally active drugs, to single neurones in the central nervous system is now well established. In almost all cases, however, it has been necessary to relate the effects on neuronal activity to the current passed and the time for which it was applied (that is to the electrical charge) rather than to the quantity of the substance released. It is therefore important to know (*a*) how much of the substance under study is released by a given quantity of electricity, (*b*) whether there is a linear relationship between the amount released and the product of current and time, and (*c*) whether variation in the size and shape of the electrode tip appreciably affects the quantity released, because this factor is important when comparing experimental results from different laboratories.

Attempts have been made to determine some of these factors using bio-assays for acetylcholine (Krnjević, Mitchell & Szerb, 1963a) and fluorescence techniques for amines (Krnjević, Laverty & Sharman, 1963b). However, these methods are subject to considerable error, particularly at lower sensitivity ranges (Krnjević *et al.*, 1963b). The availability of tritium labelling for a wide range of substances, and of sensitive methods for counting the radioactivity, have rendered such studies much more feasible and studies of the iontophoretic release of tritium-labelled

glutamic acid and γ-aminobutyric acid have recently been published (Zieglgänsberger, Herz & Teschemacher, 1969 ; Obata, Takeda & Shinozaki, 1970).

The object of the present study was to determine whether the amounts of three putative synaptic transmitters, acetylcholine, noradrenaline and 5-hydroxytryptamine (5-HT), released from multi-barrelled micropipettes, using the same current levels and time intervals as in pharmacological experiments, were detectable by scintillation counting and whether consistent and reproducible results could be obtained. The drug D-lysergic acid diethylamide (LSD 25) was also included in this study since its actions as a possible antagonist to 5-HT in the central nervous system were being investigated concomitantly (Boakes, Bradley, Briggs & Dray, 1969, 1970a, b). In addition, two types of micro-electrodes, differing considerably in size, were used.

Methods

The two types of 5-barrelled micropipettes, with heights of approximately 3 cm and 6 cm, were constructed from Pyrex glass tubing of internal diameter 0·1 mm and 4 mm, respectively. The small micropipette blanks corresponded to the type used in all previous studies in this laboratory and were prepared according to the method of Curtis & Eccles (1958), and the large blanks were obtained commercially. Both types of micropipettes were filled with glass-distilled water under reduced pressure after they had been drawn out.

Aqueous D,L-[7-^{3}H] noradrenaline hydrochloride (specific activity 3·64 Ci/mmol), aqueous [G-^{3}H] 5-hydroxytryptamine creatinine sulphate (specific activity 5·6 Ci/mmol), and solid [*Me*-^{3}H] acetylcholine chloride (specific activity 147 mCi/mmol) were obtained from the Radiochemical Centre, Amersham. The [*Me*-^{3}H] acetylcholine chloride was dissolved in distilled water to give a radioactive concentration of 1 mCi/ml, equivalent to that of the other two compounds. 25 mg of D-lysergic acid diethylamide tartrate (LSD 25, Sandoz) was irradiated with 7 Ci of tritium gas in the dark at 4° C for 14 days at the Radiochemical Centre. The [G-^{3}H] LSD 25 was supplied as a freeze-dried solid in five ampoules and the purity was determined by thin-layer chromatography using the method of Genest & Farmilo (1964).

1% solutions of D,L-noradrenaline (pH 5·5), 5-hydroxytryptamine (pH 5·5) and acetylcholine (pH 5·0) were prepared by adding the appropriate quantities of the labelled solutions to unlabelled solutions of D,L-noradrenaline hydrochloride (B.D.H.), 5-hydroxytryptamine creatinine sulphate (Koch-Light) and acetylcholine chloride (Hopkin & Williams Ltd.) respectively. A 10% solution of D,L-noradrenaline was also prepared.

To each ampoule of [G-^{3}H] LSD 25, 1 ml of deionized water was added, giving an approximate concentration of 5 mg/ml. The quantity of [G-^{3}H] LSD 25 present in each ampoule was determined by measuring the fluorescence at 445 nm, using an excitation wavelength of 325 nm in an Aminco-Bowman Spectrophotofluorimeter, of a diluted portion containing approximately 5 μg/ml of [G-^{3}H] LSD 25 and comparing this with standard solutions of unlabelled LSD 25 in deionized water. The total radioactivity of each ampoule was determined by counting the disintegration rate of a diluted portion for 10 min. The specific activity of the [G-^{3}H] LSD 25 was 2·9 Ci/mmol.

The centre barrel of each micropipette was filled with 3 M NaCl solution and one of the side barrels with the tritiated solution. They were then stored for 48 h at 4° C. Immediately before use the tips were broken to 6–8 μm and washed by dipping in distilled water. For iontophoretic release the micropipettes were held vertically with the tips immersed in 0·45 ml of 0·9% NaCl. The rest of the experimental arrangement was identical to that normally used for microiontophoresis experiments but with NaCl replacing the animal (except for the *in vivo* experiments).

The relationship between the iontophoretic release and electrical charge flowing was examined for acetylcholine, noradrenaline and 5-hydroxytryptamine by applying (i) a range of currents (25–100 nA) for a set time period (3 min) and (ii) a fixed current (50 nA) for varying periods of time (0·5–5 min). With [G-^{3}H] LSD 25, a fixed current (50 nA) was used in conjunction with time periods of up to 11 min, since these had been found necessary to produce antagonism to effects of 5-HT (Boakes *et al.*, 1969, 1970a, b). Two samples were taken for each current level and time interval. A backing current of 15 or 25 nA was applied before commencement and after the completion of each period of iontophoresis and the efficiency of the backing current was measured for each micropipette over a 3 min period. From this the spontaneous leakage was calculated for all the micropipettes over the same time periods as those used for iontophoretic release.

At the end of each period of iontophoresis, the NaCl solution was added to a scintillation vial and the container washed out with a further 0·45 ml of 0·9% NaCl; 10 ml of scintillation fluid (0·6% 2,5-diphenyloxazole, 0·012% 1,4 Di-2-(5-phenyloxazolyl)-benzene, in xylene, with 27% Triton X-100) were then added. The disintegration rate was normally counted over 10 min, but in the case of low counts, this was extended to 50 min, in a Packard Tri-Carb liquid scintillation spectrometer, with an internal standard correction, followed by deduction of the background activity.

The iontophoretic release of [*Me*-^{3}H] acetylcholine chloride was also measured *in vivo* in the cerebral cortex of three rats anaesthetized with urethane, using the same parameters as in the *in vitro* experiments. In each case, a small piece of cortex into which the tritiated compound has been released was homogenized in 0·9 ml of 0·4 N perchloric acid and 10 ml of scintillation fluid added. The disintegration rate was counted over a 10 min period.

25 μg/kg of [G-^{3}H] LSD 25 was injected into the femoral vein in three intact cats anaesthetized with halothane and four unanaesthetized decerebrate cats. Twenty minutes after the injection, the animals were killed and the brains removed. The brain stem was separated off, washed with normal saline, blotted and then cut into small pieces. Each piece of tissue was weighed and then homogenized in 0·9 ml of 0·4 N perchloric acid, 10 ml of scintillation fluid added and counted for 10 min.

Results

Iontophoretic release

The results, both individually and collectively, for each compound for the small and large micropipettes, were analysed. In calculating the iontophoretic release, the spontaneous leakage was deducted from the average result for each interval in the collective analysis and from the separate results in the individual analysis. In

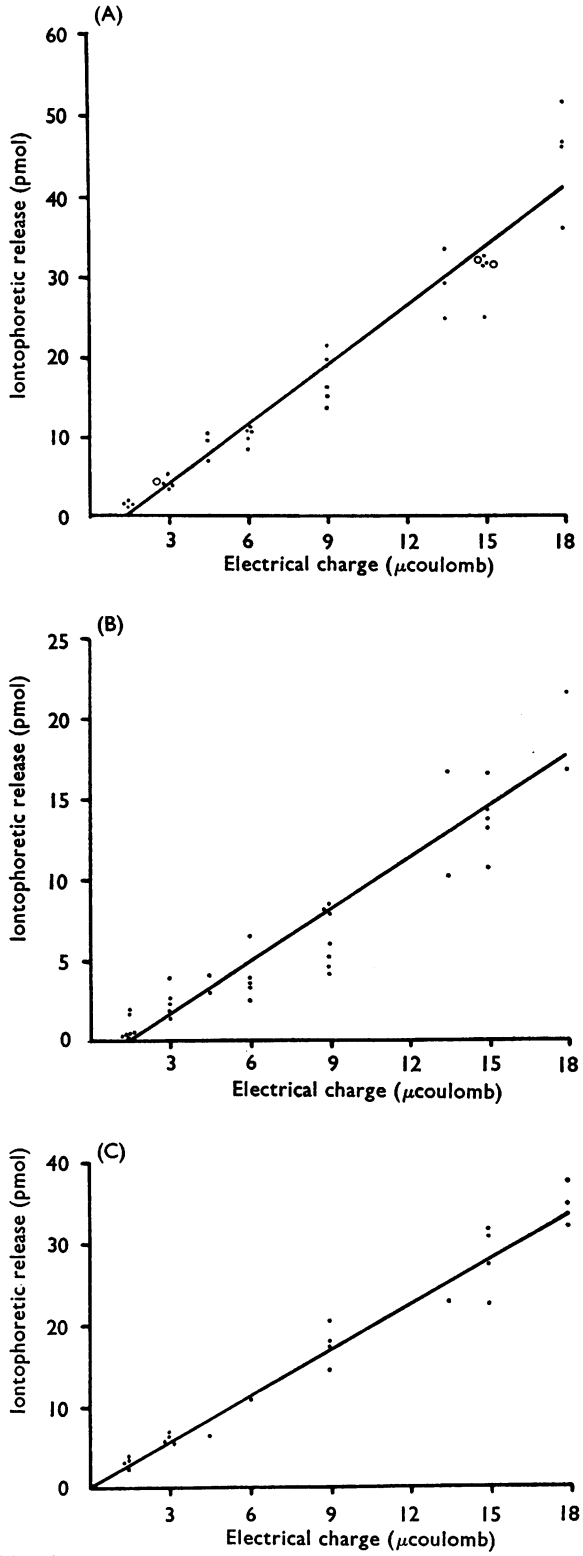

FIG. 1. Relationship between ionotophoretic release (pmol) and the electrical charge (μcoulomb) for: (A), six small micropipettes containing a 1% solution of [*Me*-^{3}H] acetylcholine chloride. The open circles show the release from three small micropipettes *in vivo*; (B), six small micropipettes containing a 1% solution of D,L-[7-^{3}H] noradrenaline hydrochloride; (C), five small micropipettes containing a 1% solution of [G-^{3}H] 5-hydroxytryptamine creatinine sulphate. The lines are the calculated regression lines. The dots refer to the *in vitro* results in all cases.

the case of [G-^{3}H] LSD 25, however, the tartrate ion was also labelled and the spontaneous release of the LSD 25 moiety could not be measured. The iontophoretic release (pmol) was plotted against the electrical charge passed (μcoulomb) and the effective transport number (t), obtained by multiplying the slope (expressed in mol/coulomb) by Faraday's number.

Acetylcholine. Six small (Fig. 1A) and four large micropipettes collectively gave highly significant regression lines ($f=17{\cdot}7$, $P<0{\cdot}001$ and $f=8{\cdot}3$, $P<0{\cdot}001$, respectively). The deviations of the lines from the origin were not significant (small micropipettes, $f=2{\cdot}26$, $P>0{\cdot}05$; large ones, $f=0{\cdot}82$, $P>0{\cdot}4$). The transport number was greater with the large micropipettes ($t=0{\cdot}48$) than with the small ones ($t=0{\cdot}24$).

The data from one small and one large micropipette fitted the calculated regression lines well ($f=21{\cdot}3$, $P<0{\cdot}001$; $f=9{\cdot}0$, $P<0{\cdot}001$, respectively). The deviations of the regression lines from the origin were not significant (small micropipette $f=2{\cdot}2$, $P>0{\cdot}05$; large micropipette, $f=1{\cdot}0$, $P>0{\cdot}3$). The transport numbers obtained for both the small ($t=0{\cdot}19$) and large ($t=0{\cdot}44$) micropipettes were in close agreement with those from the collective results.

The *in vivo* results shown in Fig. 1A agreed with the *in vitro* results. Although the *in vivo* results are few in number, they suggest that the iontophoretic release is similar to that obtained *in vitro*.

D,L-*noradrenaline.* The collective results from six small micropipettes containing 1% D,L-noradrenaline (NA) (Fig. 1B) and four containing 10% NA gave highly significant regression lines ($f=11{\cdot}3$, $P<0{\cdot}001$ and $f=13{\cdot}8$, $P<0{\cdot}001$, respectively). The deviations of the regression lines for the origin were not significant (1% NA, $f=1{\cdot}53$, $P>0{\cdot}1$, and for 10% NA, $f=1{\cdot}08$, $P>0{\cdot}3$). The transport number for the 10% NA solution ($t=0{\cdot}19$) was double that for the 1% NA solution ($t=0{\cdot}09$). The analysis of the results for one micropipette containing each concentration of NA was considered. The regression lines fitted the data well (1% NA, $f=10{\cdot}6$, $P<0{\cdot}001$ and for 10% NA, $f=13{\cdot}7$, $P<0{\cdot}001$) and did not deviate significantly from the origin (1% NA, $f=1{\cdot}7$, $P>0{\cdot}1$; 10% NA, $f=0{\cdot}17$, $P>0{\cdot}8$). The transport numbers agreed well with those from the collective results (1% NA, $t=0{\cdot}09$; 10% NA, $t=0{\cdot}15$).

5-Hydroxytryptamine creatinine sulphate. According to the analysis provided by the Radiochemical Centre, more than 98% of the tritium was in the 5-hydroxytryptamine moiety, the results could therefore be considered to be due to 5-hydroxytryptamine and the creatinine component neglected.

The collective results from five small micropipettes (Fig. 1C) and three large micropipettes gave highly significant regression lines ($f=18{\cdot}8$, $P<0{\cdot}001$ and $f=14{\cdot}4$, $P<0{\cdot}001$, respectively). The deviations of the regression line from the origin was not significant in either case (small micropipettes, $f=0{\cdot}019$, $P>0{\cdot}8$ and for the large micropipettes, $f=0{\cdot}49$, $P>0{\cdot}6$). The transport number was greater with the large micropipettes ($t=0{\cdot}31$) than with the small ones ($t=0{\cdot}18$).

The results from one small and one large micropipette gave highly significant regression lines ($f=18{\cdot}7$, $P<0{\cdot}001$, and $f=55{\cdot}4$, $P<0{\cdot}001$, respectively) and the deviations of the lines from the origin were insignificant (small micropipette, $f=0{\cdot}5$, $P>0{\cdot}6$ and for the large micropipette, $f=0{\cdot}75$, $P>0{\cdot}4$). The transport numbers

were very similar to those obtained from the collective results (small micropipette, $t=0{\cdot}17$; large micropipette, $t=0{\cdot}33$).

D-*Lysergic Acid Diethylamide Tartrate (LSD 25).* The collective results from twelve small micropipettes (Fig. 2) gave a highly significant regression line ($f=21{\cdot}4$, $P<0{\cdot}001$). The deviation of the regression line from the origin was insignificant ($f=1{\cdot}1$, $P>0{\cdot}3$). The transport number for the collective results was very low ($t=0{\cdot}02$). The release from individual micropipettes was linear, but the effective transport number was subject to variation between micropipettes. The micropipette with the highest transport number ($t=0{\cdot}05$, Fig. 2) and the micropipette with the lowest transport number ($t=0{\cdot}007$, Fig. 2) both gave highly significant regression lines ($f=22{\cdot}0$, $P<0{\cdot}001$; $f=32{\cdot}6$, $P<0{\cdot}001$, respectively) and the deviations of the lines from the origin were not significant ($f=0{\cdot}6$, $P>0{\cdot}6$; $f=0{\cdot}02$, $P>0{\cdot}9$, respectively). The duration of the backing current was kept to below 5 s before the commencement and after the completion of each period of iontophoresis, so that the release of the labelled tartrate ion would not affect the results. Measurement of the release of the tartrate ion with a backing current of 15 nA showed that this was not significant over short time intervals.

Intravenous injection of LSD 25. The intravenous injection of 25 μg/kg of [G-^{3}H] LSD 25 into three intact, and four decerebrate cats gave an average concentration in the brain stem of $22{\cdot}3 \pm 5{\cdot}4$ pmol and $22{\cdot}5 \pm 2{\cdot}9$ pmol/g of tissue, respectively. The half-life of LSD 25 in cats has been shown to be 130 min (Axelrod, Brady, Witkop & Evarts, 1957) and it is therefore reasonable to assume that the radioactivity measured 20 min after injection was due to [G-^{3}H] LSD 25.

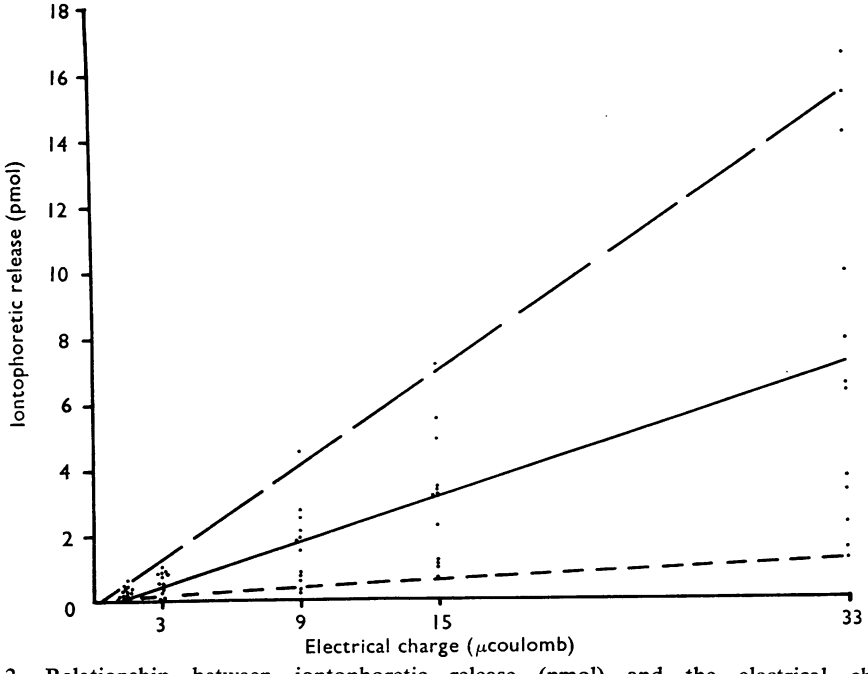

FIG. 2. Relationship between iontophoretic release (pmol) and the electrical charge (μcoulomb) for twelve small micropipettes containing a 0·5% solution of D-[G-^{3}H] lysergic acid diethylamide (LSD 25). ——, Calculated regression line for all the micropipettes; — —, calculated regression line for the micropipette with the highest transport number; - - - - -, calculated regression line for the micropipette with the lowest transport number.

Spontaneous leakage and leakage with backing current. The results shown in Table 1 are average values. The spontaneous release was small compared with the iontophoretic release, for all the micropipettes tested. Despite the higher concentration, the leakage from the micropipettes containing 10% NA did not appear to exceed that for the 1% NA solution. Surprisingly, the leakage did not vary appreciably with time, except for the large 5-HT-containing micropipettes.

The leakage over a 3 min period with a 25 nA backing current was not significantly less than the spontaneous leakage over the same interval, with the exception of the large 5-HT-containing micropipettes. A 15 nA backing current appeared to be more effective in all the cases tested and especially for the large 5-HT-containing micropipettes.

Discussion

The data presented here demonstrate that, for the collective and for the individual results, the release of ACh, NA, 5-HT and LSD 25 was directly proportional to the charge passed, in the range normally used for microiontophoretic studies with these compounds. The transport numbers obtained for ACh and 5-HT, using the large micropipettes, were approximately double those obtained with the small micropipettes. The transport number obtained with the large micropipettes with ACh agrees with that reported by Krnjević *et al.* (1963a) using micropipettes of a similar size ; however, in the case of 5-HT, a higher transport number was found in the present study. The variation in iontophoretic release of ACh, NA and 5-HT between different micropipettes of the same size and containing the same substance was surprisingly small. There was more variation in the release of LSD 25 between different micropipettes than with the other compounds tested, but as the transport number for this substance was very low, some variation under these conditions might be expected.

The high level of spontaneous release of ACh found by Krnjević *et al.* (1963a) was probably due to the high concentration used, since, in the present study with more dilute solutions, the spontaneous release was found to be small compared with the iontophoretic release (Table 1). The finding that the release with a backing current of 25 nA was not significantly different from the spontaneous release over a period of 3 min might possibly be explained by "after diffusion" (Castillo & Katz, 1957).

The finding that the release of ACh *in vitro* was similar to the amount released *in vivo* would appear to justify the use of the *in vitro* method.

TABLE 1. *Leakage from micropipettes*

Tritiated solution	Type of micropipette	Spontaneous leakage (pmol) over time periods (min) of					Leakage (pmol) over 3 min with backing currents of	
		0·5	1	2	3	5	15nA	25nA
ACh	Small	0·1	0·1	0·5	0·3	0·2	—	0·3
	Large	0·2	0·3	0·2	0·4	0·5	—	0·1
5-HT	Small	0·2	0·2	0·3	0·3	0·2	0·1	0·3
	Large	0·6	2·0	2·6	2·8	5·2	0·4	1·0
NA	Small	0·1	0·3	0·2	0·3	0·3	0·1	0·3

The very low transport number ($t=0{\cdot}02$) obtained for LSD 25 provides an explanation for the long periods of iontophoresis used for this substance (Roberts & Straughan, 1967; Boakes, Bradley, Briggs & Dray, 1970b), since the quantity of LSD 25 released by iontophoresis at 50 nA for 5 min is comparable with that of 5-HT released by 50 nA for 30 s. It is of interest that similar concentrations of LSD 25 were found in the brain stem in both intact and decerebrate cats when the drug was injected intravenously and this concentration (22·5 pmol/g or $2{\cdot}2 \times 10^{-8}$M) is comparable with the calculated concentration from iontophoresis experiments (see Boakes *et al.*, 1970b).

It is possible that the compounds tested could have been chemically changed to some extent during iontophoresis. The radioactivity assay, unlike the bioassay or flourimetric assay, measures both the unchanged compound and its breakdown products. This might explain the higher transport number obtained for 5-HT with the radioactive method compared with that obtained using the fluorimetric assay by Krnjević *et al.* (1963b).

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