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EVIDENCE FOR INTRA-CELLULAR GUANETHIDINE STORES IN THE RAT HEART REVEALED AFTER REMOVAL OF CALCIUM IONS

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Both calcium and sodium ions have been reported to be implicated in the uptake and release of noradrenaline from peripheral nerves (Huković & Muscholl, 1962; Burn & Gibbons, 1964, 1965; Bogdanski, 1965; Boullin & Brodie, 1965; Boullin, 1967; Iversen & Kravitz, 1966). Calcium is also involved in the storage and release of guanethidine (Boullin, Costa & Brodie, 1966; Boullin, 1966a) but not in guanethidine uptake (Boullin, 1966b). The present work provides further information on the part played by calcium ions in guanethidine storage and suggests that the release of guanethidine from specific intra-cellular binding sites in the isolated perfused heart is regulated by the level of calcium ions in the perfusion medium, and that non-specific binding of guanethidine is greatly diminished in the absence of the cation.

METHODS

Male Wistar rats (weight 180–220 g) were used. The hearts were labelled with radioactive guanethidine *in vivo* or *in vitro*. For experiments *in vivo* the hearts were labelled by intravenous injection of guanethidine (5 mg/kg) into the tail vein. The animals were killed 30 min later and the hearts removed for perfusion. Each organ was perfused by the Langendorff technique with Locke solution at constant flow (approximately 6 ml./min). The composition of the Locke solution was the same as that described by Boullin (1966b) with the exception that in the experiments with calcium-free solution the cation was omitted and 1 mM disodium edetate was added (tonicity being maintained by addition of NaCl).

In the experiments involving *in vitro* labelling with radioactive guanethidine the following procedure was adopted: hearts were removed from untreated animals and perfused at constant flow as described above. After an initial period of 5 or 10 min perfusion with drug-free Locke solution at about 6 ml./min, the heart was perfused with Locke solution containing guanethidine for 2–10 min. In some experiments perfusion was then continued with drug-free solution for another 10 or 30 min and 1 or 2 min samples of the cardiac effluent were collected in graduated centrifuge tubes. At the end of the experiments this fluid and the heart tissues were assayed for guanethidine as described elsewhere (Boullin, 1966b).

The number of experiments was limited by shortage of labelled material. Dr. C. I. Furst, CIBA Laboratories, Horsham, Sussex, synthesized and kindly made available three different batches of radioactive guanethidine. In experiments with ^{14}C-guanethidine the specific activity was 2.48 or 3.14 μc/mg; in some of the later experiments tritiated guanethidine (132 μc/mg) was used, when the experimental procedure was modified as described in RESULTS.

Tissue levels of guanethidine

Guanethidine levels in the heart are expressed as μg base/g tissue after deduction of the drug confined in the extra-cellular space. This was determined by perfusion of five hearts for 10 min with ^{14}C-inulin (New England Nuclear Corporation, Boston, specific activity 3.08 μc/mg) and a value of 0.38±0.03 ml./g was obtained which is similar to that given by Iversen (1965).

RESULTS

Disposition of guanethidine after labelling in vivo

Tissue levels. Seven rats received ^{14}C-guanethidine 5 mg/kg (specific activity 3.14 μc/mg) by injection into the tail vein. On killing 30 min later the heart content was 5.8 μg per organ (Table 1) or 9.05±0.52 μg/g. This was less than 1% of the injected dose.

TABLE 1

EFFECT OF CALCIUM ON THE DISPOSITION OF GUANETHIDINE IN THE HEART AND EFFLUENT AFTER *IN VIVO* LABELLING

Rats received ^{14}C-guanethidine 5 mg/kg (specific activity 3·14 μc/mg) 30 min before the hearts were removed for perfusion. The organs were perfused with Locke solution containing 0, 2·2 or 20 mM calcium for 30 min. The efflux during the first 2 min was discarded because this time was required for clearance of 95% of the extra-cellular space. Data shown are mean values (± S.E.) from three groups of five perfused hearts, and one group of seven hearts which were not perfused. * Values significantly greater than control (2·2 mM calcium) at 5% level. † Values significantly greater than control (2·2 mM calcium) at 1% level.

Calcium concn. mM	Guanethidine recovered per organ (a) In heart μg	(b) In perfusate μg	% of guanethidine recovered in perfusate	Guanethidine recovered in perfusate as % of control (2·2 mM calcium)
2·2	4·88±0·33	1·12±0·07	18·7±1·2	100
20	4·58±0·14	1·40±0·11*	23·4±1·8	125
0	2·89±0·53	3·07±0·12†	51·4±2·0	274
Not perfused	5·77±0·16	—	—	—

Pattern of efflux of ^{14}C-guanethidine from isolated perfused hearts. In other experiments, hearts from animals which had been similarly treated with ^{14}C-guanethidine 5 mg/kg intravenously were perfused for 30 min with Locke solution, containing 0, 2.2 or 20 mM calcium. The data from fifteen perfused hearts are summarized in Table 1. Of the total amount of radioactive material recovered, more than 80% remained in the heart after perfusion with Locke solution containing 2.2 mM calcium. Efflux was significantly elevated by either a nine-fold increase in calcium concentration or complete removal of this cation. Minute by minute analysis of the guanethidine content of the cardiac effluent is shown in Fig. 1. It can be seen that the pattern of efflux is complex in all three experimental situations. As is the case for the efflux of ^{3}H-noradrenaline from the isolated perfused rat heart (Kopin, Hertting & Gordon, 1962; Kopin & Gordon, 1963), each efflux curve for guanethidine can be resolved into several exponential components. The first phase, seen in all experiments irrespective of the calcium concentration, and therefore not shown in Fig. 1, was a very rapid decline in the rate of release with a half-time of 1.7 min. This is identical to the half-time for efflux of sorbitol (Morgan, Henderson, Regen & Park, 1961) and inulin (Boullin & Sullivan, unpublished observations) from the extra-cellular space. Thereafter, there was a further decline in the rate of efflux in all experiments. The pattern of efflux was similar during perfusion with 0

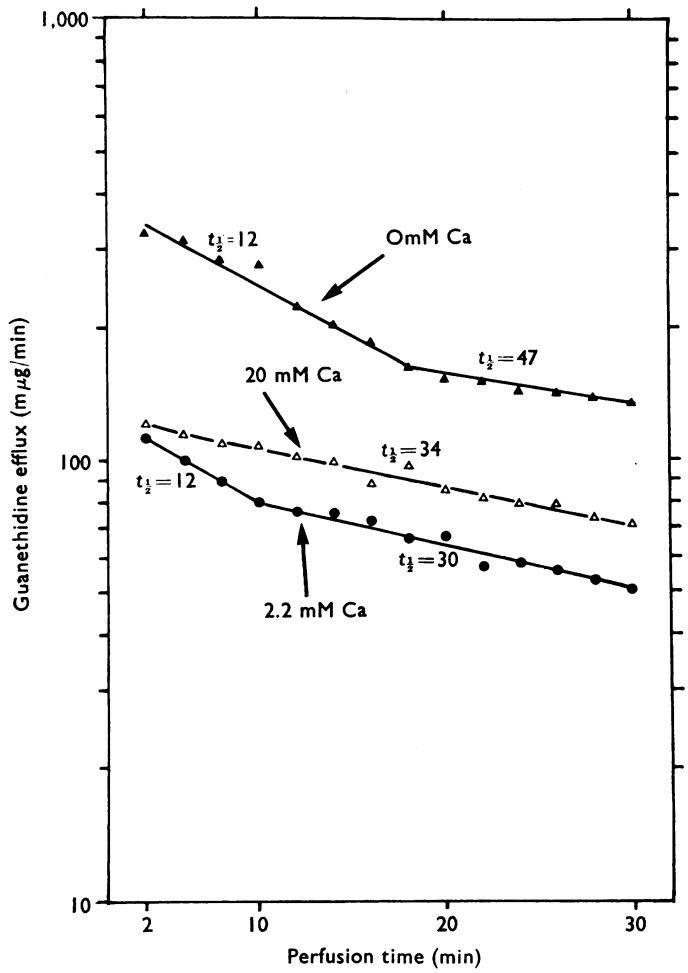

Fig. 1. Efflux of guanethidine from isolated rat hearts perfused for 30 min with Locke solution containing 2.2 mM calcium (●), 20 mM calcium (△) or calcium-free plus 1 mM disodium edetate (▲). Half-times for efflux ($t_{\frac{1}{2}}$) are shown. The efflux during the first 2 min of perfusion had a half-life of less than 2 min irrespective of the calcium concentration and is not shown. Results are the mean data from three groups of five animals (see Table 1).

or 2.2 mM calcium. In either case, two exponentials are clearly seen. With 2.2 mM calcium the first exponential has a half-time of 12 min and a duration of 8 min. With 0 mM calcium the time constant was the same but the duration of efflux was 16 min. Thereafter the decline in the rate of efflux was faster with 2.2 mM calcium (half-time 30 min) than with 0 mM calcium (half-time 47 min). The pattern of efflux during perfusion with 20 mM calcium was different. After the initial washout period, efflux followed a single exponential with a half-time of 34 min which closely approximates to the half-time of the second exponential obtained during perfusion with 2.2 mM calcium.

Before conclusions can be drawn about the significance of the data shown in Fig. 1, further analysis is necessary, because the first exponential phase of release from a two-

TABLE 2

TIME CONSTANTS OF GUANETHIDINE EFFLUX FROM STORAGE COMPARTMENTS IN ISOLATED PERFUSED HEARTS

These results are calculated from the data given in Fig. 1 according to the method of Iversen (1963).

Calcium concn. mM	Half-time for efflux (min)	
	First phase	Second phase
0	4·7	47
2·2	2·6	30
20	34·0	—

compartment system is composed of drug released from both compartments. To determine the characteristics of the efflux process from the first compartment it is necessary to extrapolate the second exponential to zero time and to subtract the values obtained from the first exponential phase (Iversen, 1963, see Fig. 4). When this procedure is carried out on the data shown in Fig. 1 corrected time constants are obtained. Thus Table 2 shows that whereas guanethidine efflux was from two stores with time constants of 2.6 and 30 min in the presence of normal calcium ion concentration, on removal of the cation the time constants of efflux were different (4.7 and 47 min), and the rate of loss was greatly increased. One possible explanation for these differences was that in calcium-free conditions guanethidine was released from one or more additional stores.

Experiments were therefore designed to investigate the role of calcium ions in guanethidine uptake and storage, with particular reference to binding at the specific sites concerned with the pharmacological action of guanethidine. For these experiments an *in vitro* labelling technique was used.

Disposition of guanethidine after labelling in vitro

Hearts from nine rats were infused with Locke solution containing 2.2 mM calcium and ^{14}C-guanethidine (specific activity 2.48 μc/mg) in concentrations of 0.1, 1.0 or 10.0 μg/ml. for 5 min. The flow rate was 5.8 ml./min, so that 2.9, 29 or 290 μg of drug passed through each heart during 5 min perfusion. The results are shown in Fig. 2. It can be seen that the uptake process is saturable because there is a progressive reduction (from 14% to 2%) in the proportion of guanethidine taken up by the heart with increasing guanethidine concentration in the perfusion fluid.

In other experiments the degree of guanethidine binding was assessed by perfusion with guanethidine-free solution for 30 min after labelling with 0.1 or 1.0 μg/ml. by infusion for 10 min. These experiments showed that the drug was tightly bound; 70–75% of the initial amount taken up was still present within the heart after washing for 30 min with Locke solution containing normal calcium (Table 3). This agrees well with the value of 80% remaining after perfusion following *in vivo* labelling (Table 1). These results showed that guanethidine was successfully taken up and retained by the isolated heart, as has been shown on heart slices by Schanker & Morrison (1965) and Boullin (1966b).

In contrast to the results obtained with normal calcium, when guanethidine was infused in calcium-free Locke solution, uptake was greatly reduced (Table 3). With 0.1 μg/ml. infusion the reduction was 70.4% (412–122 mμg) and with 1.0 μg/ml. it was 67.4% 1,960–640 mμg). Although the percentage loss was greatly different, however, the total

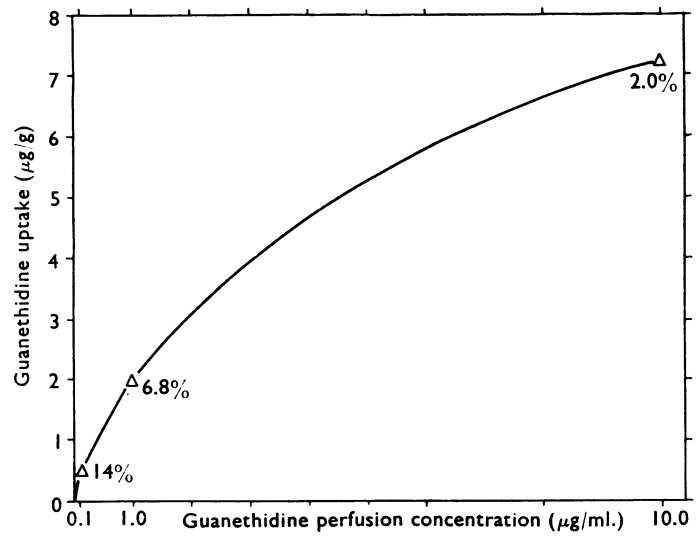

Fig. 2. Effect of perfusion concentration on the uptake of guanethidine by isolated hearts. Three groups of three hearts were infused with ^{14}C-guanethidine 0.1, 1.0 or 10 μg/ml. for 10 min and tissue content determined. Guanethidine uptake μg/g (after correction for extra-cellular space) is plotted against infusion concentration (μg/ml.). Mean values for each group are given; percentages refer to the proportion of the total dose infused that was taken up.

TABLE 3

EFFECT OF CALCIUM ON THE DISPOSITION OF GUANETHIDINE IN THE RAT HEART AND EFFLUENT AFTER *IN VITRO* LABELLING

To determine the quantity of guanethidine taken up, hearts were perfused with ^{14}C-guanethidine 0·1 or 1 μg/ml. (specific activity 3·14 μc/mg) for 5 min. The amount of guanethidine initially taken up was determined in one series of experiments by assay of heart tissues after guanethidine infusion. The amount retained was measured in a second series when labelling was followed by 30 min perfusion with guanethidine-free Locke solution. Mean data from eight groups of three hearts are shown.

Calcium concn. mM	Guanethidine concn. μg/ml.	Total guanethidine infused mμg	Guanethidine: (a) Initially taken up mμg	Guanethidine: (b) Lost after 30 min perfusion mμg	Guanethidine lost (b) as % of guanethidine taken up (a)
2·2	0·1	2,900	412	102	24·8
0	0·1	2,950	122	87	71·3
2·2	1·0	28,800	1,960	580	29·6
0	1·0	29,750	640	593	92·8

amount discharged during the 30 min wash showed a very close similarity to that lost when the calcium concentration was normal (Table 3). Because the *in vivo* experiments showed that removal of calcium ions may have resulted in the release of guanethidine from storage compartments in addition to those affected by normal calcium (Table 2), it seemed possible that the reduced uptake in these calcium-free experiments *in vitro* was caused by the elimination of calcium-dependent binding sites, and that the similar amount released came from storage sites unaffected by calcium concentration.

The final series of experiments was carried out to try to throw further light on the site and mechanism of guanethidine binding under calcium-free conditions. For these experiments, tritiated guanethidine of higher specific activity (132 μc/mg) was used.

TABLE 4

UPTAKE AND DISPOSITION OF GUANETHIDINE IN THE ISOLATED RAT HEART

The data summarize the disposition of ^{3}H-guanethidine in the heart and cardiac effluent during the last 9 min of a 10 min infusion of 1 μg/ml. guanethidine in 2·2 mM calcium or calcium-free Locke solution. Mean values from two groups of four hearts are given. The results for each minute of infusion (±S.E.) are given in Fig. 1

Calcium concn. mM	Total drug infused μg	Rate of infusion μg/min	Max. rate of uptake μg/min	Total uptake μg	Total retained after 10 min infusion μg
2·2	51·6±1·8	5·73±0·18	1·02±0·058	2·93±0·19	2·93±0·19
0	53·2±1·6	5·91±0·20	0·46±0·06	0·85±0·04	0·07±0·001

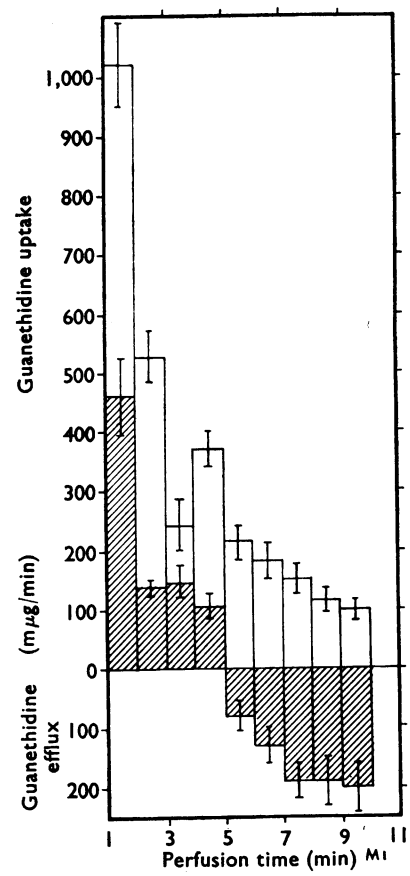

Fig. 3. Effect of calcium on the disposition of ^{3}H-guanethidine in the isolated rat heart and perfusate. Data for the last 9 min of a 10 min infusion at the rate of 5.7 to 5.9 μg/min are shown. The level of amine in the heart (guanethidine uptake) or in the venous effluent above the 29 μg/min infused (guanethidine efflux) is shown. Histograms refer to the mean level in each minute (±S.E.) obtained with four animals. Open histograms refer to experiments using hearts perfused with solution containing 2.2 mM calcium; hatched histograms refer to experiments where calcium free solution containing EDTA was used. Note that positive efflux occurred only with the latter.

The results of these experiments are shown in detail in Fig. 3 and are summarized in Table 4. The experimental procedure involved determination of guanethidine levels in heart and effluent during the 10 min course of infusion of guanethidine. Two groups of six rats were used. One group was perfused with Locke solution containing 2.2 mM calcium and the other with calcium-free solution containing 1 mM disodium edetate. After preliminary perfusion with drug-free solution for 10 or 15 min, the hearts were infused with 1 μg/ml. ^{3}H-guanethidine. The radioactivity in the cardiac inflow and outflow was monitored every minute, and guanethidine uptake was assessed by calculating the difference between the inflow and outflow.

In a separate experiment four groups of four animals were infused with guanethidine for 2, 4, 6 and 8 min and at the end of infusion the hearts were analysed for guanethidine as described in METHODS ; the results are given in Table 5.

Fig. 3 shows that when 2.2 mM calcium solution was used uptake occurred throughout the 10 min infusion period, although the rate of uptake was greater at the beginning of infusion than at the end. On the other hand in calcium-free hearts, uptake occurred during the first 5 min only. In hearts infused for longer than this time the guanethidine level in tissue extracts declined until after infusion for 10 min the calcium-free hearts were almost completely depleted (Table 5). Simultaneously, the rate of efflux of radioactivity in the cardiac effluent was increased from 5.91 ± 0.20 to 6.12 ± 0.31 μg/min, an increase over the mean influx of 10.4% (four hearts perfused for 10 min). This phenomenon was seen irrespective of whether the preliminary perfusion with drug-free solution was for 10 or 15 min before guanethidine infusion commenced, and therefore was apparently caused by guanethidine itself rather than by removal of the extra-cellular cation.

TABLE 5

EFFECT OF CALCIUM IONS ON GUANETHIDINE LEVELS IN RAT HEARTS

Ten groups of four rats were infused with ^{3}H-guanethidine 1 μg/ml. (specific activity 132 μc/mg) in Locke solution containing 0 or 2·2 mM calcium for 2, 4, 6, 8 or 10 min, and tissue levels determined. Mean values for each group ±S.E. are given

Infusion time (min)	Guanethidine level μg/g heart tissue	
	Calcium-free	Normal calcium
2	0·46±0·03	1·02±0·07
4	0·74±0·08	1·79±0·16
6	0·77±0·09	2·37±0·22
8	0·45±0·02	2·70±0·27
10	0·07±0·001	2·93±0·19

DISCUSSION

With the normal concentration of extra-cellular calcium, guanethidine was lost from the heart at two exponential rates (Fig. 1), indicating release from separate stores or compartments. The time constant of the first component was 2.6 min ; as the figure for such extra-cellular markers as sorbitol and inulin is 1.7 min (Boullin & Sullivan, unpublished observations ; Morgan *et al.*, 1961) initial guanethidine efflux may have resulted from release of loosely bound drug from blood vessels or other sites. The initial exponential phase of release was complete after 10 min and thereafter release continued from another compartment at a slower rate (time constant 30 min). It was this compartment that

was affected by excess calcium ions, as the monophasic efflux during perfusion with 20 mM calcium had a similar time constant of 34 min. The result was direct evidence that calcium ions increased the release of guanethidine from the heart, and moreover showed that the drug came from the same store as in normal physiological conditions.

In contrast, guanethidine release was even greater under calcium-free conditions. The time constants for release were 4.7 and 47 min, respectively. The latter pool was obviously large since efflux was at a high rate, with a slow rate of decline. The question arises as to whether this rapid loss represented efflux from the same store from which guanethidine was normally released when calcium ions were present, or from a separate store. It would seem most likely that the slower time constant for efflux under calcium-free conditions may describe the efflux characteristics of a different cellular compartment, but it cannot be excluded that a change in the release characteristics of the normal store was brought about by the calcium-free conditions.

It is well known from earlier work that guanethidine impairs the ability of the sympathetic nerves to store transmitter. The results described for the perfusion of guanethidine *in vitro* in calcium-free solution indicate that the drug also impairs its own storage, which suggests that the guanethidine storage site is also the synaptic vesicles. Several results in the literature accord with this view. Thus the capacity of rat heart to take up guanethidine is strictly limited (Schanker & Morrison, 1965); only a small proportion of the drug accumulated by adrenergically innervated tissues is actually involved in mediating the pharmacological effects (Chang, Costa & Brodie, 1965; Brodie, Chang & Costa, 1965) and tritiated guanethidine is released by sympathetic nerve stimulation (Boullin, Costa & Brodie, 1966) by a calcium-dependent mechanism (Boullin, 1966a). Moreover guanethidine-induced noradrenaline depletion of tissues takes several hours (Cass & Spriggs, 1961) and results in the discharge of pharmacologically inactive deaminated metabolites, formed by intra-neuronal monoamine oxidase, and only small amounts of unmetabolized catecholamine (Kopin & Gordon, 1963); however, very large amounts of guanethidine release sufficient noradrenaline to produce sympathomimetic activity (Hertting, Axelrod & Patrick, 1962). This may be the result of the inability of intra-neuronal monoamine oxidase to cope with catecholamine released in bulk, which may also explain the fact that in calcium-free conditions guanethidine causes the release of unmetabolized noradrenaline from heart at a high rate (Bogdanski, 1965).

An interaction between calcium ions and specifically bound guanethidine is evident from the report of Burn & Welsh (1967) who have recently shown that excess calcium can reverse the adrenergic neurone blocking action of guanethidine on the Finklemann preparation. Since the present results indicate that calcium ions may release guanethidine from intra-cellular stores within the heart, it is reasonable to suppose that this displacement represents loss from the pharmacological sites of action, and that these sites are the synaptic vesicles in adrenergic nerves.

The *in vitro* experiments provide further information on the role of calcium ions in guanethidine uptake and binding. Table 3 shows that guanethidine uptake is drastically reduced in the absence of calcium ions. Nevertheless, at both perfusion concentrations, the amounts of guanethidine subsequently lost after labelling were not greatly affected by removal of calcium, even though those initially taken up were vastly different. This result raises the possibility that, with normal calcium ions two stores were available to

guanethidine, but in the absence of the cation only one was available; according to the *in vivo* experiments this would be the intra-cellular store. On the basis of the experiments with tritiated guanethidine (Fig. 3) it would appear that under calcium-free conditions guanethidine uptake was strictly limited, and that the capacity of the heart to store guanethidine was abolished after guanethidine infusion for 5 min, so that the amount of drug originally taken up (850 mμg, Table 4) was thereafter almost entirely lost, even though guanethidine infusion was still continuing. Since this phenomenon was independent of the duration of the preliminary perfusion with calcium-free solution before guanethidine was infused, the conclusion may be drawn that the interference with guanethidine storage was an effect of the drug itself and was not the result of an action caused by removal of the cation.

The following propositions are therefore made to explain the results: in physiological conditions with 2.2 mM calcium, guanethidine is stored in small amounts in sympathetic nerves in an intracellular compartment which may be the synaptic vesicles as it is affected by excess calcium (Fig. 1) and calcium is known to penetrate adrenal medullary cells (Douglas & Poisner, 1962). This specific binding is, however, normally obscured by a large amount of drug that is bound non-specifically at non-neuronal sites, and not involved in the mediation of pharmacological responses. Removal of calcium ions eliminates this binding (Table 3) so that the pharmacological action of guanethidine may be investigated without interference.

If the pharmacological action of guanethidine is to alter the properties of the vesicles in some way so that their storage capacity is impaired (Fig. 3), substances in the vesicles, such as noradrenaline and guanethidine, will leak out into the neurone. When calcium is absent the initial rate of loss is rapid because of the "leakiness" of the outer neuronal membrane. When the normal concentration of extra-cellular calcium is present, however, they pass into the extra-cellular space at a slower rate.

Further experiments into the effect of guanethidine on synaptic vesicles should show a reduction in the number of vesicles and their electron density with an increase in the quantity of catecholamines and guanethidine in the non-particulate fraction of sympathetic nerves. The time course of this effect should be rapid and should correlate with the time course of the failure of sympathetic transmission occurring after administration of guanethidine. Such experiments might well be the next step in determining the validity of the hypotheses.

SUMMARY

1. Rat hearts were labelled with radioactive guanethidine by intravenous injection of 5 mg/kg, or infusion into the isolated organ of 0.1, 1.0 or 10.0 μg/ml. for 5–10 min in Locke solution. The pattern of guanethidine efflux after *in vivo* labelling was studied in isolated hearts perfused with Locke solution containing 0, 2.2 or 20 mM calcium.

2. With 2.2 mM calcium, efflux followed two exponentials indicating loss from two cellular compartments. Perfusion with 20 mM calcium enhanced efflux from one of these compartments by 25%.

3. Perfusion with 0 mM calcium enhanced guanethidine efflux by 274%. Release followed a biphasic pattern, but the drug was released from different cellular compartments which were not affected when calcium was present.

4. Hearts infused *in vitro* with 0.1, 1.0 or 10 μg/ml. took up 14% to 2% of the infused dose when 2.2 mM calcium was present. This uptake was reduced by about 70% by removal of calcium; thereafter the quantity of drug lost was not altered by removal of calcium ions.

5. In experiments where uptake and efflux were studied simultaneously during *in vitro* infusion of 1 μg/ml. for 10 min, uptake was continuous with 2.2 mM calcium but occurred during the first 5 min only when calcium was omitted.

6. The conclusion drawn from the results is that removal of calcium ions causes release of guanethidine primarily from non-specific cellular binding sites and not from the intra-neuronal store. It is suggested that the pharmacological action of guanethidine is to destroy the intra-neuronal store, and that this action may be particularly evident under calcium-free conditions.

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