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RELEASE OF [^{3}H]-NORADRENALINE FROM THE MOTOR ADRENERGIC NERVES OF THE ANOCOCCYGEUS MUSCLE BY LYSERGIC ACID DIETHYLAMIDE, TYRAMINE OR NERVE STIMULATION

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1 A method is described for labelling the neuronal noradrenaline (NA) stores of rat anococcygeus with [^{3}H]-NA and detecting subsequent release of ^{3}H from the superfused tissue by nerve stimulation or drugs.

2 Lysergic acid diethylamide (LSD) or tyramine but not barium chloride or carbachol increased the efflux of ^{3}H although each drug produced an equivalent contractile response. This confirms that LSD has an indirect sympathomimetic action.

3 LSD was found to produce a proportionately smaller reduction of the nerve-induced efflux of ^{3}H than of the accompanying contractile response.

4 The inhibition of nerve-induced contractile responses by LSD was shown to be independent of the neuronal uptake of noradrenaline and any post-junctional inhibition demonstrated to be non-specific.

Introduction

The isometric contraction of rat anococcygeus *in vitro* or *in vivo* gives a quantitative measure of the effects of agonist drugs or of stimulation of its adrenergic motor innervation (Gillespie, 1972; Gillespie & McGrath, 1973). The tissue also receives an inhibitory innervation, however, so that with the *in vitro* preparation, discrete stimulation of the motor nerves is difficult due to the mixed nature of the nerve bundles invading the tissue (MacLellan, 1973; J.S. Gillespie, personal communication). In consequence, field stimulation (Gillespie, 1972) is the simplest and most satisfactory means for achieving maximal nerve stimulation but the resulting isometric tension response is the resultant of the algebraic summation of motor and inhibitory nerve effects (McGrath, 1973; Gillespie & McGrath, 1974a). In the case of agonist drug responses, a further complicating property of rat anococcygeus is its sensitivity to indirect sympathomimetic actions even by drugs not normally noted for such effects (Gillespie & McGrath, 1975). The usefulness of the preparation could, therefore, be extended if an index of the output of neuronal noradrenaline (NA) were available.

We have now developed a superfused, *in vitro* preparation of anococcygeus in which the NA stores in the sympathetic nerve varicosities are labelled with [^{3}H]-NA. This was facilitated since the properties of the tissue with respect to the neuronal uptake of NA had been determined earlier (Nash, Gillespie & Robertson, 1974). The overflows of tritium induced by drugs or by field stimulation were measured and compared, as indices of NA output, with the accompanying isometric tension responses.

The method has been used here to investigate further effects of lysergic acid diethylamide (LSD) on the anococcygeus muscle which had previously been investigated using only contractile responses. LSD produces contraction of several smooth muscle tissues which receive a motor adrenergic innervation (Thomson, 1958; Ambache, Killick, Srinivasan & Zar, 1975; Gillespie & McGrath, 1975). In the rat and cat anococcygeus muscles and rat vas deferens this contraction is abolished by phentolamine or by pretreatment with 6-hydroxydopamine (Gillespie & McGrath, 1975) which suggests an indirect sympathomimetic effect. The effects of LSD on spontaneous ^{3}H-efflux have, therefore, been compared with those of known direct and indirectly-acting contractile agents. Secondly, LSD is known to inhibit the contractile response to field stimulation but not to NA (Ambache *et al.*, 1975; Gillespie & McGrath, 1975) suggesting

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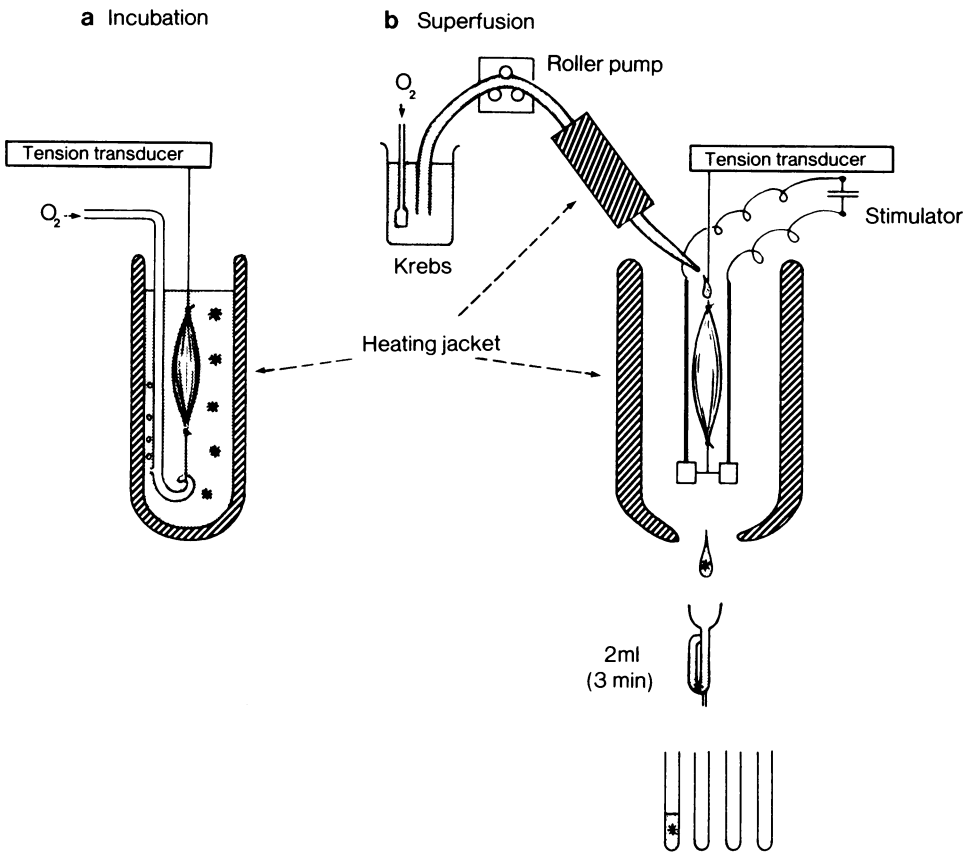

Figure 1 Apparatus used. (a) Incubation with [^{3}H]-noradrenaline. Anococcygeus held under isometric conditions. (b) Superfusion of anococcygeus, which is held isometrically in air between parallel electrodes. Asterisks denote presence of radioactivity.

a pre-junctional inhibitory effect on the motor nerves. The effects of LSD against nerve-induced ^{3}H-efflux have thus been examined to confirm this site of action. Finally the mechanism of the sympatholytic effect of LSD was shown to be independent of the neuronal uptake of noradrenaline.

A preliminary account of these results has been published (McGrath & Olverman, 1977).

Methods

Single rat or cat anococcygeus muscles were incubated in a 3 ml bath under isometric conditions at an initial resting tension of 0.5 g in Krebs bicarbonate solution at 37°C with [^{3}H]-NA (NA 0.5 μM; 5 μCi/ml (−)-[7-^{3}H]-NA acetate, Radiochemical Centre, Amersham) for 30 min (disodium edetate 1.3 μg/ml; ascorbic acid 20 μg/ml also present). The tissues were then held isometrically in air under an initial resting tension of 0.5 g and superfused with drug-free Krebs solution at 37°C at a rate of 0.66 ml/min (Figure 1). Sequential 2 ml aliquots of superfusate (corresponding to 3 min periods) were collected, 1 ml of which was mixed with 10 ml Toluene-Triton X scintillation fluid and the radioactivity counted in a liquid scintillation counter (Packard Tri-Carb, Model 3390) for 5 min and ct/min converted to d/min by external channels ratio standardization. Samples of the incubation medium and of tissue from the anococcygeus muscle (digested with Soluene at the end of the experiment) were also measured for estimation of the tissue/medium ratio for [^{3}H]-NA. Seventy-five min after starting superfusion, LSD 5 μM, tyramine hydrochloride 5 μM, carbachol 3 μM or barium chloride 8 mg/ml was added to the superfusion fluid. In experiments assessing the drug-induced ^{3}H-overflow, to minimize the influence of variation between tissues, the efflux of ^{3}H during the 3 min period before the addition of each agonist drug was given an arbitrary d/min of 100 and all the other effluxes expressed rela-

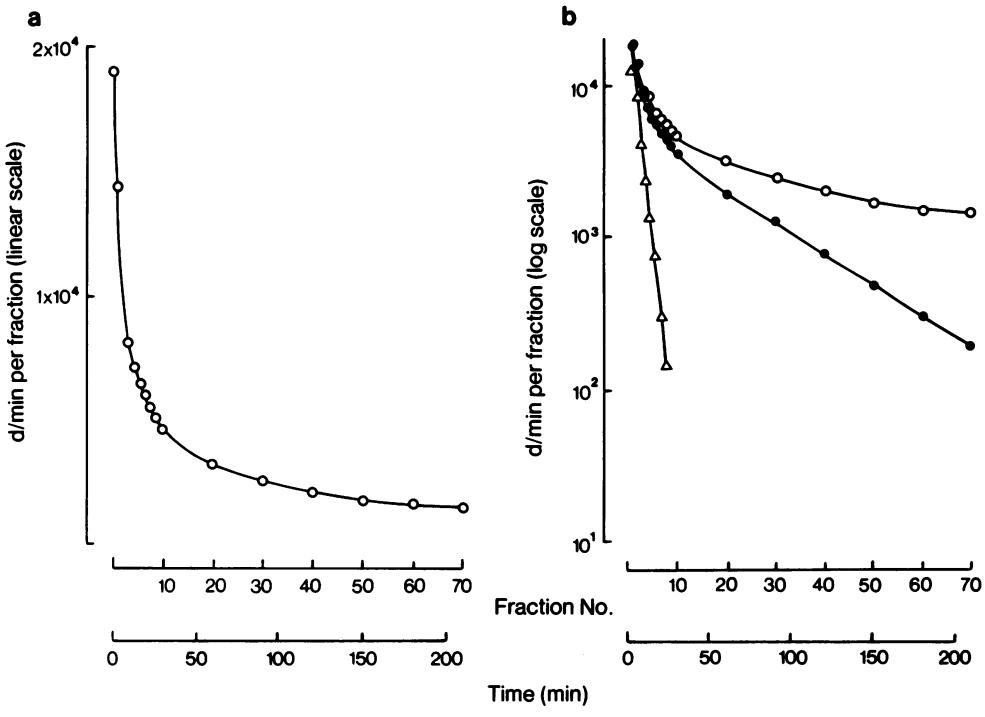

Figure 2 Radioactivity in superfusate samples after pre-incubation of anococcygeus with [^{3}H]-noradrenaline (single experiment). (a) Linear scale. (b) Radioactivity on log scale; (○): raw data as (a). This first curve reached a plateau between fractions 60 and 70. The d/min value at 70 min was subtracted from each value, and the residues replotted as a second curve: (●). This second line was log linear between fractions 20 and 70. The regression line from fractions 20 to 70 was therefore calculated and subtracted from the second curve. A residual third curve was therefore replotted and found to be log linear: (△).

tive to this. Isometric tension in the tissue was measured throughout with a Grass FT03 transducer and displayed on a Devices M2 pen recorder or Grass Model 7 polygraph.

Field stimulation of the intramural nerves was applied via Ag:AgCl wire electrodes running parallel to and on either side of the tissue (Devices isolated stimulator, 1 ms pulses, supramaximal voltage, train length and frequencies in text). Electrical conductivity between the electrodes and the tissue was maintained by a film of superfusion fluid held by surface tension. It was necessary to ensure that the electrodes were sufficiently close together so that when the tissue 'contracted isometrically' the thin sheet of muscle did not 'narrow' and consequently break the circuit between the electrodes. Nerve-induced ^{3}H-overflow in the two 3 min periods after stimulation was measured by estimation of the basal efflux which would have been found in the absence of stimulation. This was found by calculation of a linear regression line for the baseline before and after test periods (usually with a minimum of 6 points) and extrapolation to find the appropriate intermediate points. This 'basal overflow' was then subtracted from the actual values and the excess in the two periods added together to give a final figure (see also Figure 4).

In a further set of experiments rat anococcygeus muscles were not preincubated with [^{3}H]-NA but simply set up *in vitro* for field stimulation and isometric recording as described by Gillespie (1972). Frequency-response curves (F-R) to field stimulation with 100 pulses (1 ms, supramaximal voltage) or dose-response curves to NA (D-R) were constructed. Separate tissues were used for F-R and D-R. Control F-R or D-R were constructed for all tissues and subsequently contralateral tissues were treated separately as follows. (1) One muscle was exposed to cocaine 1 μM for 30 min and new F-R or D-R constructed. LSD 0.3 μM was then added to the bath and F-R or D-R repeated again. (2) The contralateral muscle was treated similarly except for the omission of cocaine.

Drugs used were, barium chloride (Analar), cocaine hydrochloride (Macarthys), lysergic acid diethylamide tartrate (Sandoz), tyramine hydrochloride (Sigma),

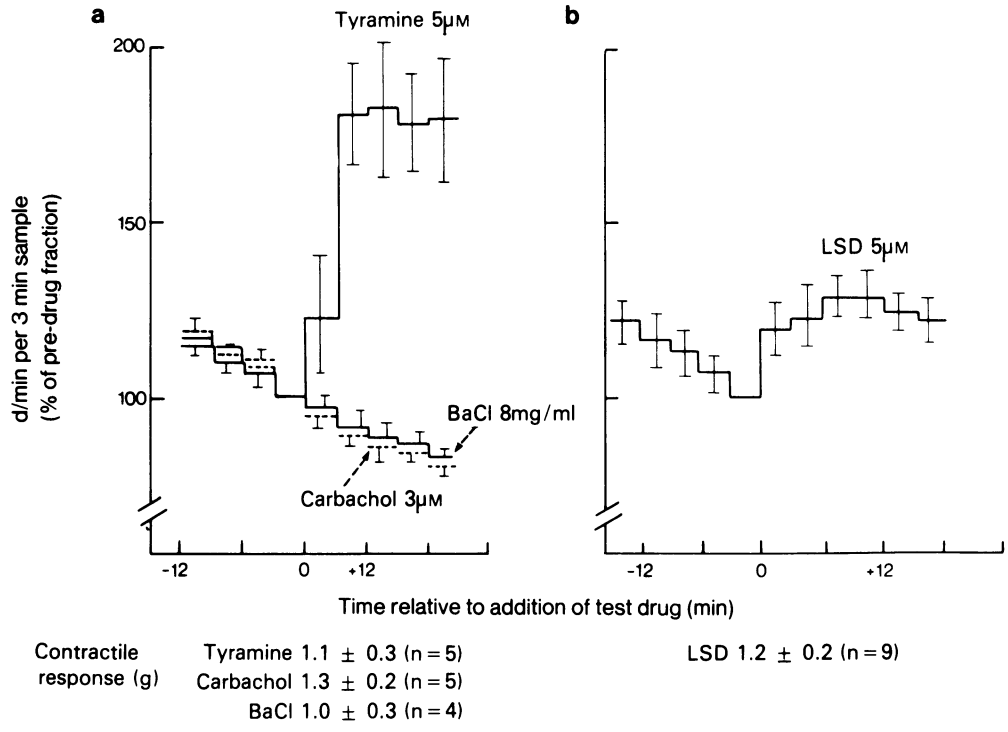

Figure 3 Effects of agonist drugs on the overflow of ^3H from rat anococcygeus muscles previously incubated with [^3H]-noradrenaline. Ordinate scales give radioactivity in samples of superfusate collected over successive 3 min periods. For each sample, radioactivity is expressed as a percentage of that in the sample prior to addition of the test drug; vertical lines show s.e. means. Abscissa scales give the time of the end of each sample period relative to addition of the test drug which in each case was 75 min after starting superfusion. Maximum contractile response produced by each test drug is indicated below each graph. (a) Tyramine, 5 μM, heavy continuous line; BaCl, 8 mg/ml, thin continuous line; carbachol, 3 μM, broken line. (b) Lysergic acid diethylamide (LSD), 5 μM.

noradrenaline bitartrate (Sigma), noradrenaline acetate (details above, Radiochemical Centre, Amersham). All drugs were dissolved in 0.9% w/v NaCl solution (saline) and added to the superfusion fluid or organ bath in a volume of 1 part per hundred or less.

Results

^3H overflow

Following incubation with [^3H]-NA, the quantity of ^3H appearing in the superfusate showed an exponential decay with time which could be separated by curve stripping into two log-linear components with half-lives respectively of 25 ± 5 and 116 ± 10 min (n = 6). An example is shown in Figure 2. Test drugs were, therefore, added 75 min after starting perfusion when the decay was essentially log-linear.

LSD 5 μM or tyramine 5 μM increased this spontaneous overflow whereas barium chloride 8 mg/ml or carbachol 3 μM did not, although each substance contracted the tissue to a similar degree (Figure 3). This suggests that contraction of the tissue *per se* does not release ^3H from the tissue.

Field stimulation of the tissue over a 15 s period with 10 or 20 Hz produced reproducible increases in the basal overflow of tritium in the 6 min period following stimulation. LSD 5 μM inhibited both this nerve-induced overflow and the accompanying contraction. However, on a percentage basis the reduction in contraction was always greater than the corresponding reduction in ^3H overflow (Figures 4 and 5).

The tissue concentration of ^3H measured at the end of several experiments divided by the ^3H content of the corresponding incubation medium gave a ratio of 4.5 ± 0.5 (n = 6). In a further 3 experiments where the tissue was digested immediately after incubation with [^3H]-NA, mean tissue/medium ratio was 5.5 ± 1.3 (n = 3).

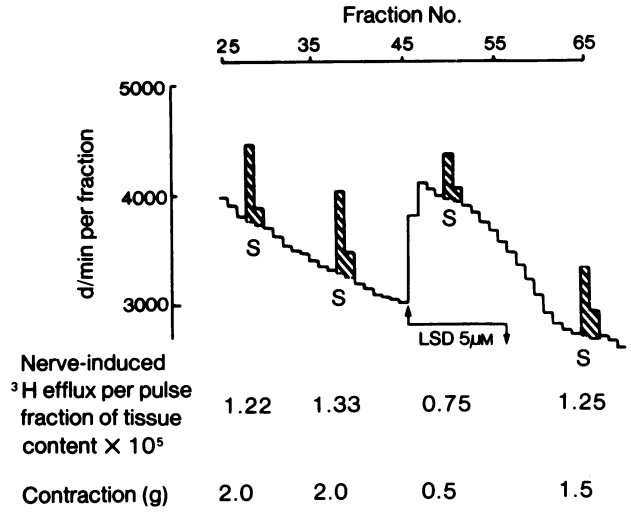

Figure 4 Effect of lysergic acid diethylamide (LSD) on the basal and nerve-induced efflux of ^3H in a single experiment. Shaded areas indicate the difference between background and nerve-induced efflux in the two (3 min) fractions following field stimulation (10 Hz, 150 pulses). Total nerve-induced efflux of ^3H following each train of pulses was calculated and expressed as the fraction of the tissue content of ^3H released per pulse.

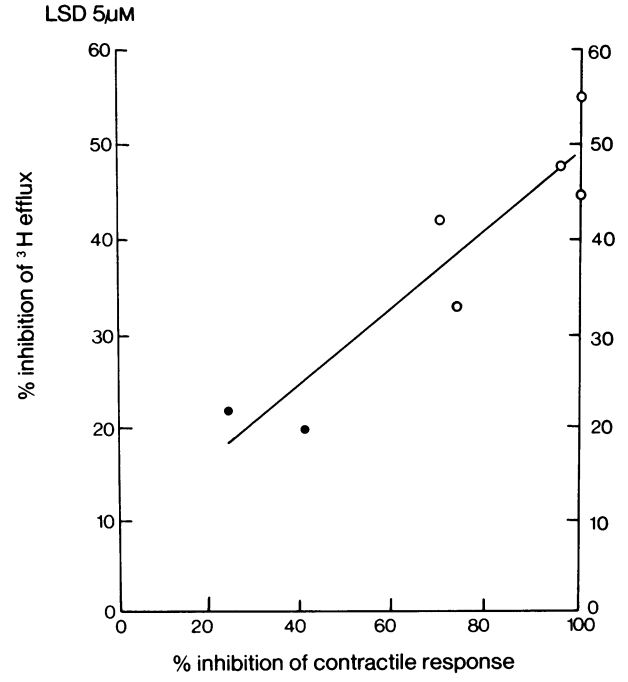

Figure 5 Relationship between the inhibitory effects of lysergic acid diethylamide (LSD, 5 μM) on the nerve-induced efflux of ^3H and the corresponding contractile response. Field stimulation for 15 s: (○), 10 Hz; (●) 20 Hz. Contraction and ^3H efflux responses to stimulation 12 min after addition of LSD were measured and expressed as percentages of the pre-LSD controls. Each point represents a single observation from a separate experiment.

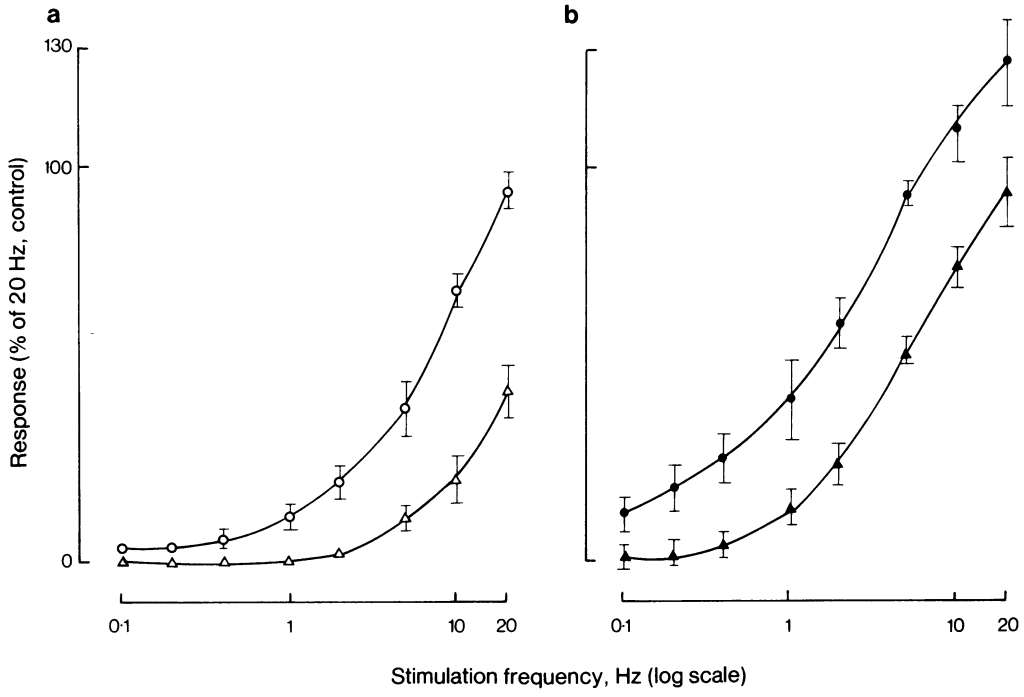

Figure 6 Effects of lysergic acid diethylamide (LSD, 0.3 μM) and/or cocaine (1 μM) on the contractile responses in rat anococcygeus produced by field stimulation (1 ms, supramaximal voltage, 100 pulses). (a) Control (○); LSD (△). (b) Cocaine (●); LSD plus cocaine (▲). Each value is expressed as a percentage of the response to 20 Hz in the first frequency-response curve (not shown). Vertical lines show s.e. means. Circles represent the second and triangles the third frequency-response curves (see Methods) (n = 6).

Contractile responses

Cocaine 1 μM displaced the F-R curve to field stimulation and the D-R curve to NA to the left but did not affect responses to carbachol indicating an increased sensitivity to endogenous or exogenous NA. LSD 0.3 μM shifted the F-R curve to the right in a parallel manner but reduced the responses to high concentrations of NA and carbachol (Figures 6 and 7). This latter non-specific depression may be a fatigue effect due to the prolonged exposure to the contractile effect of LSD. The concentration of LSD was reduced in this section to 0.3 μM in an attempt to minimize this effect but considerable contraction was still produced. Overall, however, these effects are interpreted as indicating a pre-junctional inhibition of the nerve response (as confirmed by the ^{3}H overflow), especially at low frequencies where the size of the response is comparable with those to low doses of NA and carbachol which are unaffected by LSD. In the presence of cocaine 1 μM, LSD 0.3 μM produced a similar effect on the F-R curve to that found in the absence of cocaine although each individual curve was, of course, displaced to the left (Figure 6).

Discussion

Contraction of the anococcygeus by barium chloride or carbachol which act directly on the muscle did not alter the overflow of ^{3}H. LSD and tyramine, which also contracted the tissue, increased the overflow of ^{3}H. It is concluded that LSD, like tyramine, is an indirectly acting sympathomimetic agent in this tissue. The mean overflow produced by LSD was less than that produced by tyramine. Overflow of [^{3}H]-NA from a tissue is not a reliable guide to the mount of [^{3}H]-NA actually released into the synaptic cleft due to the operation of NA uptake mechanisms into both nerve and muscle (see Hughes, 1972). When the indirect sympathomimetic activity of a substance is being analysed, therefore, its effect on these uptake mechanisms must be considered. Tyramine is known to inhibit neuronal NA uptake (Burgen & Iversen, 1965) whereas LSD appears to have no such effect in tissue slices (Dengler, Spiegel & Titus, 1969) or in the rat anococcygeus, as measured by its failure to shift to the left the dose-response curve for NA in the present study. This might explain the relatively smaller overflow induced by LSD in the anococcy-

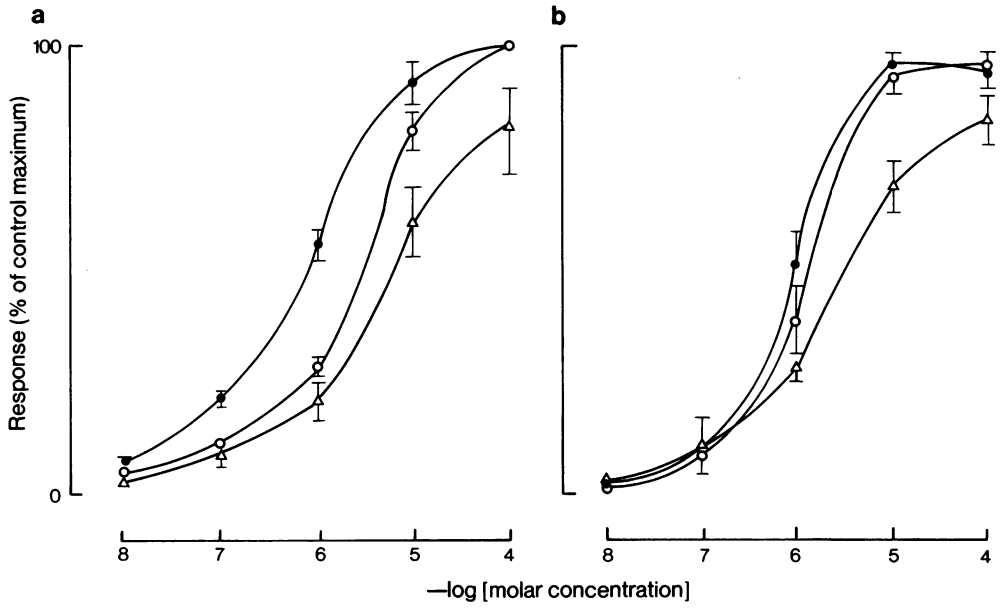

Figure 7 Effects of lysergic acid diethylamide (LSD, 0.3 μM) and cocaine (1 μM) on the dose-response curves to (a) noradrenaline or (b) carbachol in rat anococcygeus. Control (○); cocaine (●); LSD (△), expressed as percentages of control; vertical lines show s.e. means; $n = 6$.

geus and also why Hughes (1973) failed to detect the production of any significant overflow of NA by LSD in the vas deferens where uptake mechanisms have a particularly important influence on the overflow of NA (Hughes, 1972). Guanethidine (5 μM), which blocks the neuronal uptake of NA in anococcygeus (Gillespie, 1972; Foster, Shah & Small, 1978) also produced an increase in the basal efflux of ^{3}H which was greater than that produced by LSD (Olverman, 1975). It has previously been noted from data derived by indirect means that many substances, not usually noted for their sympathomimetic effect, can act as indirect sympathomimetics in anococcygeus (Gillespie & McGrath, 1974a; 1975). This method may, therefore, prove to be a sensitive assay for drug-induced release of NA. One qualification to the method is the continuing relatively high level of basal efflux after 75 min of label-free superfusion. In future the technique might be made more selective by washing for a considerably longer period, for example 2 to 3 hours.

Labelling the neuronal NA stores in the anococcygeus muscle appears to be a simple and straightforward method of studying adrenergic mechanisms. The preparation has a high endogenous NA content (Gillespie & McGrath, 1974b) achieves a relatively high tissue/medium ratio of [^{3}H]-NA, consists of a simple unidirectional layer of densely innervated smooth muscle and there is no doubt as to the adrenergic nature of the motor transmission as there is for instance in the vas deferens (Ambache & Zar, 1971).

Reproducible increases in ^{3}H efflux from the nerve terminals can be induced either by drugs or by nerve stimulation. The method allowed confirmation of the formerly indirect evidence that LSD inhibited the nerve-induced release of NA from this tissue (Ambache *et al.*, 1975; Gillespie & McGrath, 1975). In addition, the percentage reduction in ^{3}H efflux was less than the corresponding percentage reduction in the contractile response. This might be expected since field stimuli will activate the reciprocal motor and inhibitory innervation present within this tissue (Gillespie, 1972; Gillespie & McGrath, 1973) but emphasizes the difficulty in interpreting quantitatively the effects of drugs on the resultant net contractile response.

It has recently been suggested that part of the indirect sympathomimetic effect in the anococcygeus of a high concentration of guanethidine or cocaine (McGrath, 1973; Gillespie & McGrath, 1974a) might be due to potentiation, via blockade of neuronal uptake, of the effects of spontaneously released NA (Foster *et al.*, 1978). However, unlike cocaine, LSD does not block the neuronal uptake of NA as indicated by its failure to shift leftwards the D-R curve to NA. The NA liberated from the tissue by LSD appears, therefore, to be displaced from the nerves rather than released spontaneously.

Blockade of neuronal uptake of NA produced the known selective potentiation of contractions evoked by NA (Gibson & Pollock, 1973) or by field stimulation (Gillespie & McGrath, 1974; Clanachan & McGrath, 1976) but did not affect the inhibitory effect of LSD. This eliminates the possibility that the inhibitory effect of LSD might actually have been partly masked by an opposing potentiation, due to blockade of re-uptake, of the effects of the residual NA output. Caution should, however, be exercised in making a quantitative interpretation of these frequency-contractile response curves since the effect of the inhibitory nerves will be unaffected by cocaine and will be greatest at frequencies of 1 Hz and above (McGrath, 1973; Gillespie & McGrath, 1973). The contractile effect of LSD was also not significantly altered by cocaine but no attempt has been made to relate this to possible mechanisms for the sympathomimetic action of LSD due to the notorious difficulties involved in interpreting the effects of uptake blockade on the action of indirect sympathomimetic agents such as tyramine (see Trendelenburg, 1972).

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