Br. J. Pharmac. (1969), **35**, 10–28.

The inhibitory action of noradrenaline and adrenaline on acetylcholine output by guinea-pig ileum longitudinal muscle strip

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1. Noradrenaline and adrenaline reduce the output of acetylcholine by the guinea-pig ileum longitudinal strip by up to 80%, both in resting conditions and after stimulation. The effect is graded with dose, and is detectable with noradrenaline 2×10^{-7} g/ml. Adrenaline is approximately 4 times as active as noradrenaline, and its action after being washed out is more persistent.

2. If resting output is high, both amines have a proportionately greater effect and their action, as dosage is increased, is to reduce resting output to a basal level, relatively constant from strip to strip, of about 10 ng/g/min.

3. With stimulation, the effect of the amine is greater at low frequencies, when the output per volley is high, than at high frequencies. The effect is reduced by increasing the number of shocks delivered. There thus appears to be a basal output per volley, of the order of 1–2 ng/g/volley, which can be reached either by relatively rapid stimulation, by prolonged stimulation, or by treatment with these amines.

4. If noradrenaline is applied during continued stimulation at 40/min, the depression of acetylcholine output during its presence is followed by an augmented output when the drug is withdrawn. The magnitude of this "overshoot" increases with the duration of noradrenaline exposure.

5. Phenylephrine 4 μg/ml. and amphetamine 20 μg/ml. reduced the acetylcholine output, but isoprenaline 1 μg/ml., dopamine 1 μg/ml. and methoxamine 10 μg/ml. were ineffective.

6. Phenoxybenzamine reduced the resting output and increased the stimulation output. Of the two other blocking agents examined, phentolamine had no effect on either resting or stimulation output and ergotamine transiently reduced stimulation output. The effect of phenoxybenzamine was not due to a reaction with either adrenoceptive or muscarinic receptors.

7. Phenoxybenzamine, phentolamine and ergotamine abolished the effect of adrenaline and noradrenaline on both resting output and on output in response to stimulation.

8. In strips obtained from animals treated with reserpine and guanethidine, a rise in resting acetylcholine output and in stimulation output at low frequencies was found. In these conditions, noradrenaline was still effective.

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9. Reducing the hydroxytryptamine content of the strips by treatment with *p*-chloro-(±)-phenylalanine did not significantly affect acetylcholine output.

10. It is concluded that acetylcholine output by the nervous networks of the longitudinal strip is under the normal control of the sympathetic by a species of presynaptic inhibition mediated by α receptors. This implies that for a tissue under dual autonomic control, withdrawal of sympathetic control will lead to a parasympathetic response which is not only unopposed but also itself enhanced.

Since the classic work of Finkleman (1930), it has been generally accepted that the inhibition of the rhythmic activity of the intestine by sympathetic stimulation is due to release of an adrenaline-like substance, now believed to be noradrenaline. Further, evidence has accumulated that the receptive mechanism involves both α and β receptors (in Ahlquist's nomenclature, Ahlquist & Levy, 1959). The inhibitory effect on the sympathetic transmitter has usually been considered in terms of a direct action by the catecholamines on the smooth muscle concerned. Bozler (1940) showed that adrenaline and noradrenaline reduced electrical excitability of smooth muscle ; Bülbring (1954) and Bülbring & Kuriyama (1963) showed, using taenia coli, that these amines produced a hyperpolarization of the smooth muscle cell membrane ; and Jenkinson & Morton (1967a, b) have shown, on depolarized taenia coli, that noradrenaline produces an increase of potassium permeability mediated by α receptors.

There is, however, evidence that the catecholamines can depress intestinal activity also by inhibiting the nerve networks of the intestine and by inhibiting the release of acetylcholine. McDougal & West (1952, 1954) found that the peristaltic reflex of the guinea-pig ileum was inhibited by low concentrations of adrenaline and noradrenaline which did not affect the response of the ileum to added acetylcholine, and Schaumann (1958) found that these amines reduce the output of acetylcholine by resting guinea-pig ileum. There is, too, a considerable body of evidence (Marazzi, 1939 ; Lundberg, 1952 ; Eccles & Libet, 1961 ; McIsaac, 1966) that adrenaline will depress transmission in sympathetic ganglia, although other investigators have described a facilitating action (Bülbring & Burn, 1942 ; Malmejac, 1955). On the perfused ganglion, Paton & Thompson (1953) obtained evidence that part of the depressant effect was due to reduction of acetylcholine output, which according to Birks & MacIntosh (1961) was prevented by some factor in plasma.

The finding that with the longitudinal strip of guinea-pig ileum all the acetylcholine derives from the nerve network (Paton & Zar, 1965, 1968) provides an opportunity for testing the effect of catecholamines on acetylcholine release in conditions where all the acetylcholine comes from nervous tissue. This paper describes the results of such *in vitro* experiments and also deals with the effects of noradrenaline antagonists. In addition, the effect of the catecholamines on acetylcholine output was investigated on strips obtained from guinea-pigs treated with reserpine or guanethidine in order to deplete the noradrenaline stores of the tissue (Muscholl, 1965). A preliminary account of some of the results was made by Vizi (1967).

Methods

Preparation of longitudinal muscle strips

Guinea-pigs of either sex weighing between 200 and 300 g were used. The longitudinal muscle strip was prepared as described by Rang (1964) and by Paton & Zar (1968) with slight modifications in order to obtain more robust strips; the glass tubing over which the ileum was pulled had a diameter of 8 mm, and was clamped at an angle of 45°, and the longitudinal muscle layer was separated from the underlying circular muscle by stroking it away from its mesenteric attachment not only at the upper end but along the whole length (10–15 cm) of the portion of intestine. The strip was then gathered together and tied at each end with cotton thread. The strips prepared in this way weighed 50–100 mg.

The animals pretreated with reserpine or guanethidine showed pronounced diarrhoea. On opening the abdomen the ileum was more or less empty and exhibited strong spontaneous movements. It was difficult to pull the ileum over the glass pipette used for a normal ileum because of the contracted state, so a narrower glass pipette (6 mm diameter) was used.

The strip was set up in an organ bath of 3–5 ml. volume in Krebs solution at 37° C. Eserine sulphate in a concentration of 2×10^{-6} g/ml. was added and a constant stream of 95% oxygen and 5% carbon dioxide was bubbled through the fluid by a fine syringe needle in the bottom of the bath. Changes of bath fluid were made by overflow. Before collecting the first samples for assaying the acetylcholine output, the strip was allowed to equilibrate under resting conditions for 60 min.

Electrical stimulation

The method described by Paton (1963) and Paton & Zar (1968) was used. The strip was stimulated by means of two platinum electrodes, one at the top and one at the bottom of the organ bath (so-called " field " stimulation). Square-wave impulses were used of 1 msec duration at a frequency of 6 to 600/min and of an intensity giving a potential drop between the electrodes of 15 V/cm. The acetylcholine output was found to depend on the stimulus strength, so the voltage was kept constant in each experiment by continuously monitoring the potential drop between the electrodes on an oscilloscope.

Assay of acetylcholine

A length of about 8 cm from the aboral part of the ileum was used. Its oral end was ligated and its aboral end tied over an open-ended polythene tube which projected through the bottom of the organ bath, to allow the luminal contents to be extruded from the ileum without contaminating the bath. The ileum was suspended in Krebs solution at 37° C containing morphine sulphate (10 μg/ml.) in order to reduce endogenous acetylcholine release, and eserine sulphate (5 ng/ml.) to increase the sensitivity to acetylcholine. The bath, of capacity 5 ml., was aerated with a mixture of 95% oxygen and 5% carbon dioxide. The sensitivity of the preparation increased with time, normally allowing assay of concentrations of acetylcholine down to 2 ng/5 ml. The contractions of the ileum to acetylcholine were recorded by means of an auxotonic lever (Paton, 1957) writing on a smoked drum. Samples for assay, or control acetylcholine solutions freshly made up in distilled water saline,

were added to the assay bath at intervals of 1 min in volumes of 0.2 to 0.6 ml. The estimate of the acetylcholine content of donor samples was based on repeated control dose-response curves. When the donor strip was exposed to drugs, the samples were compared with control acetylcholine solutions to which the drug concerned was added to give the same final concentration in the assay bath. The drugs used in these experiments had the following actions on contraction caused by acetylcholine in the highly sensitive conditions of the assay. Noradrenaline and adrenaline diminished the response by 5 to 40%, but a normal response to acetylcholine returned immediately after washing out. This action was much greater on responses to small doses of acetylcholine than at higher concentrations. Isoprenaline was also depressant, but, as observed by Farmer & Lehrer (1966), its effect persisted for 2–3 min after washing out the drug. Amphetamine, 50–100 ng/ml., and dopamine, 50–100 ng/ml., slightly increased the effect of acetylcholine.

Both the spontaneous resting release of acetylcholine and the increased output resulting from field stimulation were measured. The resting output, in ng/g per min, was given by $\frac{R}{t} \times \frac{1000}{W}$, where R is the total output in ng as measured in the assay; w is the weight of the strip in mg measured after the experiment, excess moisture on the strip being removed by pressing the tissue between two filter papers; and t is the collection period in min. The output per volley is given in ng/g per volley by $\frac{(S-R)}{F \cdot t} \times \frac{1000}{W}$ where S is the total acetylcholine output due to stimulation together with the presumed resting output over the collection period used; R is the resting acetylcholine output in ng during the period of collection, calculated from the control resting output; F is the rate of stimulation per min; t is the duration of stimulation in min. The resting output and the output per volley were lower than found by Paton & Zar (1967), perhaps because the average weight of their strips was lower (about 20 mg.) than in the present experiments (70 ± 10 mg), or because they had used older animals. There is also the possibility of a difference in density of sympathetic innervation because with the present method of preparing the strips a greater number of blood vessels may have been preserved than in the experiments of Paton & Zar.

Drugs

Drugs used in these experiments were: acetylcholine chloride (B.D.H.); (−)-noradrenaline bitartrate (Koch-Light Laboratories Ltd.); (±)-adrenaline bitartrate (Burroughs Wellcome & Co.); isoprenaline sulphate (Burroughs Wellcome & Co.); physostigmine sulphate (Burroughs Wellcome & Co.); dibenyline (Smith Kline & French); ergotamine tartrate (B.D.H.); reserpine (Ciba); (−)-phenylephrine hydrochloride (Boots); guanethidine sulphate (Ciba); *p*-chloro-(±)-methamphetamine (Chinoin); *p*-chloro-(±)-β-phenylalanine (B.D.H.). The drugs were dissolved in distilled water or distilled water saline. Concentrations of the drugs used are expressed in terms of their salts. Reserpine for the subcutaneous injection was prepared according to the formulation found in *Martindale's Extra Pharmacopoeia* (1958, p. 745).

Reserpine was given in a daily dose of 1.5 mg/kg subcutaneously on 2 successive days: the preparation of the longitudinal strip was made 6–8 hr after the last injection. An interval of 6–8 hr was chosen, in view of the analysis of the action of reserpine by Vizi, Somogyi & Knoll (1966) and by Knoll, Vizi, Knoll & Somogyi (1966), showing that over 1–4 hr after giving reserpine the tissue is still under the

influence of released amine, and has not yet reached the pure depleted state. Guanethidine was given in a dose of 15 mg/kg subcutaneously 6 hr before preparing the strip, as in the experiments of Cass & Spriggs (1961).

Results

Effect of sympathetic amines on acetylcholine output by guinea-pig longitudinal muscle strips

Table 1 summarizes the effect of various sympathetic amines on the resting output of acetylcholine and on the output in response to electrical stimulation.

Noradrenaline. In a concentration of 1 μg/ml., noradrenaline reduced the resting output on the average by 56.8%. A just detectable reduction of resting output was obtained with 0.2 μg/ml. The effect on resting output varied with the control level of output and was proportionally greater with high than with low resting output. The result was a reduction of the resting output to the same minimum value of about 10 ng/g per min whatever the control level. This is shown in the left graph of Fig. 1 for four experiments with different control levels of output.

The effect of noradrenaline on the output in response to stimulation depended on the frequency of stimulation. As shown in Table 1 and Fig. 2, the reduction in output produced by 1 μg/ml. was: at 10/min, 80.5% ; at 20/min, 62.5% ; at 40/min, 48.6% ; at 60/min, 26.2% ; and at 180/min, 6.7%.

The action of noradrenaline is graded with dose. Table 1 gives the results of experiments in which the effect of noradrenaline 0.2–3 μg/ml. was studied on the acetylcholine output in response to stimulation at 20 shocks/min, and the curve with the black circles of Fig. 3, obtained from these results, shows the relationship between dose and effect on acetylcholine output. On increasing the dose of noradrenaline from 0.2 to 2 μg/ml. the reduction in output increases from less than 20 to more than 70%, but on further increasing the dose there is scarcely any further

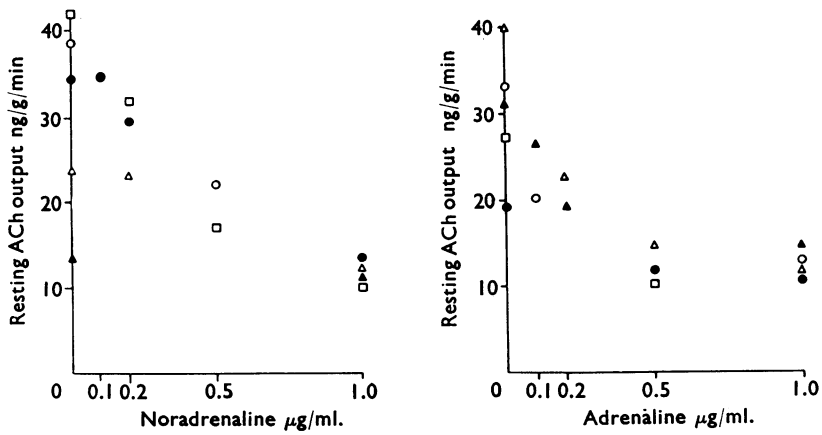

FIG. 1. Action of noradrenaline and adrenaline on resting ACh output of longitudinal muscle strip of guinea-pig ileum. Collection periods, 15 min. Experiments chosen in which the initial resting output varied.

TABLE 1. *Acetylcholine output from the longitudinal muscle strip of guinea-pig ileum. Action of different amines on resting and stimulation acetylcholine output*

Condition	Rate of stimulation or resting collection period	No. of shocks	Total control ACh output ng/g per min ± S.E. (No. of expts. in brackets)	ACh output ng/g per volley ± S.E.
Resting	15–20 min	—	27·1±2·3 (46) (range 8·3–52·9)	—
Resting	15–20 min	—	33·2 (1)	—
Resting	15–20 min	—	33·0±1·7 (5)	—
Resting	15–20 min	—	40·5 (2)	—
Resting	15–20 min	—	27·8±2·4 (6)	—
Stimulated	10/min	50	74·1±7·3 (5)	5·4±0·9
Stimulated	20/min	20	145·0±10·2 (3)	6·0±0·7
Stimulated	20/min	60	104·0 (1)	4·2
Stimulated	20/min	60	87·0 (1)	3·3
Stimulated	20/min	60	107·0 (1)	4·4
Stimulated	20/min	60	101·0±13·2 (6)	4·0±1·0
Stimulated	20/min	60	94·0 (1)	3·5
Stimulated	20/min	100	79·7±4·7 (4)	3·12±0·2
Stimulated	20/min	200	54·2±10·2 (3)	2·04±0·5
Stimulated	40/min	200	120·8±13·9 (9)	2·3±0·2
Stimulated	60/min	60	201·4±29·4 (3)	3·0±0·4
Stimulated	60/min	180	140·1±22·3 (3)	1·5±0·2
Stimulated	180/min	180	301·4±60·9 (5)	1·6±0·3
Stimulated	600/min	600	836·2±99·4 (8)	1·35±0·2
Resting	15 min	—	27·3±2·6 (6)	—
Resting	15 min	—	30·5 (2)	—
Resting	15 min	—	32·1±0·9 (5)	—
Resting	15 min	—	27·8±1·8 (6)	—
Stimulated	20/min	100	78·8 (2)	2·7
Stimulated	20/min	100	87·1±2·2 (4)	2·7±0·1
Stimulated	20/min	100	91·5 (2)	3·3
Stimulated	20/min	100	80·0 (1)	2·8
Stimulated	180/min	180	351·7±72·0 (3)	1·8
Stimulated	20/min	200	56·0±6·0 (3)	2·1±0·3
Resting	15 min	—	50 (1)	—
Stimulated	40/min	200	102·0 (2)	1·9
Resting	15 min	—	21·6±4·0 (3)	—
Stimulated	20/min	100	68·2 (2)	2·4
Resting	15 min	—	27·0 (1)	—
Stimulated	20/min	100	64·1 (2)	2·1
Stimulated	20/min	100	71·2 (1)	—

Treatment (No. of expts. in brackets)	Reduction of ACh output %	*P*
(−)-Noradrenaline 0·1 μg/ml. (1)	3·0	
(−)-Noradrenaline 0·2 μg/ml. (5)	16·7±4·0	<0·05
(−)-Noradrenaline 0·5 μg/ml. (2)	41·6	
(−)-Noradrenaline 1 μg/ml. (6)	56·8±2·5	<0·01
(−)-Noradrenaline 1 μg/ml. (5)	80·5±7·5	<0·01
	—	
(−)-Noradrenaline 0·2 μg/ml. (1)	18·7	
(−)-Noradrenaline 0·5 μg/ml. (1)	40·0	
(−)-Noradrenaline 2 μg/ml. (1)	71·3	
(−)-Noradrenaline 1 μg/ml. (6)	62·5±5·0	<0·01
(−)-Noradrenaline 3 μg/ml. (1)	73·0	
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(−)-Noradrenaline 1 μg/ml. (3)	48·6±12·0	<0·05
	—	
(−)-Noradrenaline 1 μg/ml. (3)	26·2±6·2	>0·1
(−)-Noradrenaline 1 μg/ml. (2)	6·7	
(−)-Noradrenaline 1 μg/ml. (3)	−4·14±2·6	
(±)-Adrenaline 0·1 μg/ml. (6)	29·4±3·7	<0·01
(±)-Adrenaline 0·2 μg/ml. (2)	31·2	
(±)-Adrenaline 0·5 g/ml. (5)	65·1±7·0	<0·01
(±)-Adrenaline 1 g/ml. (6)	71·2±5·7	<0·02
(±)-Adrenaline 0·1 μg/ml. (2)	40·0	
(±)-Adrenaline 0·2 μg/ml. (4)	54·3±3·7	<0·01
(±)-Adrenaline 0·5 μg/ml. (2)	72·7	
(±)-Adrenaline 1 μg/ml. (1)	75·0	<0·01
(±)-Adrenaline 0·2 μg/ml. (3)	11·4±9·0	>0·4
(±)-Isoprenaline 0·5 μg/ml. (3)	−6·9	
Phenylephrine 4 μg/ml. (1)	54·5	
Phenylephrine 4 μg/ml. (2)	33·0	
Dopamine 1 μg/ml. (3)	12·0±3·0	>0·5
Dopamine 1 μg/ml. (2)	6·0	
(±)-Amphetamine 20 μg/ml. (1)	42·0	
(±)-Amphetamine 20 μg/ml. (2)	55·5	
Methoxamine 10 μg/ml. (1)	Nil	

increase in the reduction. A maximum possible reduction of slightly over 70% is thus achieved by a noradrenaline concentration of 2–3 μg/ml.

Figure 4 shows how the output of acetylcholine was affected when noradrenaline (1 μg/ml.) was applied for various periods of time during maintained stimulation at a frequency of 40/min. When applied for 5 min (at *a*) the output fell by 42.5% but after withdrawal of the noradrenaline it rose to a level higher than before the

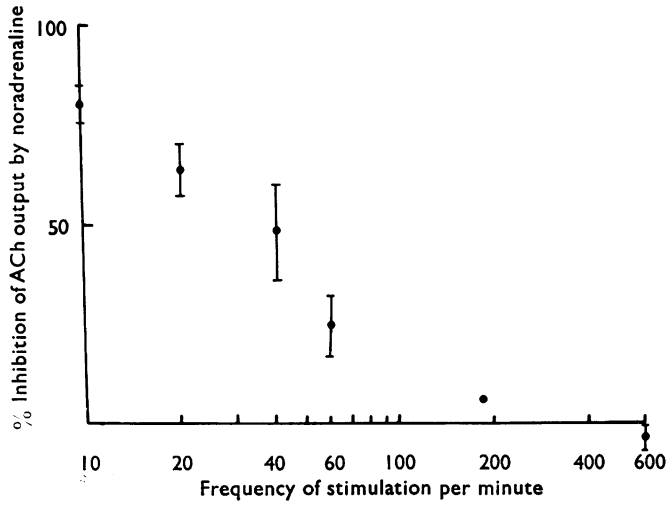

FIG. 2. Inhibition by noradrenaline, 1 μg/ml., of ACh output induced by stimulation at various frequencies. Noradrenaline was given 5 sec before stimulation began. Number of shocks at 10/min, 50; at 20/min, 60; at 40/min, 200; at 60/min, 180; at 180/min, 180; at 600/min, 600.

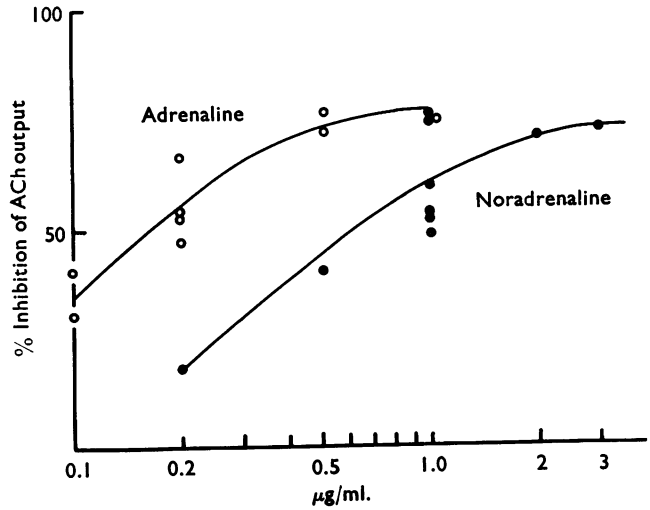

FIG. 3. Log-dose-response curve of inhibitory effect of adrenaline and noradrenaline on ACh stimulation output at a frequency of 20/min. Ordinate, % inhibition; abscissa, log concentration of amine. Number of shocks: 100 for adrenaline, 60 for noradrenaline. The drugs were given 5 sec before stimulation began. Potency ratio of adrenaline to noradrenaline approximately 4.

noradrenaline exposure. This " overshoot," which was at its maximum about 10 min after the withdrawal, amounted to 160 ng/g acetylcholine. This was close to the value of 190 ng/g by which the output had been suppressed during the 5 min exposure. When the noradrenaline was applied for 15 min (at *b*), the output was depressed during the whole period of exposure, but after the withdrawal there was again an excess output equivalent to 190 ng/g as compared with 540 ng/g, the amount by which the output had been suppressed during the exposure. The experiment *c* in which the noradrenaline was applied for 50 min finally shows that the depression of acetylcholine output during the response can be maintained for at least 50 min, although there was some indication of partial recovery during this period. After withdrawal of the noradrenaline there was again an " overshoot "; the excess output amounted to 375 ng/g, which, though greater than that following the shorter periods of exposure, was far less than the 1730 ng/g by which the output had been suppressed during the exposure.

Adrenaline. The effect of adrenaline was similar to that of noradrenaline in that it depressed the resting output of acetylcholine as well as the output in response to electrical stimulation. It differed from noradrenaline in that it was about 4 times as potent, that the depression of output continued for some time after its withdrawal, and that no " overshoot " was detected.

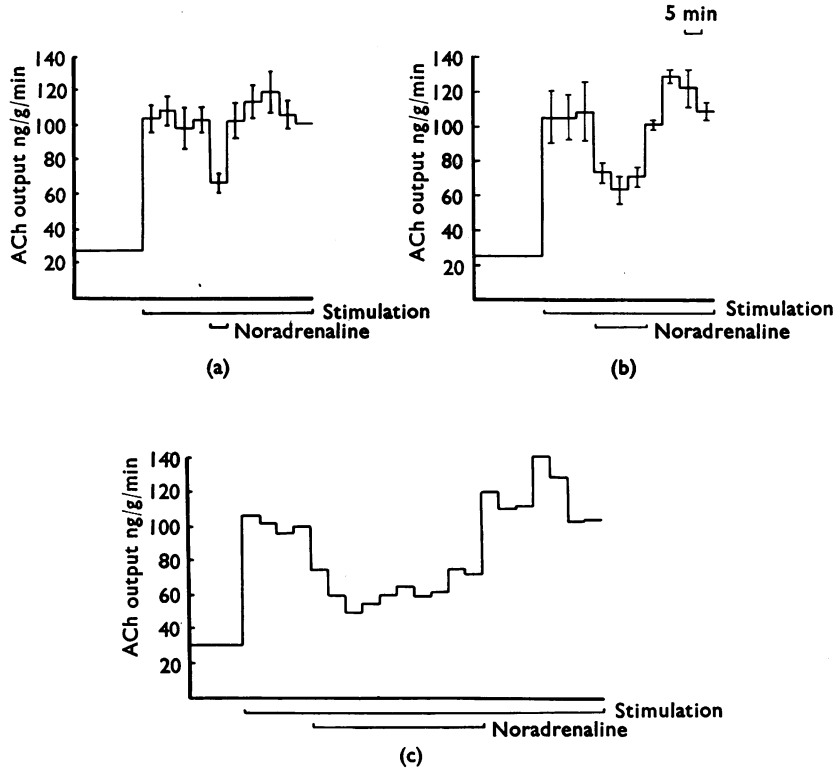

FIG. 4. Output of ACh during and after exposure to noradrenaline 1 μg/ml. for 5, 15 and 50 min during maintained stimulation at the frequency of 40/min. Mean values of (a) seven, (b) three and (c) two experiments; vertical bars give $\pm$S.E.

As shown in Table 1, adrenaline 0.1 μg/ml. reduced the resting output by 29.4%, 0.2 μg/ml. by 31.2%, and 0.5 mg/ml. was apparently sufficient to produce an almost maximal reduction, for it reduced the output by 65.1% and 1 μg/ml. caused a reduction of the same order (71.2%). The values for similar concentrations of noradrenaline were: 0.1 μg/ml., 3%; 0.2 μg/ml., 16.7%; 0.5 μg/ml., 41.5%; 1 μg/ml., 56.8%. The greater potency of adrenaline is also evident from a comparison of the curves for adrenaline and noradrenaline in Fig. 3, and the delay in the recovery of the resting output after withdrawal of the adrenaline is shown in Fig. 5.

As in the experiments with noradrenaline, the effect on the resting output varied with the control level and was proportionally greater with high than with low resting output. The same minimum value was attained with both amines, whatever the control level (Fig. 1).

The experiment in Fig. 5 shows that when stimulation and adrenaline (1 μg/ml) were applied simultaneously for 5 min, the increased output in response to the stimulation was prevented and afterwards there was a reduction in output. The last part of Fig. 5 shows the graded response to 1 and 0.5 μg/ml. adrenaline applied during maintained stimulation. In both instances recovery of output after withdrawal of the adrenaline was delayed, and there was no sign of an excess output as in the experiments with noradrenaline. As with noradrenaline, the effect on output diminished as frequency of stimulation increased (Table 1).

Other amines. Intestinal smooth muscle differs from other tissues in that action both on α and on β receptors is believed to produce a single effect, relaxation (Ahlquist & Levy, 1959). The question therefore arises as to which type of receptor system is involved in the inhibition of acetylcholine output. *Isoprenaline* in a concentration of 1 μg/ml., which is sufficient to reduce the response to added acetylcholine, had no significant effect on output, either on resting or on stimulation output. In fact, as seen in Table 1, in each of the three tests with stimulated preparations there was a small increase of output (mean 6.9%). *Phenylephrine* was ineffective at 1 μg/ml., but at 4 μg/ml. the output was reduced. As seen from Table 1, the resting output was reduced 54.5% and the stimulation output 33%. Higher concentrations were not tested because they interfered with the assay.

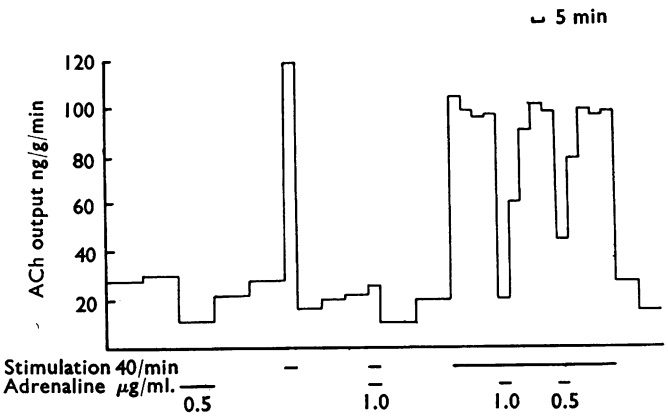

FIG. 5. Effect of adrenaline (0.5 or 1.0 μg/ml.) on resting output and on output evoked by both brief and sustained stimulation, in one experiment.

Dopamine 1 μg/ml. did not affect either resting or stimulation output. *Amphetamine* 20 μg/ml. reduced resting output 42% and stimulation output 55.5% as shown in Table 1. *Methoxamine* 10 μg/ml. had no effect on stimulation output. The significance of this result is not clear; methoxamine has an unusual chemical structure, and although usually classified as a stimulant of α receptors, has been shown to be a β-blocking agent (Karim, 1965). The inactivity of isoprenaline and the effectiveness of phenylephrine support the view that the effect of the amines on acetylcholine output is through α receptors.

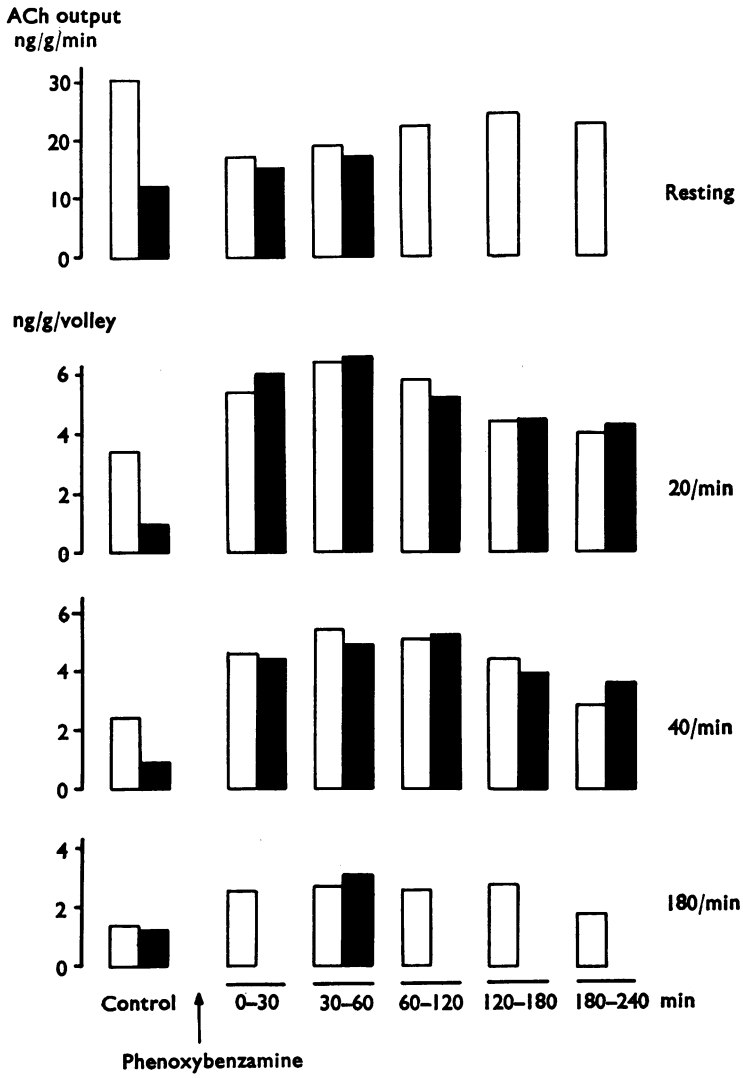

FIG. 6. Action of phenoxybenzamine (1 μg/ml. for 20 min) on resting ACh output and on volley output at frequencies of stimulation of 20, 40 and 80/min. Empty columns, in absence of noradrenaline; filled columns, in presence of noradrenaline 1 μg/ml. Ordinates, ACh output in ng/g/min or volley; abscissae, periods of time after the exposure to phenoxybenzamine during which outputs were measured.

Action of noradrenaline antagonists

Phenoxybenzamine was found to reduce resting output and to increase output in response to stimulation. The drug was applied by exposing the tissue to a concentration of 1 μg/ml. for 20 min and then washing it out, since its strong antimuscarinic action would interfere with the assay. As shown in Fig. 6, depression of resting output and augmentation of stimulation output were present in the samples collected during the first 30 min, after phenoxybenzamine removal. The reduction in resting output decreased during the following hours. The augmentation of the stimulation output was at or near its maximum in the first 30 min sample, sometimes increased in the succeeding one or two samples, and then decreased gradually but was still present after 3–4 hr. If phenoxybenzamine was re-applied after the effect had worn off, the effect was reproduced. Previous exposure to phentolamine before the addition of phenoxybenzamine did not abolish its effect.

The fact that the two other blocking agents examined, phentolamine and ergotamine (see next paragraph), did not show the augmentation of the stimulation output and that previous exposure to phentolamine did not abolish the effect, suggests that it is not mediated by adrenergic receptors. A second possibility was that it was due to the blocking of acetylcholine receptors by phenoxybenzamine. In a concentration of 1 μg/ml., phenoxybenzamine had a strong antimuscarinic action, giving a dose ratio after 20 min of about 80 against acetylcholine. The antagonism declines much faster than that to histamine or to catecholamines, and had fallen to 4-fold in 2 hr and continued to decline further. This time course did not correspond to that of the effect on acetylcholine output. A more specific test of the effect of occluding the acetylcholine receptors on acetylcholine output was made using the irreversible atropinic substance benzilylcholine mustard (Gill & Rang, 1966), which is known to be virtually free of adrenaline antagonism, but is believed to undergo a reaction in the tissues not unlike that of phenoxybenzamine. Exposure of the strip to benzilylcholine mustard (BCM) 5 ng/ml. for 20 min, a procedure sufficient to inactivate 99% of the receptors, had only a minor and transient effect on acetylcholine output. The cause of rise in output with phenoxybenzamine is therefore still obscure. Although the effects of phenoxybenzamine and BCM are, in low doses, reversible,

TABLE 2. *Acetylcholine output after different α-blocking agents in the presence and absence of noradrenaline 1 μg/ml.*

Blocking agent	ACh output before noradrenaline (ng/g/min)		% change in ACh output with noradrenaline	
	Resting	Stimulation	Resting	Stimulation
None	29·6±3·9 (8)	114·0±9·6 (8)	−55±6 (4)	−64·2±8·1 (7)
Phenoxybenzamine 10^{-6} g/ml.	16·9±4·0 (4)	206·0±15·3 (6)	−5±3 (3)	−5·5±2 (5)
Phentolamine 10^{-6} g/ml.	31·5 (1)	103·0 (1)	—	—
3×10^{-6} g/ml.	26·5±5·0 (3)	120·0±12·0	—	−10±2 (6)
Ergotamine 10^{-5} g/ml.	18·3 (2)	75·6 (4)	—	−9 (1)

After the exposure (20 min) to α-blocking drugs the strips were washed out with 10 times the volume of the organ bath. Collection periods for the resting acetylcholine output, 15 min, and for the stimulation output, 5 min. Field stimulation, 40 shocks/min. Mean values of output given, ± standard error, with number of experiments in brackets.

TABLE 3. *Acetylcholine output of longitudinal strip of guinea-pig ileum after reserpine treatment*

Condition	Rate of stimulation or resting collection period	No. of shocks	Total control ACh output ng/g per min±S.E. (No. of expts. in brackets)	ACh output ng/g per volley ±S.E.	Treatment (No. of expts. in brackets)	Reduction of ACh output %	*P*
Resting	15 min	—	*a* 36·6±5·1 (5)		(±)-Adrenaline 0·5 μg/ml. (5)	62·0±3·6	<0·01
Stimulated	6/min	30	72·5 (1)	6·0			
Stimulated	20/min	20	279·0 (2)	12·5			
Stimulated	20/min	100	*b* 164·4±2·4 (11)	6·3±0·1	(±)-Adrenaline 0·5 μg/ml. (2)	67·1	<0·01
Stimulated	40/min	40	452·5 (2)	10·4			
Stimulated	40/min	200	*c* 231·0±20·7 (4)	4·8±0·4			
Stimulated	60/min	60	*d* 410·0±51·0 (3)	6·2±0·7			
Stimulated	180/min	180	*e* 500·3±81·6 (3)	2·5±0·5	(±)-Adrenaline 1 μg/ml. (3)	16·5	>0·1
Stimulated	600/min	600	*f* 893·2±75·0 (4)	1·4±0·2			

Increase in output after reserpine compared with corresponding control values in Table 1:

a Resting		+35%	*P*<0·01
b Stimulation	20/min	+106·2%	*P*<0·01
c Stimulation	40/min	+91·2%	*P*<0·01
d Stimulation	60/min	+104%	*P*<0·01
e Stimulation	180/min	+65·9%	*P*<0·05
f Stimulation	600/min	+6·8%	Not significant

Reserpine was given in a daily dose of 1·5 mg/kg subcutaneously on two successive days. The acetylcholine output was measured 6–8 hr after the last injection.

higher doses (BCM 5 μg/ml., phenoxybenzamine 15 μg/ml.) depressed output both at rest and in response to stimulation, and no recovery took place.

Phentolamine had no significant action of its own on acetylcholine output. *Ergotamine* reduced stimulation output detectably, and at a concentration of 10 μg/ml. depressed it transiently by up to 50%. All the three antagonists, phenoxybenzamine, phentolamine and ergotamine abolished the effect of noradrenaline in a concentration of 1 μg/ml. on both resting output and output in response to stimulation (Table 2). In a single experiment, phenoxybenzamine was also found to abolish the depression of output in response to stimulation at 40/min by adrenaline 0.5 μg/ml. These results indicate again that α receptors are involved in this action of the catecholamines.

Effect of noradrenaline depletion on acetylcholine release

Reserpine. Table 3 shows the acetylcholine outputs, at rest or in response to stimulation, by strips from reserpinized animals. The results have been compared, at the foot of the table, with those described for normal animals. Resting output in reserpinized animals was increased from the control mean value of 27.1 ng/g per min to 36.6, a rise of 35%. The output in response to stimulation was also increased, much more so at low frequencies than at high ; thus with stimulation for 1 min at 600/min, the increase in minute output was only 6.8%, an insignificant rise. Figure 7 has been derived from these and the data of Table 1, to show the pattern of output under normal conditions and after reserpine.

In this comparison between outputs at different frequencies, the number of shocks applied varied. But it had earlier been found with whole gut (Paton, 1963) that the volley output dwindled as number of shocks increased, so that, even when the outputs for equal durations of stimulation are compared, the fraction of output due

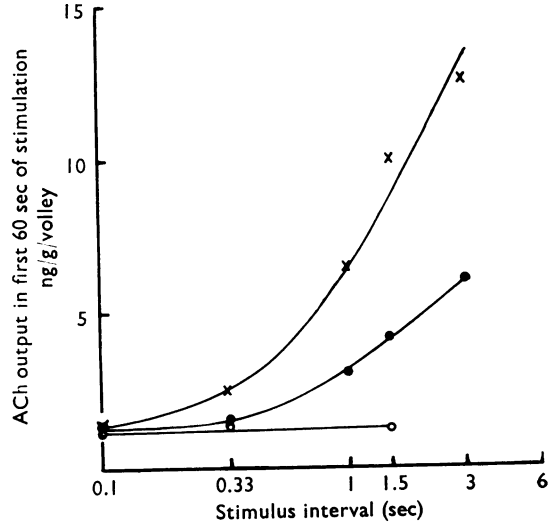

FIG. 7. Comparison of the effects of reserpine pretreatment (×——×) and of noradrenaline 1 μg/ml. (○——○) on average ACh volley output over the first minute of stimulation at various frequencies. Control, ●——●.

to high initial output per volley varies with the frequency of stimulation used. A second comparison was therefore made, measuring output at a fixed frequency, 40/min, for 4, 10, 20, 40 and 200 shocks. The results were analysed in terms of average output per volley over successive periods ; thus if total outputs from 4, 10 and 20 shocks are known, the output attributable to the stimulation by shocks 5–10 and by shocks 11–20 can be calculated. Figure 8 shows the output per volley so obtained in preparations from normal and from reserpinized animals, as well as in preparations treated with noradrenaline 1 μg/ml. In each experimental condition, the output falls with successive shocks from an initial high value to a steady plateau, so that an initial " reserve " can be defined, consisting of the amount by which output in response to the first 40 shocks exceeds the steady state output. With the normal preparation, the reserve amounted to 167 ng/g, and had declined by a half after 8 shocks. With the reserpinized preparation, it amounted to 380 ng/g, and declined by a half in 14 shocks. In the presence of noradrenaline 1 μg/ml., the reserve, 4 ng/g, was only just detectable, and had disappeared after 4 shocks.

After treatment with reserpine, the effect of added noradrenaline and adrenaline was unchanged ; these amines continued to reduce output at rest or at low rates of stimulation by about 60%, and, as before, were much less effective at higher rates of excitation (Table 3). The results quoted were all obtained with preparations from animals treated 6–8 hr earlier with reserpine (see **Methods**). In a single experiment when reserpine was given only 3 hr earlier, the increase in output of acetylcholine at rest or on stimulation was not seen.

Strips of ileum from reserpinized guinea-pigs were occasionally used for assay purposes. They proved to be more sensitive to acetylcholine than normal strips, but because their spontaneous activity was also increased, no advantage for assay purposes resulted.

Guanethidine. In three experiments, pretreatment with guanethidine was found to have the same effect on acetylcholine output as reserpine. Resting output was

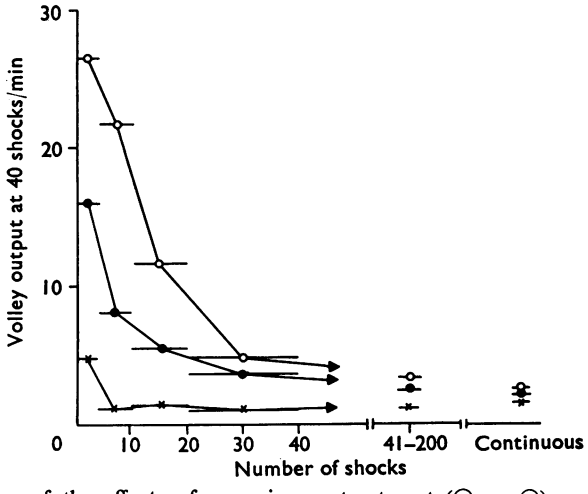

FIG. 8. Comparison of the effects of reserpine pretreatment (○——○) and of noradrenaline 1 μg/ml. (×——×) on acetylcholine output per volley, at a constant stimulation rate of 40/min, for varying number of shocks delivered. Normal, ●——●.

increased about 40% ; the output at 20 or 40/min was increased about 100%, with no change in output at 10/sec. The effect of adrenaline was as usual ; for instance a concentration of 1 μg/ml. reduced the output at 40/min from 131 ng/g per min to 41 ng/g per min.

p-Chlorophenylalanine and p-chloroamphetamine. It could be argued that the effects of reserpine and guanethidine just described are attributable not only to catecholamine depletion, but also to mobilization of hydroxytryptamine (HT) with a rise in free HT (Brodie, 1958). HT is known to stimulate cholinergic nerve endings. Brownlee & Johnson (1965) showed that exposure for 45 sec to HT at a concentration of 500 ng/ml. increased the resting output of acetylcholine from guinea-pig ileum by about 0.1 ng/g ; this suggests that the effect, if present, is relatively small. Opportunity for a further test of this possibility is provided by two drugs which selectively reduce HT content of a tissue. First, *p*-chloro-$\pm$-phenylalanine (*p*-CPA) has been shown to reduce the HT content of blood, brain, colon and spleen in the rat by 60% or more with only slight reduction in noradrenaline content (Koe & Weissman, 1966). To test its action on acetylcholine output, *p*-CPA was given in a subcutaneous dose of either 316 mg/kg 72 hr before test, or 100 mg/kg on each of 3 successive days. These regimes, tested in three experiments, increased output at rest by not more than 27% and the volley output in response to stimulation at low frequency by not more than 35%. Second *p*-chloromethamphetamine (*p*-CMA) is known to lower the HT level of guinea-pig brain (Pletscher *et al.*, 1964) and to possess psychomimetic and sympathomimetic effects lasting 6 hr after administration (Knoll, Vizi & Ecseri, 1966). In a single experiment, given in a subcutaneous dose of 25 mg/kg 16 hr before test, *p*-CMA had no effect on acetylcholine output either at rest or stimulation. These experiments give no support to the view that HT plays a part in reserpine or guanethidine's action on acetylcholine output.

Discussion

The ability of noradrenaline and adrenaline to reduce the acetylcholine output from the nervous tissue of the longitudinal strip of the guinea-pig ileum could result from interference either with synthesis leading to reduced output, or directly with the process of release. A number of observations make it unlikely that the amines interfere with synthesis. They lose their action when tested during high rates of stimulation, although it is in this condition that synthesis is likely to be rate-limiting. With low rates of stimulation the output produced by the first four shocks given after noradrenaline administration is already greatly reduced. This contrasts with the delayed action of synthesis inhibitors. For instance, with hemicholinium it is necessary to wait for hours before signs of transmission failure appear. Further, on prolonged exposure to noradrenaline, its effect does not increase, but if anything diminishes slightly, whereas with hemicholinium, the action increases progressively. Nor do the speed of recovery and the increase in output immediately after withdrawal of the noradrenaline point to transmitter depletion by the amine. It is therefore concluded that the catecholamines interfere in some way with the process of release.

Although it is clear that this action must be on nervous tissue, because that is the source of the acetylcholine (Paton & Zar, 1968), it is still not possible to specify fully the sites at which the amines act. The acetylcholine could originate either

from preganglionic nerve endings or from postganglionic nerve trunks and termination. As Langley (1927) pointed out, there is a great excess of ganglion cells in the myenteric plexus over extrinsic preganglionic fibres, so that a substantial proportion of any acetylcholine output must be postganglionic in origin. In addition, the fact that a single shock delivered to the strip in the presence of hexamethonium or nicotine will release enough acetylcholine to produce a twitch not far short of the maximal response of the longitudinal muscle (Paton & Zar, 1968) shows that the output per volley from the postganglionic nervous tissue must be considerable. Selective excitation of preganglionic fibres, unaccompanied by excitation of postganglionic fibres, has not yet proved possible. Experiments by Emmelin & Muren (1950) on the perfused submaxillary gland indicated that about 75% of the acetylcholine released by preganglionic stimulation came from the postganglionic nervous tissue. With the strip, no more can be said than that a similar figure would be compatible with the evidence available.

The effect of the amines appears to be mediated through the so-called α receptors, as evidenced by the ineffectiveness of isoprenaline, and by the antagonism by phentolamine, phenoxybenzamine and ergotamine. The action of phenylephrine is not fully clear, because it was active against resting output, but compared with noradrenaline was proportionally less active against the output in response to stimulation. The higher potency of adrenaline compared with noradrenaline could be attributed to the lower rate of its uptake by tissues adjacent to the site of action (compare Iversen, 1967). This is supported by the finding that its action was more persistent.

It is known that noradrenaline and adrenaline increase potassium permeability by an action on α receptors (Jenkinson & Morton, 1967a, b). If this occurred at a cell membrane the potential of which fell short of the potassium equilibrium potential, the membrane potential would increase. Hyperpolarization by adrenaline has in fact been described for mammalian C fibres (Goffart & Holmes, 1962) and for sympathetic ganglia (Lundberg, 1952; De Groat & Volle, 1966) and this hyperpolarization is mediated via the α receptor (De Groat & Volle, 1966). The reduction in resting output could thus at least in part be due to hyperpolarization of the nerve endings; the amine-resistant fraction of the output would then represent the frequency of quantal release associated with a membrane potential at the potassium equilibrium point. For the output in response to stimulation, it is suggested that the increase in potassium permeability leads to an abbreviation of the action potential, with perhaps an enhanced positive after-potential, leading again to a reduction in quantal release. The failure of amine action at higher frequencies would then be due to accumulation of potassium in the periaxonal spaces, lowering the potassium equilibrium potential. Another possibility would be that the amines reduce the permeability to some ion. Interference with sodium entry, however, is excluded, for Paton & Thompson (unpublished) found that, even with desheathed frog nerve, exposed to reduced sodium concentration in the bathing fluid and stimulated at high frequencies, noradrenaline at 1 mg/ml. had no effect on nerve conduction, and adrenaline a detectable effect only at 300 μg/ml.

From the depressant action of the catecholamines on acetylcholine output, it can be concluded that *in vivo* circulating catecholamines control neurogenic intestinal activity. But the increased acetylcholine output, at rest as well as in response to

stimulation, which occurred after catecholamine depletion by reserpine and guanethidine, suggests a local control of cholinergic function as well. The possibility arises that the reserpinized animals happened, by chance, to be different from the normals. But at high rates of excitation, the output per volley was indistinguishable in normal and catecholamine-treated strips, as well as in strips from animals which had been treated with reserpine or guanethidine. It is therefore concluded that the increases in acetylcholine output after catecholamine depletion truly represent the partial removal of an adrenergic restraint. This conclusion provides complete functional corroboration of the anatomical demonstration by Norberg (1964), Jacobowitz (1965) and Norberg and Sjöqvist (1966) that the adrenergic fibres embrace the ganglia of the myenteric plexus, without directly innervating the smooth muscle. There is some discussion as to how far the effects of sympathetic stimulation on the alimentary tract are mediated directly on smooth muscle as well as indirectly through nervous tissue (compare Gershon, 1967 ; Jansson & Martinson, 1966). There seems no logical reason why, in particular cases, released amine should not spill over, in some degree, from exclusively neural surroundings so as to reach smooth muscle and exert a direct influence ; and it would remain, in each case, to test whether or not this was occurring.

Functionally, the direct interaction of the sympathetic with the parasympathetic nervous system must be of considerable importance, and may explain some otherwise puzzling phenomena. Thus the intensity of diarrhoea of animals under reserpine may seem out of proportion to the expected effect of merely removing a sympathetic inhibition of smooth muscle ; but if at the same time a direct restraint on parasympathetic nervous activity is removed, so that parasympathetic action is now not only unopposed at the effector level but itself enhanced, the signs of parasympathetic action must become very striking. Indeed, the role of direct inhibitor action of the sympathetic on certain effectors is now being seriously questioned (for example, Jansson & Martinson, 1966) and such doubt must extend to any doubly innervated organ. Sympathetic control of acetylcholine output can be viewed as a kind of presynaptic inhibition, and when compared with an antagonism at the effector level, offers the physiological advantage of economy in transmitter release.

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(*Received December* 20, 1967)