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AN ANALYSIS OF THE DIRECT AND INDIRECT ACTIONS OF DRUGS ON THE ISOLATED GUINEA-PIG ILEUM

BY

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The responses of the isolated guinea-pig ileum to coaxial stimulation of its nerves, to histamine, acetylcholine, bradykinin, nicotine, tetramethylammonium, 1,1-dimethyl-4-phenyl-piperazinium iodide and 5-hydroxytryptamine were studied, before and during anoxia, cooling, or exposure to hyoscine, phenoxybenzamine hydrochloride, morphine or hexamethonium. Dose ratios were used to determine the amount of block induced by these procedures. With the response to coaxial nerve stimulation as an indication of the excitability of the nervous tissue, it was found that anoxia or cooling abolished the response to single shocks. Under these conditions the response of the ileum to histamine, acetylcholine and bradykinin was hardly affected, indicating a direct action of these substances on the muscle fibres. The effects of nicotine, tetramethylammonium, dimethylphenylpiperazinium and 5-hydroxytryptamine were reduced to various degrees, and we have concluded that their main actions are indirect, through stimulation of cholinergic nerve fibres. When these indirect actions were prevented, increasing the dose revealed a direct action, a larger increase in dose being required for 5-hydroxytryptamine and dimethylphenylpiperazinium than for tetramethylammonium and nicotine. Exposure of the ileum to hyoscine and phenoxybenzamine showed that these direct actions of nicotine and tetramethylammonium were not only on acetylcholine receptors but also on receptors insensitive to hyoscine but sensitive to phenoxybenzamine. The main action of 5-hydroxytryptamine was on nervous elements, yet treatment of the ileum with phenoxybenzamine gave a higher dose ratio for 5-hydroxytryptamine than did treatment with morphine. The meaning of this result is discussed in relation to the general belief that receptors sensitive to morphine are in nervous tissue and receptors sensitive to phenoxybenzamine are in smooth muscle. We have concluded that morphine is only a partial antagonist of 5-hydroxytryptamine receptors in nervous tissue and that phenoxybenzamine antagonizes more 5-hydroxytryptamine receptors than those in smooth muscle.

Drugs can contract smooth muscle of isolated preparations either by a direct action on the muscle fibres or indirectly through excitation of the intrinsic network of nerves. Most methods used to distinguish between these two kinds of action have involved the inactivation of nervous tissue in such a way that the responses of the muscle itself are as little altered as possible. These methods include making the tissue anoxic (Gross & Clark, 1923 ; Garry, 1928 ; West, Hadden & Farah, 1951 ; Blair & Clark, 1956), cooling the tissue (Blair & Clark, 1956 ; Innes, Kosterlitz &

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Robinson, 1957), the use of ganglion-blocking agents (Collins, 1948 ; Feldberg, 1951), of drugs like atropine (Gaddum & Hameed, 1954 ; Garven, 1956 ; Gaddum & Picarelli, 1957 ; Kosterlitz & Robinson, 1958) and of botulinum toxin (Ambache & Lessin, 1955). Much of this work was with the guinea-pig ileum, and the conclusions have been that the effects of 5-hydroxytryptamine, nicotine and 1-1-dimethyl-4-phenylpiperazinium iodide are mediated, wholly or partly, through the excitation of cholinergic nerve fibres, whereas histamine and acetylcholine act mainly through the direct excitation of smooth muscle. None of these experiments provided direct evidence that the nervous elements had been selectively and completely inactivated. This evidence can be obtained by the technique of Paton (1955) in which the post-ganglionic nerve fibres in the ileum are selectively stimulated through coaxial electrodes. In the present experiments the reactions of the guinea-pig ileum to electrical excitation of the nerves and to drugs have been compared before and after exposing the ileum to procedures which modified preferentially either the nervous activity or the response of the smooth muscle fibres to the nervous transmitter.

METHODS

Pieces 4 to 6 cm long were cut from the guinea-pig ileum about 5 cm above the ileo-caecal valve. A piece of ileum was suspended in a 20 ml. bath containing Krebs solution at 37° C, gassed with 95% O_2 and 5% CO_2. The ileum was stimulated electrically through coaxial electrodes (Paton, 1955) with supramaximal (usually about 30 V) rectangular wave pulses of 1 msec duration at a rate of 6 shocks/min. Repetitive stimulation (5 shocks/sec for 5 to 15 sec) was used to produce complete tetani.

In some experiments the ileum was taken, together with a piece of mesentery containing an artery and its periarterial network of nerves supplying the gut. After the ileum had been set up for coaxial stimulation, the mesentery was pulled into a tube containing two electrodes connected to a second stimulator. In this way the effects of periarterial nerve stimulation could be superimposed on contractions of the ileum produced by coaxial stimulation. The contractions of the ileum were recorded on a smoked drum either with an isotonic lever or with a pendulum lever (Paton, 1957a and b), each giving a magnification of 8:1. The initial load on the tissue was 2.5 g. In some experiments an isometric system recorded the responses of the ileum. This system consisted of a moving anode transducer (RCA 5734) as one part of a balanced bridge, the output of which was amplified and fed into a "Record" moving coil milliammeter.

A series of responses was obtained to coaxial stimulation and to drugs in doses chosen to give contractions of similar height, namely 50 to 75% of the maximal. The drugs were usually injected into the bath in 0.2 to 0.5 ml. of saline ; very large doses were injected in volumes of up to 1.0 ml.

In order to deprive the ileum of oxygen it was bathed in Krebs solution made with water which had been boiled for 20 min, and the solution was bubbled with 95% N_2 and 5% CO_2. A plastic cover was placed over the top of the bath to minimize diffusion of oxygen from the air. Oxygen lack abolished in 5 to 15 min the effect of electrical stimulation, but the responses tended to return on washing out the bath (see Figs. 1 and 2). For this reason no drug was tested during the period of anoxia until the responses to the maximum electrical stimulation obtainable (90 to 100 V) had been almost or completely abolished. The contractions of the ileum in response to histamine were used as an index of the responsiveness of the muscle to direct stimulation, and other drugs were tested only when the response to histamine was constant. The ileum was deprived of oxygen for 1.5 to 2.0 hr ; the oxygen supply was then restored and the effects of the same drugs and of coaxial stimulation were retested. In similar experiments the effects of antagonists were investigated, both alone and during anoxia.

The responses to drugs and to coaxial stimulation were also compared at 37° C and at lower temperatures. At any given temperature the preparation was allowed to stabilize for 30 min.

The effects of anoxia, cooling or antagonists were expressed in terms of the dose ratio (Gaddum, Hameed, Hathway & Stephens, 1955). This is the ratio of equi-active doses of the stimulant drug before and after the change of conditions. For calculating the dose ratios the peak height of the contraction was measured and not the plateau which sometimes followed it.

Doses of the following drugs are expressed as weights of bases: acetylcholine perchlorate, histamine acid phosphate, 5-hydroxytryptamine creatinine sulphate, nicotine hydrogen tartrate, tetramethylammonium bromide. The following are expressed as weight of salt: 1,1-dimethyl-4-phenylpiperazinium iodide, hexamethonium bromide, morphine sulphate, phenoxybenzamine hydrochloride (Dibenzyline), methysergide, bretylium tosylate and hyoscine hydrobromide. Bradykinin was left in contact with the ileum for 1 min, and all other drugs for 30 sec unless otherwise stated.

RESULTS

Effects of anoxia

Fig. 1, A, shows the responses of a piece of ileum to repetitive stimulation (5 shocks/sec), to a succession of single shocks, to histamine, tetramethylammonium, bradykinin and to 5-hydroxytryptamine. The ileum was then deprived of oxygen and its response to single shocks, after an initial slight potentiation (also seen in several other experiments), was gradually abolished (Fig. 1, B). The first dose of histamine produced a contraction similar in height to that before anoxia. After the bath had been washed out the ileum responded once more to single shocks but only for about 3 min. The next dose of histamine was then given. In this and in all other experiments the responses to histamine of the ileum during the first 20 to 40 min of anoxia were irregular; because of this, these responses were not used for calculating the dose ratio. After this period the effects of histamine were much more regular (Fig. 1, C).

The responses to other drugs were considerably reduced by anoxia (Fig. 1, B). During the 30 min interval between sections B and C of Fig. 1, the drugs were given in higher doses to try to match the responses shown in Fig. 1, A. It was found that 640 ng of bradykinin was as active as the original dose of 400 ng, i.e., the dose ratio was 1.6. Fig. 1, C, shows attempts to match contractions due to the other substances. Whereas the dose ratio for 5-hydroxytryptamine was 20 (i.e., the dose had to be increased twenty-fold to produce the same contraction as in the control period), the dose ratio for histamine was less than 1 (i.e., the response to 200 ng of histamine was actually potentiated) and for tetramethylammonium was between 1 and 1.7. Fig. 1, C, also shows that the response of the ileum to repetitive stimulation at 5 shocks/sec was abolished by anoxia.

In a similar experiment (Fig. 2) histamine, bradykinin and 5-hydroxytryptamine were used. Fig. 2, B, is a tracing made 40 min after the ileum had been deprived of oxygen. The dose ratio for 5-hydroxytryptamine was more than 62, for histamine 2 and for bradykinin 3. With restoration of the oxygen supply, the original sensitivity of the ileum to the three substances gradually returned (Fig. 2, C); this showed that the reduction in response to 5-hydroxytryptamine during anoxia was not due to tachyphylaxis.

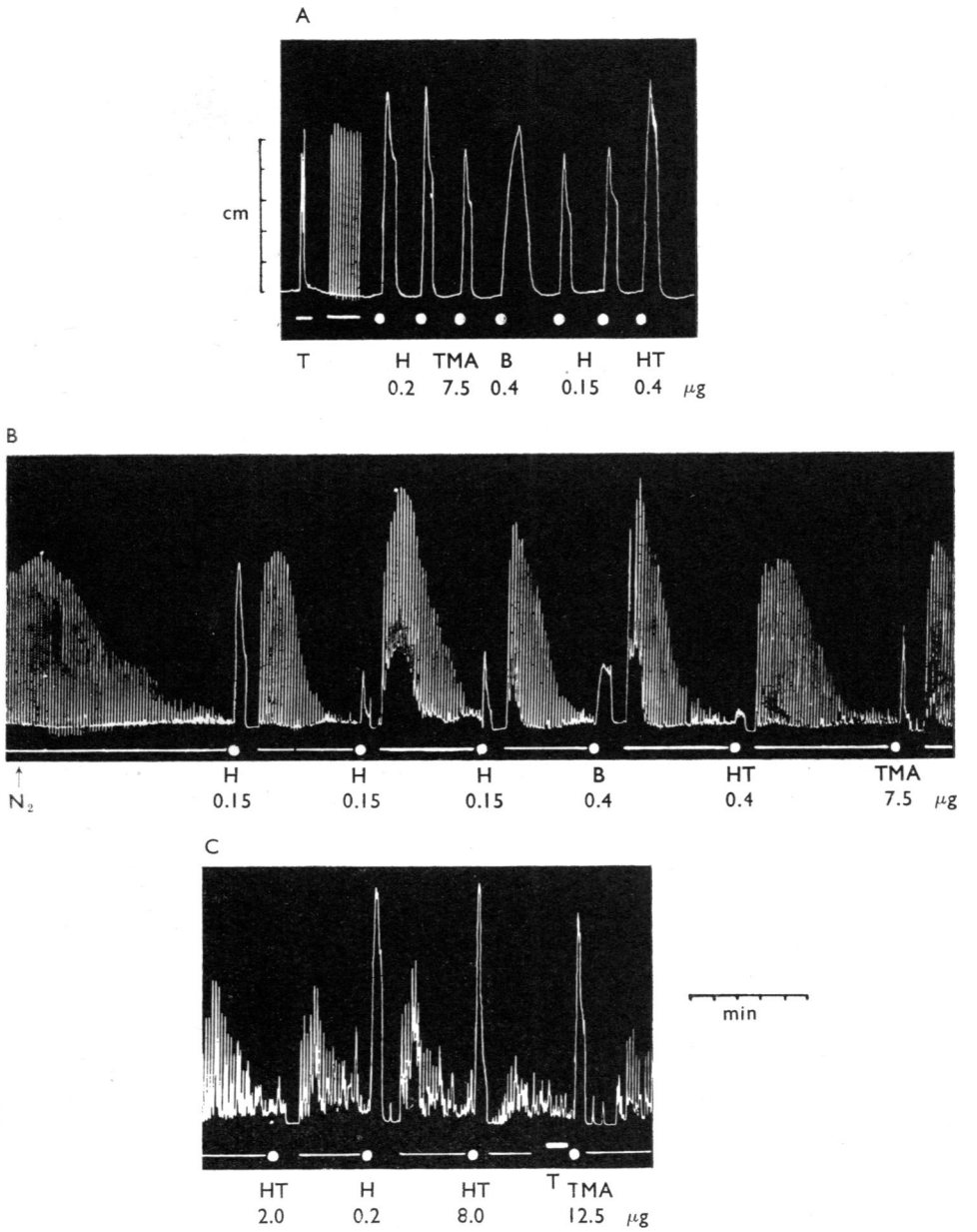

Fig. 1. Guinea-pig ileum. Responses to coaxial stimulation and to drugs, A before, and B and C during anoxia. Electrical stimulation indicated by bars beneath tracing; single shocks 6 per min, and tetanus (T) 5 shocks/sec. Responses are to histamine (H), tetramethylammonium (TMA), bradykinin (B) and 5-hydroxytryptamine (HT). Doses are given as μg in a 20 ml. bath. Start of anoxia (N_2) indicated by arrow. B shows reduction in response to drugs during first 35 min of anoxia and abolition of response to single shocks. C shows that increased doses are required to restore response after 1 hr of anoxia. Time tracing in min. For dose ratios, see text.

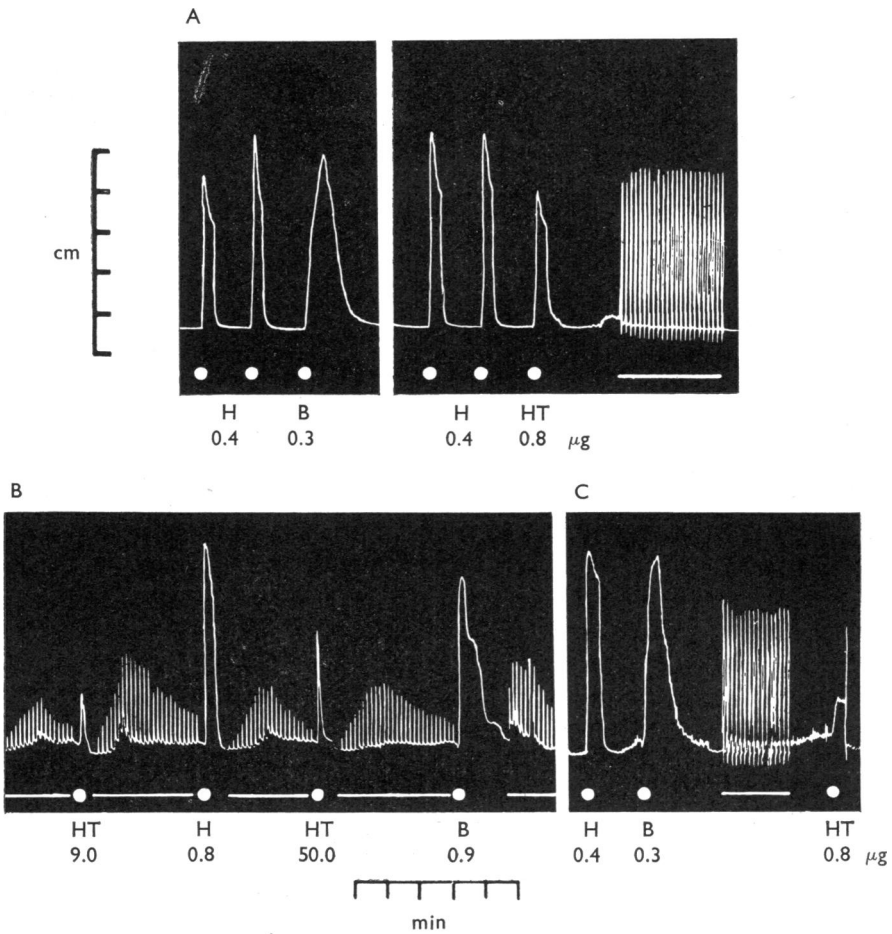

Fig. 2. Guinea-pig ileum. Records as in Fig. 1. A is a record made before anoxia, B is after 90 min of anoxia, and C is immediately after restoration of the oxygen supply. For dose ratios see experiment 6 in Table 1.

Table 1 summarizes the results of fifteen experiments. The contractions produced by histamine were least affected by anoxia and dose ratios varied from less than 1 to 2. The dose ratios were similar for acetylcholine (from less than 1 to 3), bradykinin (from 1 to 3), and tetramethylammonium (from 1.3 to 3). In any single experiment, the response to 5-hydroxytryptamine (dose ratios from 8 to more than 500) was more affected by lack of oxygen than the response to any other substance, with the possible exception of dimethylphenylpiperazinium (dose ratios from 5 to 60). Fig. 3 shows dose/response curves for 5-hydroxytryptamine and nicotine before and during anoxia. The dose ratio for 5-hydroxytryptamine is greater than that for nicotine throughout the graph.

Gaddum & Picarelli (1957) suggested that in the guinea-pig ileum 5-hydroxytryptamine acts on two types of tryptamine receptor: the M receptors, probably in

TABLE 1

INCREASE IN DOSE REQUIRED DURING ANOXIA TO GIVE RESPONSE OF THE GUINEA-PIG ILEUM EQUAL TO THAT OBTAINED UNDER CONTROL CONDITIONS

Values are dose ratios (dose during anoxia divided by control dose)

P=response to same dose potentiated. Expts. 1 to 8 were with an isotonic lever, expts. 9 to 14 were with a pendulum lever, and expt. 15 was with an isometric lever. TMA=Tetramethylammonium. DMPP=Dimethylphenylpiperazinium. 5-HT=5-Hydroxytryptamine

Expt.	Histamine	Acetylcholine	Bradykinin	Nicotine	TMA	DMPP	5-HT
1	1·3	1·0	2·0	>4·5	—	—	>300
2	P	P	—	5·0	—	—	250
3	P	—	1·0	—	—	—	>8·0
4	P	—	1·7	—	—	—	50
5	P	P	—	4·0	—	—	14
6	2·0	—	3·0	—	—	—	>60
7	P	—	1·6	—	1·3	—	20
8	2·0	2·0	—	—	—	5·0	10
9	1·5	3·0	—	—	3·0	—	33
10	P	1·5	—	1·8	—	—	10
11	1·0	—	—	—	—	60	>500
12	1·0	—	—	9·0	—	—	25
13	2·0	2·0	—	—	3·0	—	110
14	1·5	—	—	—	—	>20	25
15	—	P	—	—	—	—	140

nervous tissue, the effects of which are blocked by morphine, and the D receptors in smooth muscle which are blocked by phenoxybenzamine. The effects of anoxia on these M and D receptors were therefore investigated.

Effects of morphine and anoxia

Kosterlitz & Robinson (1958) showed that concentrations of morphine as low as 5×10^{-8} g/ml. inhibit the responses of the guinea-pig ileum to 5-hydroxytryptamine and nicotine, and that increasing the concentration of morphine 400-fold did not increase this antagonism. Other workers, using similar concentrations of morphine

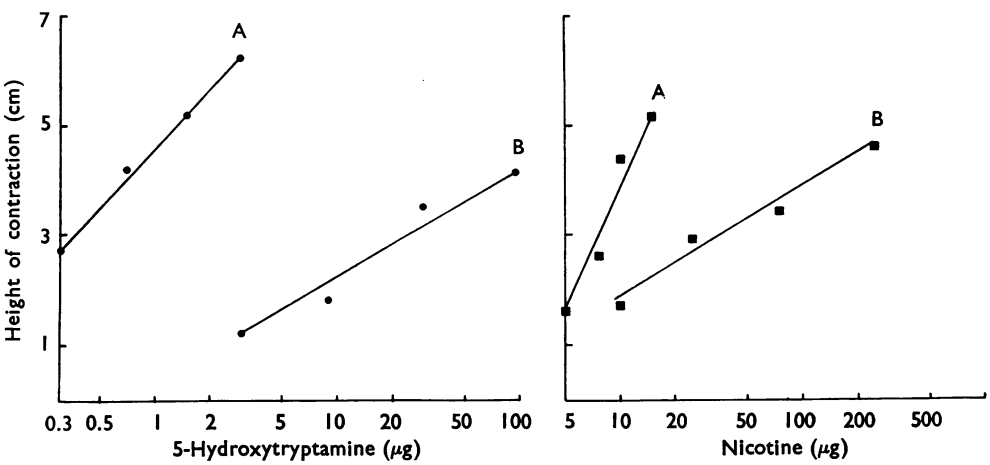

Fig. 3. Guinea-pig ileum. Log dose/response curves with 5-hydroxytryptamine (left) and nicotine (right); A before and B during anoxia. Auxotonic lever.

TABLE 2

DOSE RATIOS BEFORE AND IN THE PRESENCE OF MORPHINE, AND BEFORE AND IN THE PRESENCE OF MORPHINE TOGETHER WITH ANOXIA

TMA=Tetramethylammonium; DMPP=dimethylphenylpiperazinium; 5-HT=5-hydroxytryptamine; P=response potentiated

	Dose in presence of morphine / Control dose					Dose in presence of morphine+anoxia / Control dose				
Expt.	Histamine	Nicotine	TMA	DMPP	5-HT	Histamine	Nicotine	TMA	DMPP	5-HT
15	1	15	—	—	2·5	1·5	<15	—	—	66
16	1	13	—	—	10	1·5	<13	—	—	266
17	1	—	4	—	10	<2·0	—	<6	—	400
18	1	—	4	—	5	1·0	—	2·5	—	>22
19	1	—	—	12	2·3	1·5	—	—	>45	10
20	1	—	—	35	13	2·0	—	—	>45	83
21	P	10	—	—	—	—	—	—	—	—
22	1	15	—	—	—	—	—	—	—	—

(Gaddum & Picarelli, 1957; Barlow & Khan, 1959), also obtained maximum inhibition. In the present experiments we used concentrations of morphine between 10^{-7} and 5×10^{-6} g/ml.; the lower concentration was just as effective as the higher. Table 2 shows the antagonism by morphine of the responses to histamine, 5-hydroxytryptamine, nicotine, tetramethylammonium and dimethylphenylpiperazinium. When the ileum treated with morphine was deprived of oxygen, the dose ratios for histamine and tetramethylammonium were hardly increased (Fig. 4), but were considerably

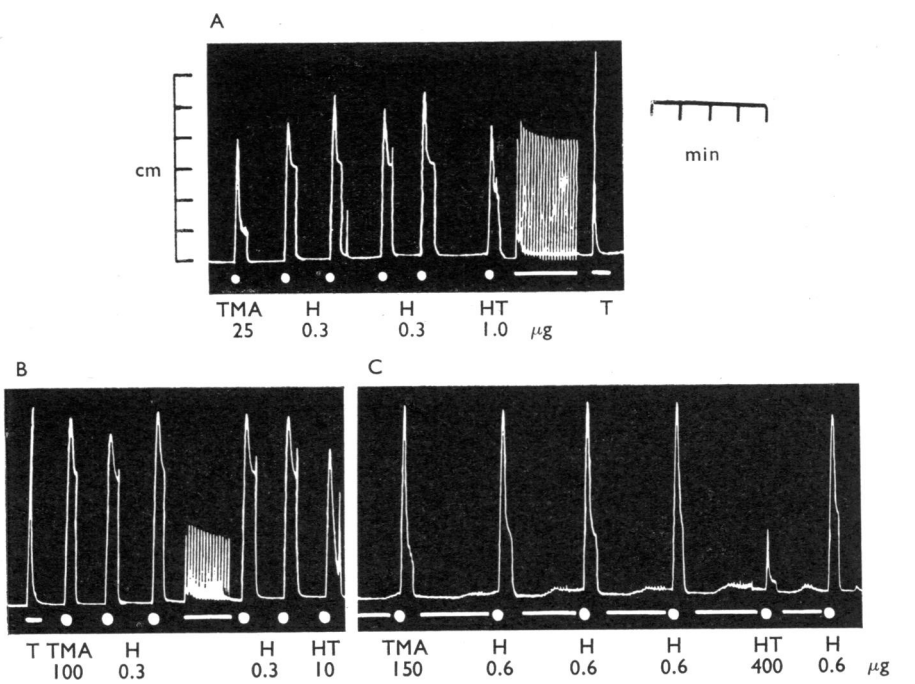

Fig. 4. Guinea-pig ileum. Records as in Fig. 1. A, before; B, in the presence of morphine (10^{-7} g/ml.); and C, during anoxia in addition to morphine. For dose ratios see experiment 17, Table 2.

increased for 5-hydroxytryptamine and dimethylphenylpiperazinium. Thus, the dose ratio was further increased by a factor of less than 2 for histamine, but by up to 40-fold for 5-hydroxytryptamine. Table 2 shows that the increase in dose ratio for 5-hydroxytryptamine varied greatly from one experiment to another. On the other hand, the dose ratios for nicotine were *decreased* when the morphine-treated ileum was deprived of oxygen. It was possible that in the presence of morphine the high doses of nicotine required to stimulate the ileum were also exciting an adrenergic mechanism which reduced the size of the contraction, but that this reduction was abolished by anoxia. The following experiment was carried out to test this possibility.

Effects of morphine and bretylium

The ileum was set up for periarterial nerve as well as for coaxial stimulation. When the periarterial nerves were stimulated at frequencies of 1 to 25 shocks/sec the twitch induced by coaxial stimulation was diminished (Fig. 5, A); this diminution was used as an indication of sympathetic nervous activity. In the presence of morphine (10^{-7} g/ml.) the twitch in response to coaxial stimulation was reduced, as was the effect of nicotine (Table 2, experiment 21, dose ratio 10). Periarterial nerve stimulation was still effective (Fig. 5, B). Bretylium (10^{-5} g/ml.) was added to the bath for 30 min; the effects of periarterial nerve stimulation were then gradually reduced, showing that the drug was inhibiting the response to sympathetic nervous activity. After washing out the bretylium, the responses to periarterial nerve stimulation remained greatly reduced (Fig. 5, C). At this time the dose ratio for nicotine was between 1.6 and 4, which was much less than that with morphine alone. Contractility of the muscle fibres was unaffected by treatment with morphine and bretylium, since the responses to histamine were slightly potentiated by these drugs. We can conclude that in the presence of morphine the contraction produced by nicotine was diminished by the simultaneous excitation of sympathetic nerves.

Effects of phenoxybenzamine and anoxia

Treatment of the ileum with phenoxybenzamine (10^{-7} g/ml.) more or less completely destroys the D receptors (Gaddum & Picarelli, 1957). When this concentration of phenoxybenzamine was left in contact with the ileum for 30 min and then washed out, the response to repetitive stimulation was reduced by about 10% and the response to single maximal shocks by 40 to 60%. Fig. 6 shows this effect and also that the phenoxybenzamine reduced the responses to all the drugs; the dose ratios were 66 for histamine, 16 for 5-hydroxytryptamine, 3.3 for acetylcholine and 1.3 for nicotine. When this ileum was deprived of oxygen (Fig. 6, C) the dose ratios for histamine and acetylcholine remained the same whereas those for nicotine and 5-hydroxytryptamine were considerably increased, as shown in Table 3 (experiment 23). Table 3 also shows that the dose ratios for tetramethylammonium and dimethylphenylpiperazinium, like those for nicotine and 5-hydroxytryptamine, were further increased by depriving the phenoxybenzamine-treated ileum of oxygen.

Since the effects of acetylcholine were also antagonized by phenoxybenzamine (Table 3), an attempt was made to protect the acetylcholine receptors from this

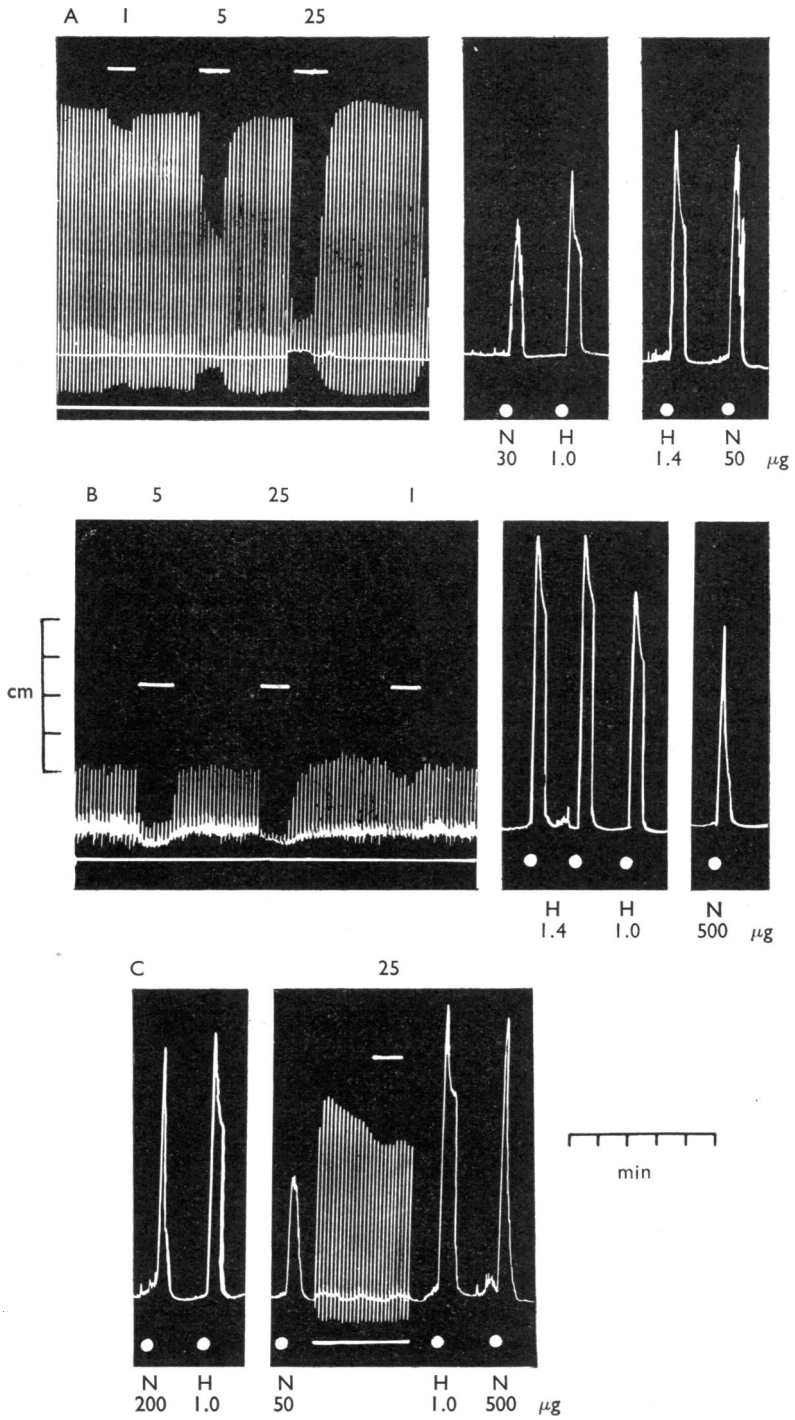

Fig. 5. Guinea-pig ileum. Records as in Fig. 1. The bars above tracings (1, 5 and 25) represent periarterial nerve stimulation at 1, 5 and 25 shocks/sec. A before, B in presence of morphine (2×10^{-7} g/ml.) and C in the presence of morphine and after bretylium (10^{-5} g/ml.) had been in the bath for 30 min and then washed out. For dose ratios, see text.

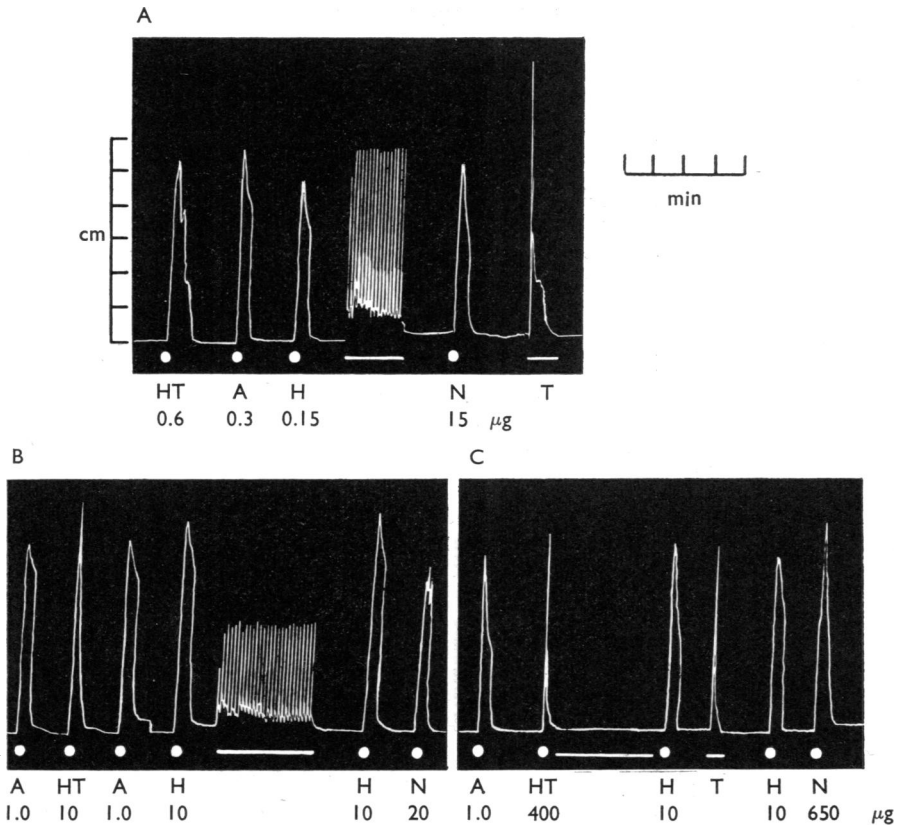

Fig. 6. Guinea-pig ileum. Records as in Fig. 1. A, before; B, after phenoxybenzamine (10^{-7} g/ml.); and C, during anoxia and after phenoxybenzamine. A=acetylcholine and N=nicotine. For dose ratios, see experiment 23, Table 3.

antagonism, using the technique of Furchgott (1954). Two adjacent pieces of ileum from a guinea-pig were set up under similar conditions and dose/response curves obtained for acetylcholine and 5-hydroxytryptamine. Acetylcholine (1 to 3×10^{-6} g/ml.) was added to one bath to "protect" the acetylcholine receptors and left for 3 min. Phenoxybenzamine (1×10^{-7} g/ml.) was then added to both baths. After 30 min it was washed out and the two pieces of ileum were allowed to recover for 30 min, during which time the baths were washed out frequently. Dose/response curves were again obtained for acetylcholine and 5-hydroxytryptamine. Treatment with phenoxybenzamine reduced the sensitivity of both preparations to acetylcholine and 5-hydroxytryptamine, and no significant difference was found between the responses of the "acetylcholine-protected" preparation and the control.

Effects of methysergide

Since phenoxybenzamine antagonized acetylcholine as well as 5-hydroxytryptamine, methysergide was used to find out whether it would antagonize 5-hydroxytryptamine without affecting the response to acetylcholine.

TABLE 3

DOSE RATIOS BEFORE AND AFTER EXPOSURE TO PHENOXYBENZAMINE (10^{-7} G/ML.), AND BEFORE AND AFTER EXPOSURE TO PHENOXYBENZAMINE TOGETHER WITH ANOXIA

Ach=Acetylcholine; DMPP=dimethylphenylpiperazine; TMA=tetramethylammonium; 5-HT=5-hydroxytryptamine

	Dose after phenoxybenzamine / Control dose						Dose after phenoxybenzamine+anoxia / Control dose					
Expt.	Histamine	Ach	Nicotine	DMPP	TMA	5-HT	Histamine	Ach	Nicotine	DMPP	TMA	5-HT
23	66	3·3	1·3	—	—	16	66	3·3	<43	—	—	666
24	20	2·0	1·3	—	—	16	60	2·0	<66	—	—	>4,000
25	50	5·0	—	—	2·0	17	100	6·6	—	—	20	>830
26	37	6·0	—	—	1·3	17	37	6·0	—	—	8·3	<444
27	50	16·0	—	<1·6	—	20	100	20·0	—	66	—	1,500
28	33	4·0	—	1·0	—	8	50	5·0	—	>198	—	1,000
29	—	3·4	—	—	—	12	—	—	—	—	—	—
30	—	6·0	—	—	—	32	—	—	—	—	—	—
31	—	3·0	—	—	—	9	—	—	—	—	—	—

After treatment of the ileum with methysergide (10^{-6} g/ml.) for 1 hr, the dose ratio for acetylcholine was less than 1 and that for 5-hydroxytryptamine was 2. However, the dose/response curves for 5-hydroxytryptamine before and in the presence of methysergide tended to converge, showing that there was little or no antagonism with the higher doses of 5-hydroxytryptamine.

Effects of hyoscine and phenoxybenzamine

Gaddum & Picarelli (1957) considered that atropine had an effect similar to morphine on the actions of histamine, 5-hydroxytryptamine and nicotine on the ileum. In our experiments hyoscine, a more specific antagonist of acetylcholine than atropine (Paton & Rosales, unpublished), was used alone and together with phenoxybenzamine to affect the responses of the ileum to coaxial stimulation and to drugs. Hyoscine (10^{-7} g/ml.) immediately abolished the responses to single shocks. The response to repetitive stimulation at maximum voltage was reduced by 30 to 80%. The responses to histamine and bradykinin were unaffected. The dose ratio for acetylcholine was from 500 to 1,000, for 5-hydroxytryptamine from 4 to 12.5, for tetramethylammonium 10, for nicotine 50 and for dimethylphenylpiperazinium 90 (Table 4).

Phenoxybenzamine (10^{-7} g/ml.) was then added to the bath for 30 min and washed out. The response of the ileum to repetitive stimulation was unchanged by phenoxybenzamine in two experiments and reduced in two. The responses to acetylcholine and bradykinin were hardly affected (for dose ratios, see Table 4). The dose ratios were increased greatly for histamine (60- to 100-fold) and for 5-hydroxytryptamine (130- to 2,000-fold). There was a small increase in dose ratio for dimethylphenylpiperazinium and nicotine (2- to 5-fold), and a rather larger one for tetramethylammonium (10-fold).

Effects of 5-hydroxytryptamine together with acetylcholine

In experiments where no antagonist was used, the M and D receptors for 5-hydroxytryptamine were presumably excited simultaneously so that the resultant contractions were a combination of the direct effects on D receptors of 5-hydroxytryptamine and of the direct effects of acetylcholine released by the actions of 5-hydroxytryptamine on M receptors. If this simultaneous excitation of two different types of receptor led to more than a simple additive effect, i.e., a synergistic one, then dose ratios with morphine or phenoxybenzamine would be complicated.

To test this possibility, different doses of acetylcholine and 5-hydroxytryptamine were first given separately and the sizes of the contractions measured. These contractions were then compared with those produced by various combinations of the doses of acetylcholine and 5-hydroxytryptamine. This whole procedure was then repeated in the presence of morphine (10^{-7} g/ml.).

To test the effect of 5-hydroxytryptamine in combination with intrinsically released acetylcholine, a number of different doses of 5-hydroxytryptamine were given at the same time as coaxial stimulation, using voltages from sub-threshold to supramaximal strength, and frequencies from 6 to 10 shocks/sec. In most experiments, the combined effects of 5-hydroxytryptamine with added or released acetylcholine

TABLE 4

DOSE RATIOS BEFORE AND IN PRESENCE OF HYOSCINE (10^{-7} G/ML.), AND BEFORE AND IN PRESENCE OF HYOSCINE AFTER EXPOSURE TO PHENOXYBENZAMINE (10^{-7} G/ML.)

Hist=Histamine; Ach=acetylcholine; Brad=bradykinin; Nic=nicotine; DMPP=dimethylphenylpiperazinium; TMA=tetramethylammonium; 5-HT=5-hydroxytryptamine; P=response potentiated

		Dose in presence of hyoscine / Control dose						Dose in presence of hyoscine and after phenoxybenzamine / Control dose						
Expt.	Hist	Ach	Brad	Nic	DMPP	TMA	5-HT	Hist	Ach	Brad	Nic	DMPP	TMA	5-HT
32	1·0	500	1	50	—	—	11	60	375	1·0	200	—	—	>5,000
33	1·0	666	1	50	—	—	12	100	666	P	250	—	—	>1,000
34	P	500	1·2	—	90	—	7·5	33	333	1·0	—	>150	—	1,000
35	1·0	1,000	1·0	—	—	10	4·0	60	3,000	2·0	—	—	100	8,000

were no greater than the larger of the contractions produced by the same doses of the two stimulants given separately. In a few instances, the combination of 5-hydroxytryptamine and either added or released acetylcholine led to a contraction, the height of which was equal to the sum of the heights of the contractions produced by the same doses of the two stimulants given separately. In none of these experiments, either before or in the presence of morphine, was any synergism observed between the actions of 5-hydroxytryptamine and acetylcholine.

Effects of cooling

The responses of the guinea-pig ileum were slower at temperatures below 37° C; therefore, the frequency of single shocks was reduced from 6 to 1 or 2 shocks/min. The responses to all drugs had an increased latent period (usually about 30 sec) and the drugs had to be left in contact with the tissue for up to 3.5 min to obtain a maximum effect. At 20° C, the only substance causing a smaller effect than at 37° C was 5-hydroxytryptamine; the effects of histamine, acetylcholine and bradykinin were potentiated, and the effects of nicotine, tetramethylammonium and dimethylphenylpiperazinium were unaltered. At 15° C (Fig. 7) the response to single shocks was abolished, except in one experiment in which the temperature had to be reduced to 12° C before this occurred. At 15° C the effects of histamine, acetylcholine and bradykinin were potentiated and nicotine, tetramethylammonium

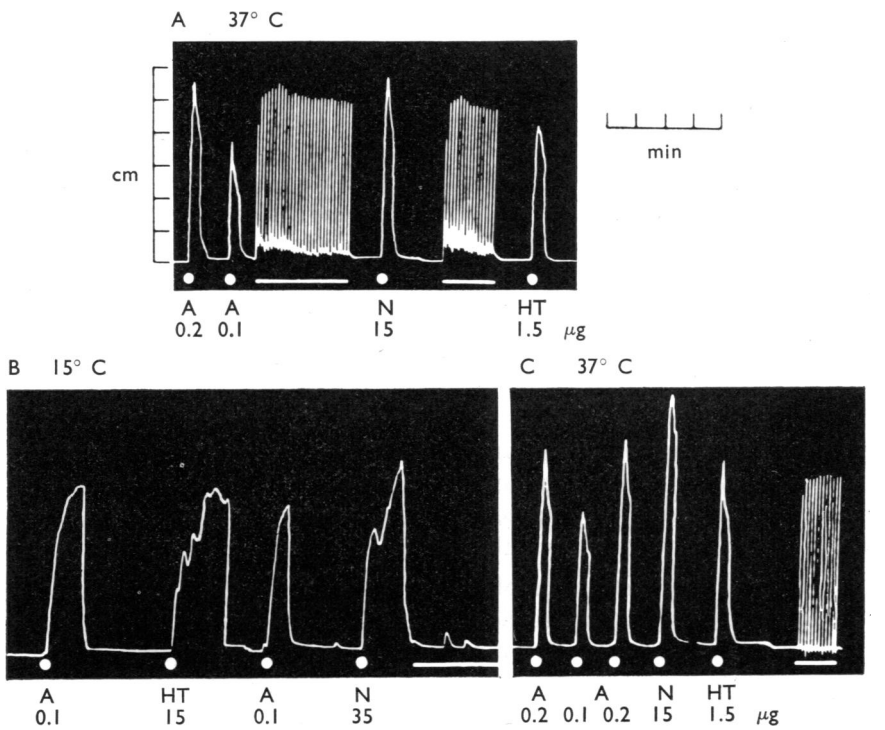

Fig. 7. Guinea-pig ileum. Records as in Figs. 1 and 6. A, at 37° C; B, at 15° C; and C, at 37° C. For dose ratios, see experiment 39, Table 5.

and dimethylphenylpiperazinium required a small increase in the dose to restore the response. 5-Hydroxytryptamine was most affected by the lower temperature, the dose ratios varying from over 7 to less than 100 (Table 5).

TABLE 5

EFFECTS OF COOLING. RATIOS OF DOSES NEEDED TO GIVE EQUAL CONTRACTIO NS AT 15° AND 37° C

*=Temperature, 12° C; P=response potentiated. DMPP=Dimethylphenylpiperazinium; TMA=tetramethylammonium; 5-HT=5-hydroxytryptamine

Expt.	Hist-amine	Acetyl-choline	Brady-kinin	Nicotine	DMPP	TMA	5-HT
36	P	P	—	—	2	—	33
37	P	P	P	—	1·3	—	>7
38	P	P	—	>5	—	—	>30
39	1	P	—	2·3	—	—	10
40*	P	1	—	—	—	<4	<100
41	P	P	—	—	—	1·5	33

The ileum was kept at 15° C for 2 hr, after which the temperature was raised to 37° C; responses to all drugs and to electrical stimulation returned to the control values or remained slightly potentiated (Fig. 7, C). Reduction of the responses to acetylcholine and histamine immediately upon rewarming, which was described by Innes *et al.* (1957), was not observed in our experiments.

Effects of hexamethonium

In three experiments the responses to 5-hydroxytryptamine and to acetylcholine were either unaffected or slightly potentiated by hexamethonium (5×10^{-5} g/ml.). This result agrees with those of Feldberg (1951), Rocha e Silva, Valle & Picarelli (1953), Robertson (1953), Gaddum & Hameed (1954), and Kosterlitz & Robinson (1958). Hexamethonium affected most the response to dimethylphenylpiperazinium (dose ratios 20 and 23); the response to nicotine (dose ratios 4 and 16) was less affected and that to tetramethylammonium was least affected (dose ratios 1.5 and 3).

DISCUSSION

When the ileum was deprived of oxygen, electrical excitation of the nerves by single shocks gradually became less effective and was eventually abolished, even when the stimulus strength was increased. We have assumed that when the postganglionic nerve fibres can no longer be excited by electrical stimulation they can also no longer be excited either by preganglionic fibre activity or by the action of drugs anywhere along the nerves.

If a drug produces a contraction of the ileum normally through excitation of nerve fibres its effect is abolished by anoxia. If during anoxia the drug in higher concentrations still produces a contraction it must be by a different mechanism, presumably by acting directly on the smooth muscle receptors. Since two different types of receptors are involved, the slopes of two dose/response curves may also be different. Because of this we have calculated the dose ratio for responses in the *middle range* of the dose/response curve.

We have made the assumption that, when only one type of receptor is involved, the dose ratio gives a measure of the percentage of receptors removed from the total population. Thus a dose ratio of 2 means that 50% of the receptors are blocked and a dose ratio of 20 means that 95% of the receptors are blocked. If two types of receptor are involved, such as the M and D receptors with 5-hydroxytryptamine, similar calculations can be made, since (*a*) our results show that simultaneous excitation of the receptors does not lead to synergistic effects and (*b*) it is reasonable to assume that one M receptor leads to the same effects as *k*D receptors, so that the receptor population can be put into the terms of one type. The results with the different drugs will now be discussed with these considerations in mind.

Histamine

The response to histamine was affected by anoxia less than were the other drugs ; nor was it reduced by morphine, morphine plus anoxia, hyoscine, hexamethonium or cooling. This agrees with the conclusion that histamine acts directly on the smooth muscle cells of the guinea-pig ileum (Emmelin & Feldberg, 1947 ; Feldberg, 1951 ; Ambache & Lessin, 1955 ; Kosterlitz & Robinson, 1958 ; Innes *et al.*, 1957). Lewis (1960) found that morphine reduced the responses of the ileum to histamine and to acetylcholine. We did not find this, but in our experiments the bathing solution, temperature and lever system were all different from those used by Lewis. Since histamine had this direct action on the smooth muscle, the small changes in dose ratio during anoxia presumably represent changes in the sensitivity or contractility of the muscle fibres. Thus, if anoxia affects the response of another drug to a similar or slightly greater extent than histamine, the action of the drug is likely to be direct ; but from this evidence alone a small indirect action cannot be excluded.

5-Hydroxytryptamine

Gaddum & Picarelli (1957) defined two types of receptors for 5-hydroxytryptamine in the guinea-pig ileum: the M receptors which are blocked by morphine, and the D receptors which are blocked by phenoxybenzamine. The M receptors are generally assumed to be in nervous tissue and the D receptors in smooth muscle.

It is interesting to work out the relative contribution of each of these types of receptor to the normal response of the ileum. When morphine was given the dose ratios were between 2.3 and 13, indicating that receptors sensitive to morphine must have contributed to the normal response. However, in the presence of morphine, coaxial stimulation still produced a contraction, suggesting that morphine only partly blocked the nervous receptors. This interpretation was supported by the fact that anoxia, which completely inactivated the nervous tissue, led to much higher dose ratios (8 to 500). Moreover, even when the response of the tissue to 5-hydroxytryptamine had been maximally reduced by morphine, the dose ratio was further increased by anoxia (10 to 400). Thus it can be argued that the activation of nervous receptors accounts for most of the normal response since, after their block when 5-hydroxytryptamine could be acting only on muscle receptors, the dose had to be increased up to 500-fold to obtain the same response. It is difficult to imagine 1/500 of this dose having any significant effect on muscle receptors in the normal

tissue. From these results, the effects of phenoxybenzamine as an antagonist should be relatively small; yet when the ileum was treated with phenoxybenzamine alone, the dose ratios for 5-hydroxytryptamine were between 8 and 32, suggesting that phenoxybenzamine, like morphine, antagonized receptors which accounted for most of the normal response.

This implies that one of the antagonists is not specific. The results of the experiments with anoxia, morphine and phenoxybenzamine in various combinations (Tables 1, 2 and 3) make it likely that phenoxybenzamine rather than morphine is non-specific and affects both types of receptor. This could happen in several ways. Phenoxybenzamine could prevent the interaction of 5-hydroxytryptamine with the nervous receptors; we have no evidence for or against this. It could prevent acetylcholine released from the nerve endings from reacting with acetylcholine receptors, a possibility supported by the experiments in which phenoxybenzamine reduced both the effects of acetylcholine and those of electrical excitation of the nerves. Methysergide is a potent antagonist of the direct actions of 5-hydroxytryptamine on smooth muscle. However, methysergide was remarkably ineffective on the guinea-pig ileum, supporting the view that 5-hydroxytryptamine acts mainly through nerves and that phenoxybenzamine antagonizes both the direct and indirect effects.

In the presence of hyoscine the dose ratios for 5-hydroxytryptamine were between 4 and 12.5, similar to those with morphine but much smaller than those with anoxia. The effects of repetitive coaxial stimulation were reduced but not abolished by hyoscine, although the effects of single shocks were completely abolished. These results suggest that hyoscine, like morphine, does not completely abolish the indirect response to 5-hydroxytryptamine. This might mean that part of the indirect action is mediated either by non-cholinergic nerve fibres or by cholinergic nerve fibres which are not blocked by hyoscine or morphine.

Another possibility remains, that anoxia not only suppressed the effect of 5-hydroxytryptamine mediated via the nerve fibres but also its direct effect on smooth muscle receptors. In other words 5-hydroxytryptamine, but none of the other drugs, would need the presence of oxygen in order to react with the smooth muscle receptors. If this were so, cooling, which also abolished the response to nerve excitation, should have led to lower dose ratios for 5-hydroxytryptamine than those during anoxia. The mean of the dose ratios for cooling was indeed lower than that for anoxia, but the scatter was so wide that no conclusion can be drawn.

Our results do not allow a localization of the site of action of 5-hydroxytryptamine on nerves; it may act through hexamethonium-resistant stimulation of ganglia, as shown for the inferior mesenteric ganglion (Gyermek & Bindler, 1962), or at some part of the axon peripheral to the ganglion.

To summarize, 5-hydroxytryptamine contracts the longitudinal muscle of the guinea-pig ileum mainly through receptors in nervous tissue; some of this action is sensitive to morphine. Smooth muscle receptors are of negligible importance unless the neuronal mechanisms have been inactivated. Phenoxybenzamine blocks smooth muscle receptors, but also antagonizes some of the effects of 5-hydroxytryptamine on nerves; methysergide is a more specific antagonist for the muscle receptors. Since the responses to 5-hydroxytryptamine of the stomachs of the guinea-pig, kitten and

rat are unaffected by morphine or hyoscine (Vane, 1957 ; Paton & Vane, 1963), the proportion of nerve receptors to muscle receptors probably varies not only from species to species but also from one part of the intestine to another ; the guinea-pig ileum may well have an exceptionally high proportion of nervous receptors. The terms " M " and " D " receptors should be used only in the sense of the original definition and should not be quantitatively equated with nervous and smooth muscle receptors.

Acetylcholine

The response of the ileum to acetylcholine was unchanged by morphine and was potentiated by cooling, confirming observations by earlier workers. Only small increases in the dose of acetylcholine were needed to restore the response during anoxia. Since we have assumed that the small dose ratios with histamine before and during anoxia were due to interference with the contractile process of the smooth muscle, the similar small dose ratios for acetylcholine do not necessarily indicate an effect on nervous elements. However, in two experiments, the dose ratios for acetylcholine were higher than those for histamine ; this may be evidence for a slight indirect action, perhaps through stimulation of ganglion cells (Feldberg, 1951). Phenoxybenzamine antagonized acetylcholine (dose ratios 2 to 16), but had little or no additional effect when hyoscine was present. This suggests that phenoxybenzamine acted at the same acetylcholine receptors as hyoscine.

Nicotine

The most interesting results with nicotine were the unusually high dose ratios with morphine (10 to 15) compared with anoxia (2 to 9). A possible explanation for this paradox is that in the presence of morphine the high doses of nicotine needed to produce a contraction excited an adrenergic mechanism which diminished the contraction. This explanation was supported by the finding that the dose ratio with nicotine was decreased when the morphine-treated ileum was deprived of oxygen and when the effects of sympathetic nerve stimulation were reduced by bretylium. This supports the view (Rocha e Silva *et al.*, 1953) that, in analysing the response of the ileum to nicotine, the inhibition produced by the stimulation of adrenergic mechanisms, perhaps of adrenergic nerve endings (Thompson, 1958), must be taken into account. Ambache & Lessin (1955) found that the excitation of cholinergic nerve fibres by nicotine was completely suppressed by botulinum toxin. Because ten-fold increases in the dose of nicotine did not lead to contraction, they assumed that nicotine had no direct action on the smooth muscle. However, these large doses of nicotine may well have been exciting inhibitory adrenergic mechanisms to such an extent that any direct actions of nicotine on the smooth muscle were completely masked. Indeed Ambache & Lessin (1955) showed that, after botulinum toxin, the rabbit ileum was relaxed by nicotine in doses which previously contracted it.

Our experiments indicate that nicotine does have a direct action on smooth muscle for, during anoxia or cooling which presumably abolished both stimulant and inhibitory nervous mechanisms, a fourfold increase in the dose of nicotine still

contracted the ileum. Similarly, when morphine was used to inhibit cholinergic mechanisms and bretylium to inhibit adrenergic mechanisms, the dose ratio for nicotine was again 4.

The direct action of nicotine on smooth muscle, which was best seen during anoxia, was partly through an effect on acetylcholine receptors, for hyoscine reduced the actions of nicotine (dose ratio 50) much more than did anoxia or cooling. In the presence of hyoscine, the dose ratio for nicotine was even further increased after treatment with phenoxybenzamine. This result suggests that nicotine also has a lesser action on receptors (other than those for acetylcholine) which are sensitive to phenoxybenzamine ; these might be the receptors for histamine or 5-hydroxytryptamine. When phenoxybenzamine was given *first*, the dose ratio for nicotine was hardly increased, showing once more that the major effect of nicotine was on receptors insensitive to phenoxybenzamine. Deprivation of oxygen then gave dose ratios of around 43 and 66, instead of the usual dose ratio of about 4. Therefore the receptors sensitive to phenoxybenzamine are relatively unimportant as long as nicotine can act upon the nervous tissue ; once this action is abolished the receptors sensitive to phenoxybenzamine become important. If they have been blocked, much greater increases in the dose of nicotine are required to produce contraction.

We conclude that the primary action of nicotine on the guinea-pig ileum is indirect, through a hexamethonium-sensitive excitation of a cholinergic mechanism. When this action is abolished, a less pronounced direct action on acetylcholine receptors and an even smaller direct action on receptors sensitive to phenoxybenzamine can be seen, as long as the excitation by nicotine of an inhibitory adrenergic mechanism does not predominate.

Tetramethylammonium

Tetramethylammonium, like nicotine, has both indirect and direct actions on the ileum. With all the procedures used to reduce or abolish the effects of nervous activity, the dose ratios were small (1.3 to 4). Tetramethylammonium differed from nicotine in that the increase in dose required to restore its effects during anoxia was similar to the increase in dose required in the presence of morphine or hexamethonium. This indicates that tetramethylammonium had less action than nicotine on the inhibitory adrenergic mechanisms.

The fact that hyoscine gave a higher dose ratio (10) than anoxia (2.4) suggests that tetramethylammonium acted directly on acetylcholine receptors, but because phenoxybenzamine further increased the dose ratio (from 10 to 100) tetramethylammonium must also have acted directly on other receptors, perhaps those for histamine or 5-hydroxytryptamine. Indeed the relative effects of adding phenoxybenzamine to the hyoscine-treated preparation were much greater for tetramethylammonium than for nicotine, suggesting that the direct action of tetramethylammonium involved a greater proportion of receptors other than those for acetylcholine.

Since tetramethylammonium is a simple quaternary salt it might be expected to react with the binding sites for nitrogen groups of several different types of receptor, such as those for acetylcholine, histamine or 5-hydroxytryptamine.

We conclude that the action of tetramethylammonium is mostly on nervous tissue, but a strong action on smooth muscle receptors, some sensitive to hyoscine and some to phenoxybenzamine, is revealed when the nervous effects are reduced or abolished.

Dimethylphenylpiperazinium

Dimethylphenylpiperazinium had a much greater indirect action than did nicotine or tetramethylammonium, as shown by the high dose ratios (21 to over 30) with anoxia, hexamethonium and morphine. The effect of cooling, however, was unexpectedly small (dose ratio less than 2), a result that is difficult to explain. Again, there was evidence that dimethylphenylpiperazinium could exert a slight direct action on acetylcholine receptors, for hyoscine gave a higher dose ratio (90) than anoxia or morphine. The small further increase in dose ratio with phenoxybenzamine suggests a very weak direct action on receptors other than those for acetylcholine. As with 5-hydroxytryptamine, morphine did not completely abolish the indirect actions of dimethylphenylpiperazinium because, after treatment with morphine, anoxia diminished the contractions even more. Although we have assumed that this partial action of morphine is simply an incomplete suppression of the release of acetylcholine from cholinergic nerves, it is of course possible that dimethylphenylpiperazinium, like 5-hydroxytryptamine, contracts the ileum by exciting nerves which are insensitive to morphine.

We conclude that dimethylphenylpiperazinium has a primary stimulant action on the guinea-pig ileum through an excitation of nerves and a weak direct action on acetylcholine receptors.

Bradykinin

All our evidence supports the view that the action of bradykinin on the ileum is wholly direct and that it does not stimulate nerves.

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