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ANTIBIOTIC-ASSOCIATED COLITIS: AN *in vitro* INVESTIGATION OF THE EFFECTS OF ANTIBIOTICS ON INTESTINAL MOTILITY

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1 Nine antibiotic compounds in common use were studied to determine their ability to affect intestinal motility *in vitro*, in the guinea-pig ileum and rabbit colon.

2 Ampicillin, doxycycline, mecillinam and metronidazole were without effect over a concentration range which included typical serum levels found when these drugs are used therapeutically.

3 Clindamycin, gentamicin, kanamycin, pivmecillinam and trimethoprim were all found to inhibit evoked and reflex responses of the guinea-pig ileum but only clindamycin and trimethoprim also affected evoked responses of the rabbit colon.

4 Kanamycin and gentamicin appeared to have a predominantly pre-junctional action, pivmecillinam and trimethoprim a predominantly post-junctional action. Clindamycin had a pre-junctional action at low concentrations and long exposure times, and a post-junctional action at high concentrations and short exposure times.

5 The concentration of each antibiotic required to inhibit the peristaltic reflex of the guinea-pig ileum was less than that required to inhibit its responses to electrical stimulation or exogenous acetylcholine or histamine but greater than the serum levels associated with their respective use in therapeutic doses.

6 A sequence of events whereby antibiotic-induced alterations in gastro-intestinal motility could lead to the development of pseudomembranous colitis is proposed.

Introduction

Although the effects of antibiotic drugs on somatic neuromuscular transmission have been extensively investigated, little consideration has been given to the possible effects of these compounds on autonomic neuro-effector transmission in the gastro-intestinal tract. Since no one hypothesis successfully accounts for the often severe gastro-intestinal side effects observed during antibiotic therapy, antibiotic-induced alterations in gastro-intestinal motility by an action on neuro-effector transmission were considered as possible factors in the causation of antibiotic associated colitis (AAC).

Current evidence suggests that impaired gastro-intestinal motility favours the multiplication of the bacterium *Clostriduim difficile* (Pittman, 1979) whose associated toxins are now considered to be major contributory factors in the development of AAC (Bartlett, Moon, Chang, Taylor & Onderdonk, 1978).

Results obtained by other workers in the last 15 years concerning antibiotics and gastro-intestinal motility are conflicting and incomplete, no consideration having been given to either the possible effects of antibiotic administration on noradrenaline release from sympathetic nerves in the gastro-intestinal tract or the concentration of these compounds found in the body fluids following therapeutic administration.

Since at least part of the mechanism of action of the aminoglycoside antibiotics (gentamicin, kanamycin, neomycin, streptomycin) at the somatic neuromuscular junction is a calcium-dependent inhibition of acetylcholine release (Pittinger & Adamson, 1972) and because neomycin has been shown to decrease noradrenaline output from the electrically stimulated rat anococcygeus muscle (Wright & Collier, 1977), effects of antibiotic compounds on both sympathetic and parasympathetic innervation of the gastro-intestinal tract required study.

Methods

Electrically stimulated preparations

Male albino guinea-pigs weighing between 250 and 500 g were killed by cervical fracture. The ileum was removed and the 10 cm nearest the ileo-caecal junction discarded, owing to its differing pharmacological properties (Munro, 1953). Luminal contents were removed by gentle flushing with Krebs solution at

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37°C. Segments approximately 5 cm long were then suspended under 1 g tension, oral end uppermost, according to the method of Paton (1955) for transmural electrical stimulation. A 20 ml organ bath was used and the bathing medium was Krebs solution (normal Krebs) of the following composition (mM): NaCl 118.5, KCl 4.75, $CaCl_2$ 2.54, KH_2PO_4 1.19, $MgSO_4$ 1.19, $NaHCO_3$ 25, glucose 11, gassed with a mixture of 95% O_2 and 5% CO_2 and maintained at 37 ± 0.5°C. In later experiments, the bathing medium was altered, either by altering the $CaCl_2$ concentration to 5.08 mM with an appropriate adjustment in NaCl concentration, or by the addition of neostigmine (32.9 nM). Electrical stimuli were produced by a Grass S88 stimulator, and the responses of the longitudinal muscle were recorded with an isometric transducer (Pye Ether type UF1) connected to a Linseis pen recorder (LS 24). The stimulus parameters used were 0.1 Hz, 0.5 ms duration and a supramaximal voltage.

Dose-response curves

Ileal segments prepared as described above, were suspended, oral end uppermost, in 20 ml organ baths under approximately 1 g tension. Antibiotic compounds found to produce inhibition in the electrically stimulated preparations were investigated for possible anti-cholinoceptor or anti-histaminic properties. Adjacent segments of ileum were exposed to either acetylcholine or histamine to obtain control dose-response cuves. The segments were then exposed to an antibiotic for 15 min and a second dose-response curve obtained in the continuous presence of the antibiotic. The segments were then washed every 3 min for 15 min (or until complete recovery was achieved), exposed to a higher concentration of antibiotic for 15 min and a third dose-response curve obtained, again in the continuous presence of the antibiotic. Recordings of isometric responses were made as described above.

Trendelenburg preparation

Ileal segments were connected to a previously calibrated Trendelenburg (1917) apparatus. A 40 ml organ bath was used, and the system was maintained at 37 ± 0.5°C in a thermostatically regulated water bath. Recordings were made by use of Washington Isotonic Transducers, and the responses displayed on a Grass Model 7D polygraph.

Doubly-innervated rabbit colon

Male New Zealand White rabbits weighing 1.7–2.0 kg were stunned and bled. A segment of descending colon with both sympathetic and parasympathetic nerves intact was then removed, according to the method of Garry & Gillespie (1955). To minimize damage to the segment, following removal, it was left in aerated Krebs solution at 37°C to expel its contents by muscle action. The extrinsic nerves were then attached to electrodes, and the whole preparation mounted in a 70 ml organ bath under a 2 g load. The bathing medium as previously was Krebs solution at 37°C ± 0.5 gassed with 95% O_2 and 5% CO_2. Electrical pulses were delivered from a Grass S88 stimulator, and changes in length measured with a Washington Isotonic transducer connected to a Linseis LS 24 pen recorder. The parasympathetic nerves of the preparation were stimulated once per minute for 10 s at 10 Hz with pulses of 1 ms duration. Sympathetic nerves were stimulated for 30 s at 50 Hz each pulse being 1 ms in duration. Supramaximal voltages were used throughout.

Drugs

The antibiotic compounds used in this study were ampicillin sodium (Beecham Laboratories Ltd.), clindamycin hydrochloride (Upjohn Ltd.), doxycycline hydrochloride (Pfizer Ltd.), gentamicin sulphate (Nicholas Laboratories Ltd.), kanamycin sulphate (Bristol Laboratories Ltd.), mecillinam hydrochloride dihydrate (Leo Laboratories Ltd.), metronidazole (May and Baker Ltd.), pivmecillinam hydrochloride (Leo Laboratories Ltd.), trimethoprim lactate (Wellcome Foundation Ltd.). All antibiotics were dissolved in distilled water and added to the organ baths in volumes not exceeding 1% of the total bath volume. Fresh antibiotic solutions were made each day. Histamine acid phosphate (Sigma Ltd.) was dissolved and serially diluted in a modified Krebs solution of the following composition (mM): NaCl 143, KCl 4.75, $CaCl_2$ 2.54. Acetylcholine chloride (Sigma Ltd.) was dissolved in a 5% NaH_2PO_4 solution, and serially diluted with Krebs solution taken to pH 4.0 with preservative-free HCl. Noradrenaline bitartrate (Sigma Ltd.) was dissolved in modified Krebs solution, and serially diluted with modified Krebs solution to which had been added ascorbic acid (14.2 μM). Fresh dilutions of these drugs were made daily, and stock solutions were kept at −20°C when not in use. Antibiotic concentrations are expressed in μg/ml for comparison with the blood levels found in human subjects. Final bath antibiotic concentrations are expressed as antibiotic (less salt) added to the bath, with the exception of gentamicin, where the concentrations given refer to the sulphate (potency 56.7%, manufacturer's information). All calculations were based on information supplied by the manufacturers.

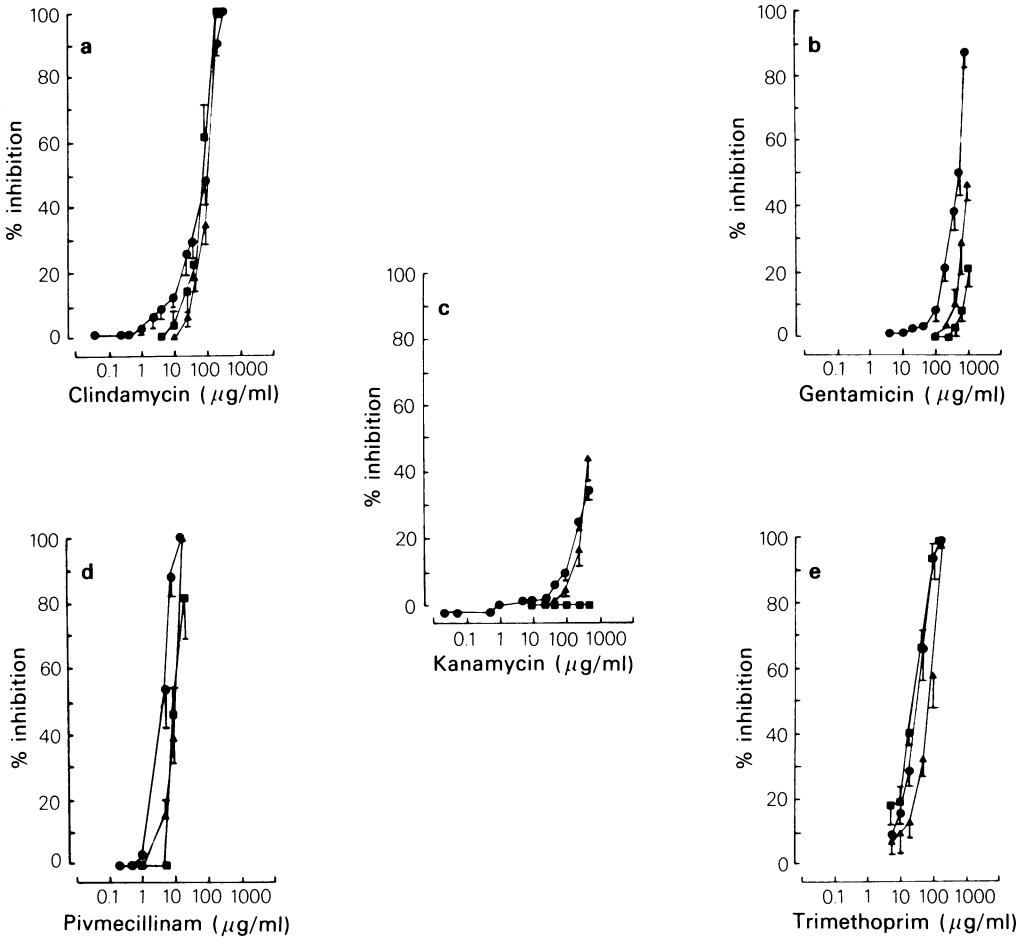

Figure 1 The inhibition produced in transmurally stimulated isolated ileum of the guinea-pig by the named antibiotics, in the following bathing media; (●) normal Krebs; (▲) normal Krebs + 33 nM neostigmine; (■) 5.08 nM Ca^{2+}-Krebs. Values shown are the means of at least 9 experiments for controls (Normal Krebs), and 5 experiments in each bathing medium; vertical lines show s.e.mean.

Results

Electrically stimulated preparations

Of the nine antibiotics studied, four (ampicillin, doxycycline, mecillinam, metronidazole) were found to be inactive within the concentration range examined (Table 1).

With the exception of kanamycin, the remainder, clindamycin, gentamicin, pivmecillinam and trimethoprim produced only inhibition of contraction of the preparations (Figure 1). Kanamycin was inhibitory at high doses, but produced some potentiation of the response at lower doses.

Table 2 summarizes the essential features of the inhibition produced by each antibiotic in the three different bathing media used.

Dose-response curves

The antibiotic concentrations chosen for this section were those producing approximately maximal and half maximal inhibition of contraction of the electrically stimulated guinea-pig ileum, except in the case of gentamicin where only the dose producing maximum inhibition was used, and pivmecillinam where the lower dose chosen was greater than that producing half maximal inhibition, due to the steepness of the dose-response (% inhibition) curve.

With the exception of kanamycin, which appeared to have no post-junctional anti-cholinoceptor or anti-histamine receptor action at the concentrations chosen (Figures 2c and 3c) all the antibiotics that had inhibitory effects in the electrically stimulated ileum exerted an apparent concentration-dependent inhib-

Table 1 Antibiotics found to be without activity *in vitro*

Drug and Concentration range	*Peak human plasma levels**	*Observations*
Ampicillin 1–800 μg/ml	3–6 μg/ml‡	Inactive on electrically stimulated preparations
Doxycycline 1–20 μg/ml	3 μg/ml‡	In Krebs solution at concentrations greater than 20 μg/ml doxycycline formed an insoluble precipitate
Mecillinam 1–800 μg/ml	6 μg/ml†	Inactive on electrically stimulated preparations
Metronidazole 0.5–200 μg/ml	7 μg/ml‡	,,

†Manufacturer's information. ‡Martindale, The Extra Pharmacopoeia 27th Ed.
*Peak human plasma levels vary with dose and route of administration. Figures quoted here are based on 'mid-range' therapeutic doses, given intramuscularly in the case of mecillinam and orally for the others.

ition of the tissue response to applied acetylcholine or histamine. The lower of the concentrations chosen (for any antibiotic) had little effect on the contraction produced by acetylcholine or histamine if the response was approximately 40% or less of maximum, i.e. up to 4 nM acetylcholine or 9 nM histamine. Above this, the responses of the tissue were reduced; only in the case of gentamicin could 100% of the control maximum contraction be achieved by increasing the acetylcholine or histamine concentration beyond that giving the control maxima (Figures 2b and 3b). Further increases in the antibiotic concentration reduced the tissue response to acetylcholine or histamine at all doses examined. Clindamycin (500 μg/ml) (Figures 2a and 3a) pivmecillinam (20 μg/ml) (Figures 2d and 3d) and trimethoprim (200 μg/ml) (Figures 2e and 3e) reduced or abolished the spontaneous activity of the preparations, and reduced the duration of contraction in response to either acetylcholine or histamine.

Trendelenburg preparation

Since it was noted that the peristaltic reflex of the guinea-pig ileum was inhibited by antibiotic concentrations lower than those required to abolish the contraction evoked by electrical stimulation, all 9 antibiotics were studied in this preparation.

The following antibiotics were found to be inactive in the concentration ranges examined. Ampicillin (1–800 μg/ml) mecillinam (1–800 μg/ml) and metroniadazole (1–200 μg/ml) had no effect on (a) the resting tension (RT) of the longitudinal or circular muscle, (b) the spontaneous activity (SA) of either muscle layer, or (c) the threshold distension (TD) required to initiate the peristaltic reflex. In 1 of 3 preparations, doxycycline (1–20 μg/ml) appeared to increase slightly the resting tone of the longitudinal muscle for between 50 and 70 s at each concentration used but as previously noted did not affect the other parameters studied.

Clindamycin (0.05–50 μg/ml) at concentrations up to 5 μg/ml was without effect on RT, SA or TD, in any preparation. When inhibition of the peristaltic reflex was seen (at 50 μg/ml, in 5 of 5 preparations) it was not accompanied by any prior alterations in RT, SA or TD. On filling, the preparation simply 'ballooned', i.e. the circular muscle relaxed completely and failed to contract. Contractions on the longitudinal muscle were, therefore, severely reduced or abolished, since the 'ballooning' effect caused a 'passive' shortening of the longitudinal muscle such that the preparation maintained a constant volume.

Gentamicin (1–400 μg/ml) at doses below 20 μg/ml (1 of 5) and 100 μg/ml (4 to 5) had no effect on RT, SA or TD. Above 20 μg/ml and 100 μg/ml respectively it caused a decrease in the RT and SA of the longitudinal muscle of about 1 min duration but was without effect on the circular muscle or TD. In 1 of 5 preparations at 100 μg/ml, 1 of 5 at 200 μg/ml and 2 of 5 at 400 μg/ml the response of the circular muscle to luminal filling was considerably reduced (or in one case completely abolished) with little effect on the contractions of the longitudinal muscle. In the one remaining preparation these effects were seen at 600 μg/ml, although the longitudinal muscle response was more depressed than in the other four. Gentamicin, reduced or abolished the response of the circular muscle to luminal filling but in contrast to clindamycin, did not affect the tone of the muscle, i.e. its ability to resist filling. In one preparation, exposure to 100 μg/ml gentamicin for 30 min had no measurable effect on the peristaltic reflex. In all cases, the effects of gentamicin were reversible with a single wash.

Kanamycin (50 μg/ml–500 μg/ml) was without effect on the RT or SA of either muscle layer and the TD was unaltered. At 500 μg/ml, without altering

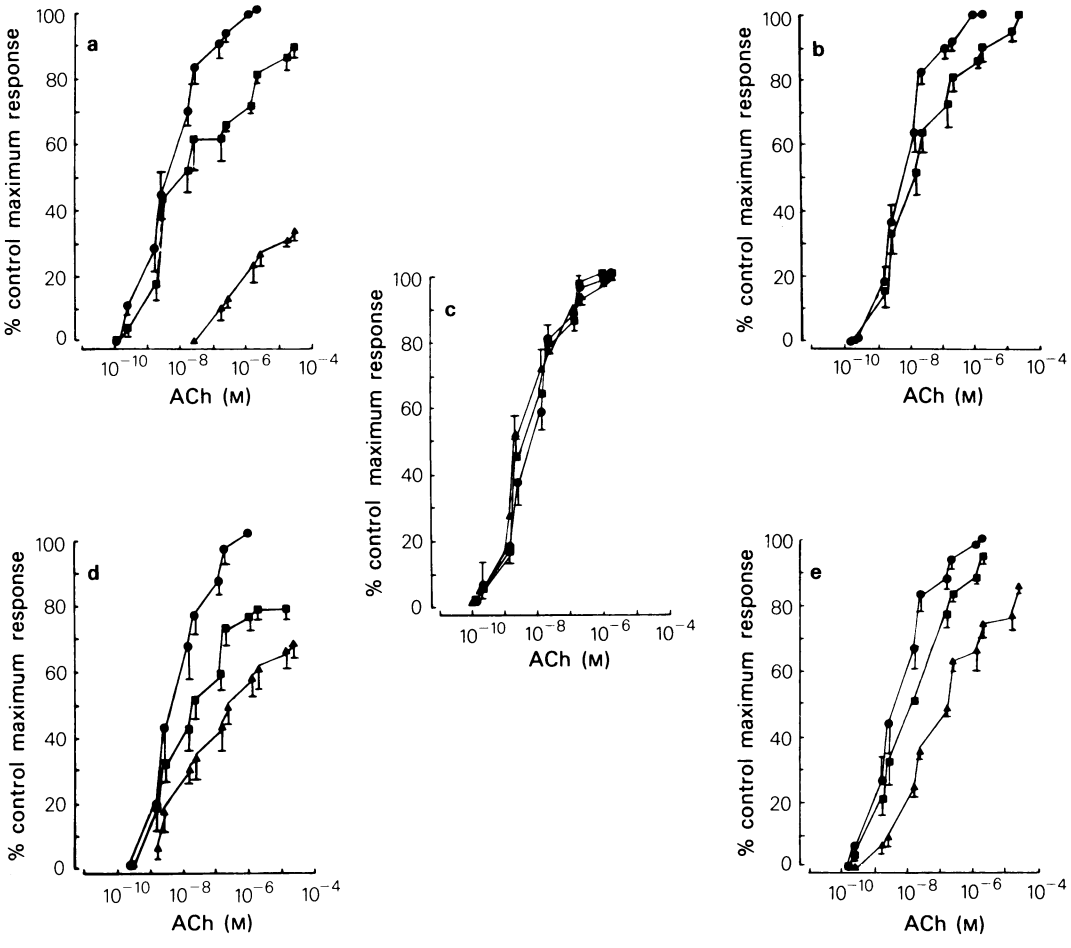

Figure 2 Log dose-response curves of the isolated ileum of the guinea-pig to acetylcholine, obtained in the continuous presence of the following antibiotics: (a) clindamycin (■) 100 μg/ml, (▲) 500 μg/ml; (b) gentamicin (■) 1 mg/ml; (c) kanamycin (■) 100 μg/ml, (●) 500 μg/ml; (d) pivmecillinam (■) 10 μg/ml, (▲) 20 μg/ml; (e) trimethoprim (■) 50 μg/ml, (▲) 200 μg/ml. All values are expressed as a percentage of the control (●) maximum response to acetylcholine in the absence of antibiotic, and are the means of 5 experiments; vertical lines show s.e.mean.

either the RT or SA, kanamycin abolished the response of both muscle layers to luminal filling. This effect was seen within 3 min of adding the drug and was reversed on washing the preparation once. In this type of preparation, exposure to higher doses of kanamycin did not produce the irreversible changes in resting tone seen in 2 of 11 electrically stimulated ileum preparations.

Pivmecillinam (0.1 μg/ml–1 μg/ml) did not affect the SA, RT or TD at concentrations up to 0.5 μg/ml. After a 1 min exposure to a concentration of 1 μg/ml both muscle layers failed to respond to luminal filling, although as previously RT and SA were not affected. These effects were reversed after a single wash, but in 1 of 5 preparations the responses of both muscle layers were modified as a result of exposure to this drug.

In 4 of 5 preparations trimethoprim (5–20 μg/ml) produced a partial inhibition of the peristaltic reflex following a 1 min exposure of the preparation to a concentration of 10 μg/ml. This was not accompanied by any alteration in RT or SA. In these preparations the inhibition was total if the concentration of trimethoprim was raised to 20 μg/ml, although RT and SA remained unaffected. In the remaining one preparation these effects were not seen until the concentration of trimethoprim was raised to 50 and 100 μg/ml. However, in this one

Table 2 Effects of antibiotics on electrically stimulated isolated ileum of the guinea-pig in normal Krebs solution, Krebs solution containing 5.08 mM Ca^{2+} and Krebs solution containing neostigmine (33 nM)

Drug and concentration range	*Maximum concentration used*	*Peak human* plasma levels*	*Bathing medium*	*Magnitude of depression* §	*Time to maximum depression*	*Magnitude of spontaneous Recovery* §	*Time taken for recovery*	*Observations*
Clindamycin 0.05–500 μg/ml	500 μg/ml	2.5 μg/ml‡	Normal Krebs	100%	82 ± 19 s (n=10)	Zero	—	At lower concentrations the effects of this drug were reversed by a single wash, but with increasing concentrations this was less easily achieved. Complete inhibition was also observed at 250 μg/ml (3 of 10).
	250 μg/ml	"	5.08 mM Ca^{2+} Krebs	100%	206 ± 55 s (n=5)	Zero	—	—
	250 μg/ml	"	33 nM Neostigmine Krebs	100%	810 ± 184 s (n=5)	Zero	—	In neostigmine, spontaneous recovery of 85 ± 5% (n=4) occurred in 315 ± 78 s, and complete recovery could be achieved at all doses by washing the preparation once (See Figure 1a).
Gentamicin 5 μg–mg/ml "	1 mg/ml	7 μg/ml‡	Normal Krebs	86.4%	52 ± 5 s (n=10)	82 ± 3%	321 ± 63 s (n=4)	In the range 1–200 μg/ml gentamicin always produced an inhibition without affecting the resting tension. Above 200 μg/ml, gentamicin increased (2 of 10), decreased (4 of 10) or did not affect the resting tension (4 to 10).
	1 mg/ml	"	5.08 mM Ca^{2+} Krebs	21 ± 6%	146 ± 41 s (n=5)	88 ± 2%	274 ± 86 s (n=4)	—
	1 mg/ml	"	33 nM Neostigmine Krebs	47 ± 4%	71 ± 5 s (n=5)	83 ± 3%	370 ± 63 s (n=4)	— (see Figure 1b)

Table 2 (*continued*)

Drug and concentration range	*Maximum concentration used*	*Peak human* plasma levels*	*Bathing medium*	*Magnitude of depression* §	*Time to maximum depression*	*Magnitude of spontaneous Recovery* §	*Time taken for recovery*	*Observations*
Kanamycin 0.025–500 μg/ml	500 μg/ml	30 μg/ml‡	Normal Krebs	34 ± 8%	45 ± 7 s (n=9)	Zero	—	In all 9 preparations in concentrations of up to 1 μg/ml, kanamycin produced a potentiation of contraction of between 2–10%. Although this was seen in every preparation at least once it was not a concentration-dependent effect.
	"	"	5.08 mM Ca^{2+} Krebs	Zero	—	—	—	No potentiation of response was seen under these conditions.
	"	"	33 nM Neostigmine Krebs	44 ± 7%	45 ± 2 s (n=4)	86 ± 2%	345 ± 75 s (n=4)	" (see Figure 1c)
Pivmecillinam 1–20 μg/ml	20 μg/ml	5 μg/ml†	Normal Krebs	100%	52 ± 8 s (n=5)	Zero	—	—
	"	"	5.08 mM Ca^{2+} Krebs	81 ± 12%	88 ± 14 s (n=5)	100% (1 of 5 only) Zero (4 of 5)	1620 s (n=1)	In this medium at concentrations up to 5 μg/ml pivmecillinam produced alterations in the resting tension of the preparation causing either cyclical (1 of 5) or continuous increases (2 of 5).
	"	"	33 nM Neostigmine Krebs	100%	242 ± 61 s (n=5)	74 ± 17%	1866 ± 256 s (n=4)	— (See Figure 1d)
Trimethoprim 5–200 μg/ml	200 μg/ml	1–5 μg/ml†	Normal Krebs	98 ± 1%	67 ± 6 s (n=9)	Zero	—	The inhibitory effects of trimethoprim were difficult to reverse by washing, particularly at higher concentrations
	"	"	5.08 mM Ca^{2+} Krebs	100%	57 ± 11 s (n=5)	Zero	—	In 4 of 5 preparations, trimethoprim 20 μg/ml and above caused an increase in the resting tension of the preparation. When, under these conditions contractions to electrical stimuli were abolished, the preparation then produced a brief relaxation of rapid onset when stimulated.
	"	"	33 nM Neostigmine Krebs	97 ± 2%	170 ± 14 s (n=5)	20 ± 3%	257 ± 10 s (n=4)	— (see Figure 1e)

*Figures quoted here are based on 'mid-range' therapeutic doses given intramuscularly in the cases of gentamicin and kanamycin and orally for the others. †Manufacturers' information. ‡Martindale, The Extra Pharmacopoeia 27th Ed. §All values are expressed as a percentage of the control maximum.

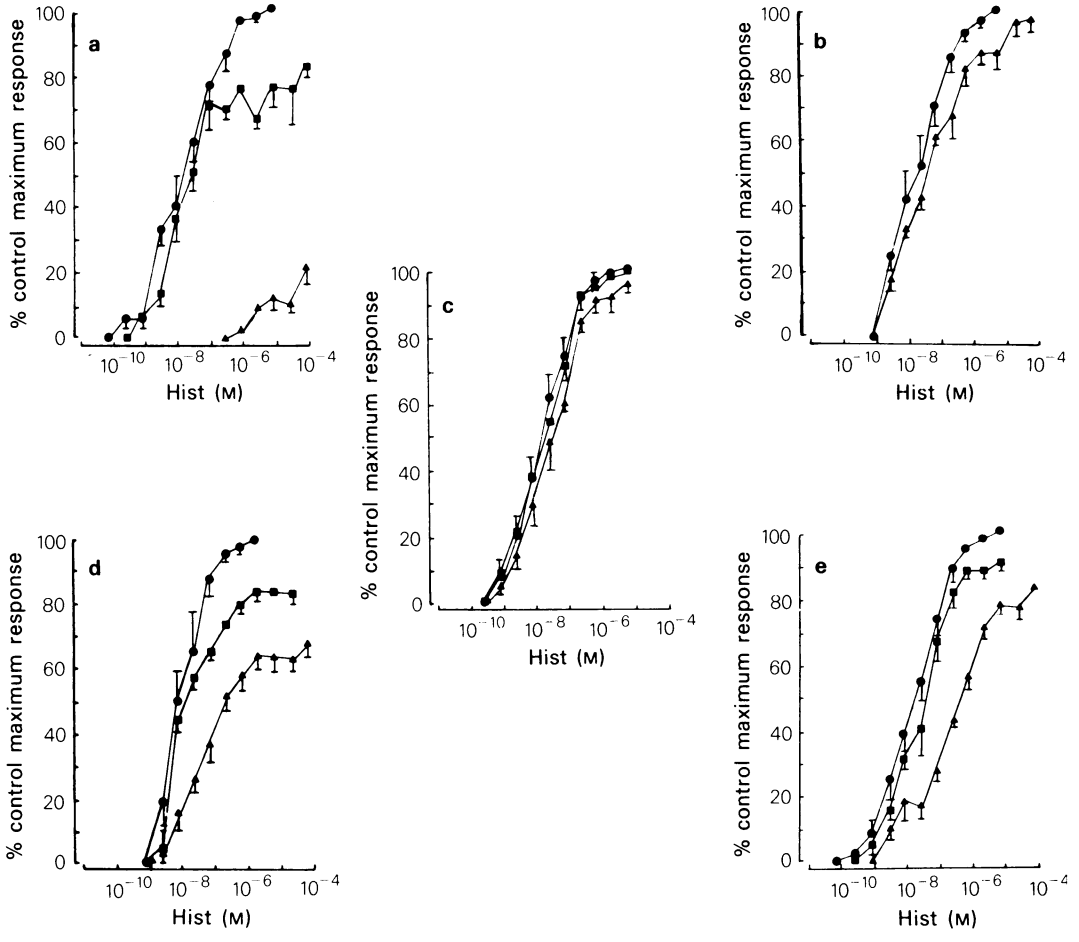

Figure 3 Log dose-response curves of the isolated ileum of the guinea-pig to histamine, obtained in the continuous presence of the following antibiotics: (a) clindamycin (■) 100 μg/ml, (▲) 500 μg/ml; (b) gentamicin (■) 1 mg/ml; (c) kanamycin (■) 100 μg/ml, (▲) 500 μg/ml; (d) pivmecillinam (■) 10 μg/ml, (▲) 20 μg/ml; (e) trimethoprim (■) 50 μg/ml, (▲) 200 μg/ml. All values are expressed as a percentage of the control (●) maximum response to histamine in the absence of antibiotic, and are the means of 5 experiments; vertical lines show s.e.mean.

preparation, RT was lowered, and SA reduced at concentrations of 5 μg/ml trimethoprim and above. In all five preparations, the inhibition was reversed by a single wash.

Doubly-innervated rabbit colon

It was found that with an exposure time of 5 min, clindamycin did not affect this preparation except at a concentration of 500 μg/ml in which case the response to stimulation of either the parasympathetic or sympathetic nerves was abolished and did not re-appear despite washing the preparation several times. It was then noted that following a minimum of 15 min exposure to clindamycin at only 50 μg/ml, the response to stimulation of the parasympathetic nerves was abolished, spontaneous activity and resting tone increased and responses to stimulation of the sympathetic nerves modified (but not abolished until 40–45 min after addition of the drug). Once this inhibition occurred, several washes over a further 30–50 min were required to obtain recovery. Acetycholine (200–300 nM) produced a contraction similar in magnitude to that obtained in response to stimulation of the parasympathetic nerves. When contractions in response to this stimulation were abolished by 50 μg/ml clindamycin the response of the preparation to applied acetylcholine was unchanged (Figure 4a). Similarly, the relaxation seen in response to stimulation of the sympathetic nerves

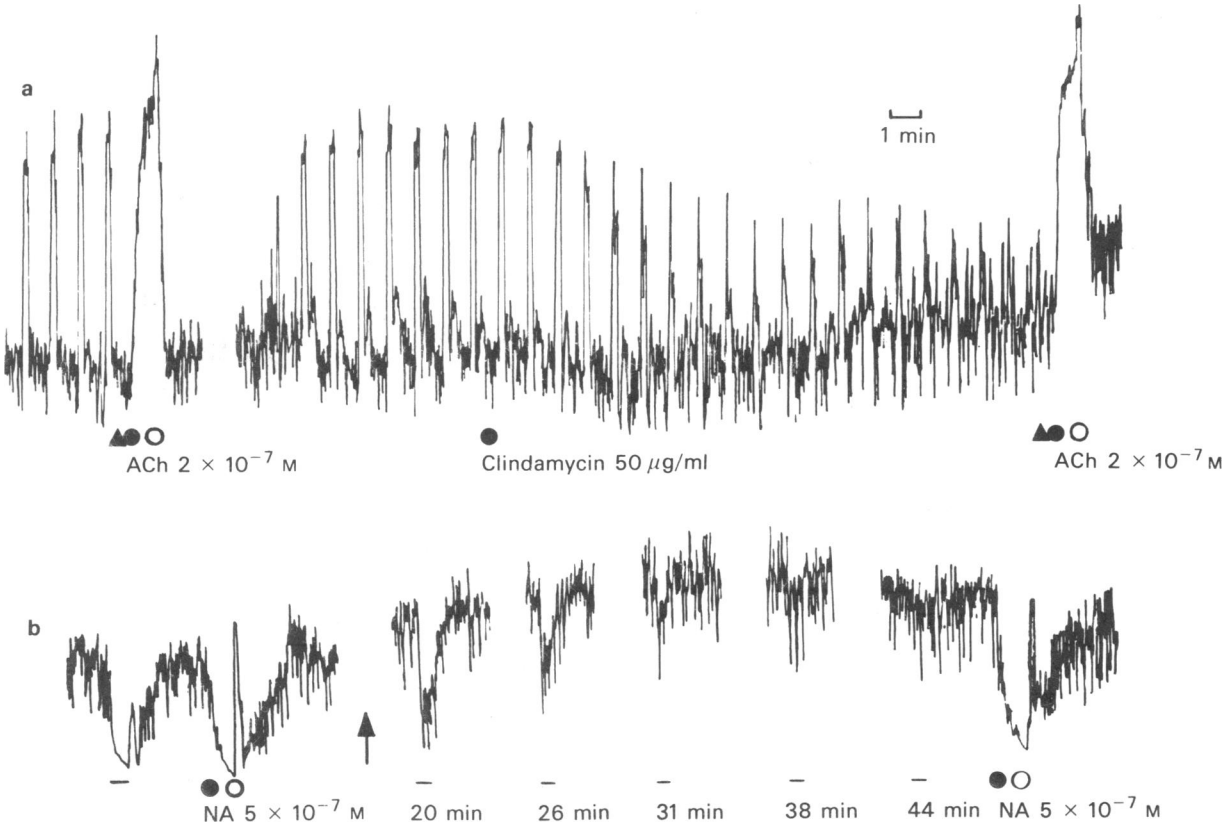

Figure 4 (a) Control responses of the isolated colon of the rabbit to electrical stimulation of the parasympathetic nerves at minute intervals (for 10 s at 10 Hz, 1 ms duration). Following cessation of electrical stimulation (▲) the tissue was exposed to acetylcholine (ACh) 2×10^{-7} M (●) which was washed out after 30 s (○). On recovery, control responses to parasympathetic stimulation were again obtained, and the tissue exposed to clindamycin 50 μg/ml (●) for 20 min, with electrical stimulation maintained throughout. At 20 min electrical stimulation was stopped, and the tissue again exposed to acetylcholine 2×10^{-7} M for 30 s. (b) Control response to electrical stimulation of the sympathetic nerves of the rabbit isolated colon for 30 s, at 50 Hz, 1 ms duration (horizontal bar). A response of similar magnitude was then obtained by exposure of the tissue to noradrenaline (NA) 5×10^{-7} M for 30 s. The sympathetic nerves were stimulated as previously 20, 26, 31 and 44 min after the addition of clindamycin 50 μg/ml (arrow). At 45 min the tissue was again exposed to noradrenaline 5×10^{-7} M for 30 s in the continued presence of clindamycin.

could be mimicked by noradrenaline (300–500 nM) and, when this response to electrical stimulation was abolished by 50 μg/ml clindamycin, the response to noradrenaline was not affected (Figure 4b).

Only in 1 of 3 experiments was gentamicin found to have an effect on the responses of the preparation to stimulation of the parasympathetic nerves. This was seen following exposure of the preparation to a concentration of 500 μg/ml and above, for 5 min. However, in two further preparations exposure to 1 mg/ml for 5 or 25 min was without effect, although a 56% reduction in contraction size was seen within 8 min of applying the drug at a concentration of 2 mg/ml. In all preparations examined, exposure to gentamicin did not affect the onset of the relaxation produced by sympathetic nerve stimulation, but increased the time taken for recovery to control level resting tone and spontaneous activity. At 500 μg/ml this effect was reversed by a single wash, but at 1 mg/ml this was less easily achieved.

At concentrations up to and including 500 μg/ml and exposure times (at 500 μg/ml) of between 5 and 50 min, kanamycin was without an observable effect on the responses of this preparation to stimulation of either the parasympathetic or sympathetic nerves, although in 1 of 9 preparations it appeared to increase slightly the time taken for the preparation to recover following sympathetic stimulation. Spontaneous activity and resting tone were similarly unaffected.

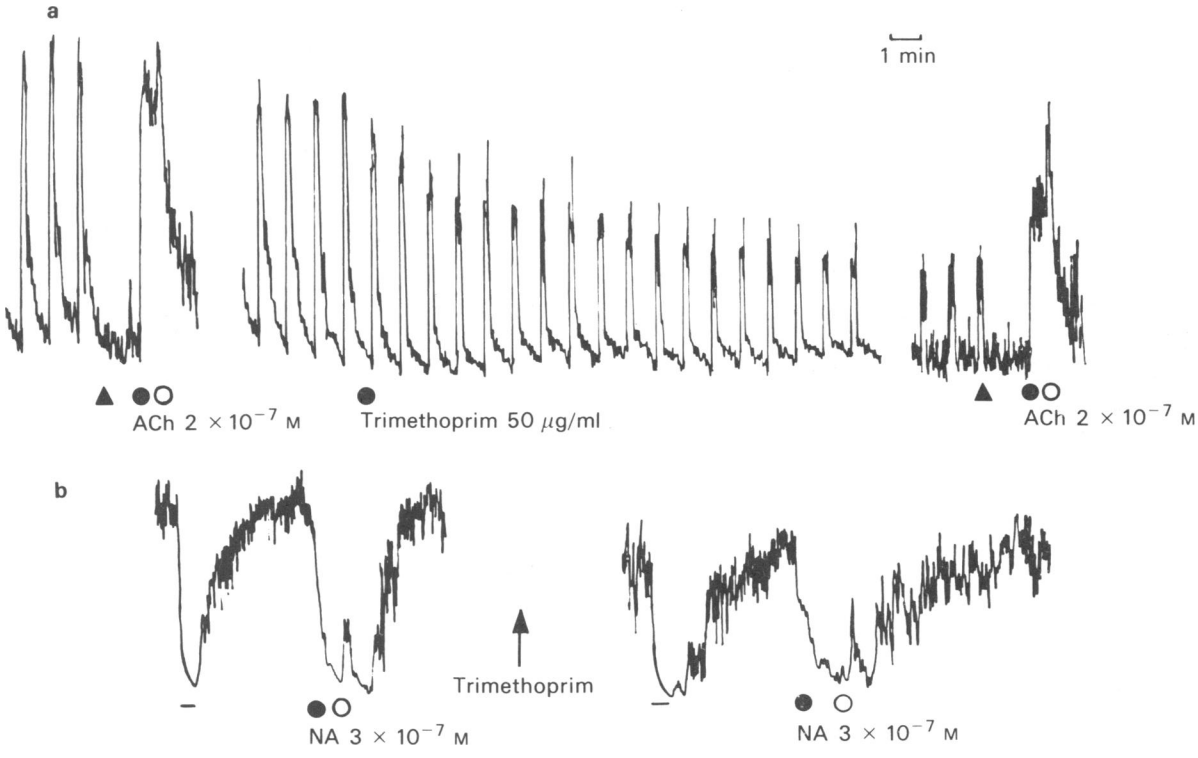

Figure 5 (a) Control responses of the isolated colon of the rabbit to electrical stimulation of the parasympathetic nerves at minute intervals (for 10 s at 10 Hz, 1 ms duration). Following cessation of electrical stimulation (▲) the tissue was exposed to acetylcholine (ACh) 2×10^{-7} M (●) which was washed out after 30 s (○). On recovery, control responses to parasympathetic stimulation were again obtained, and the tissue exposed to trimethoprim 50 μg/ml (●), with electrical stimulation maintained throughout. The first 17 min after addition are shown, and the trace continues at 43 min. At 45 min, electrical stimulation was stopped and the tissue again exposed to acetylcholine 2×10^{-7} M for 30 s. (b) Control response to electrical stimulation of the sympathetic nerves of the rabbit isolated colon, for 30 s at 50 Hz, 1 ms duration (horizontal bar). A response of similar magnitude was then obtained by exposure of the tissue to noradrenaline (NA) 3×10^{-7} M for 30 s. The preparation was exposed to trimethoprim 50 μg/ml (arrow) for 48 min, and washed 3 times in the following 20 min. The trace continues at 69 min after the addition of trimethoprim. At 70 min the sympathetic nerves were stimulated as previously, and on recovery the tissue exposed to noradrenaline 3×10^{-7} M for 30 s.

Pivmecillinam (1–30 μg/ml) had no effect on any of the parameters measured, including spontaneous activity and resting tone either when exposure times were long e.g. 15 μg/ml for 15 min, 20 μg/ml for 30 min, 30 μg/ml for 20 min (a dose higher than that completely abolishing all responses of the guinea-pig ileum to transmural electrical stimulation).

At a concentration of 10 μg/ml, and with a contact time of 5 min, trimethoprim had no effect on the contraction produced in response to stimulation of the parasympathetic nerves. However, when the sympathetic nerve stimulation was stopped, the characteristic 'rebound contraction' was absent or considerably reduced and the recovery time slightly increased in comparison to control valves. Only when the concentration of trimethoprim was in the range 50–100 μg/ml (15 min contact time) was an effect also seen on the responses to parasympathetic nerve stimulation, and in this range the effects of trimethoprim were not easily reversed by washing (Figure 5b). Increasing the exposure time to 15 min at 10 μg/ml did not produce an effect; at 50 μg/ml with an exposure time of 5–45 min contractions were often completely abolished or very much reduced. However, subsequent administration of trimethoprim at the same concentration and for the same length of time produced a lesser inhibition of contraction than the initial exposure. The inhibition produced by trimethoprim was sometimes accompanied by a slight increase in the spontaneous activity of the preparation. The effects on the resting tone varied between an increase (3 of 10), a decrease (1 of 10) and no

change (6 of 10). The reduced effect of a second exposure to trimethoprim was not evident when the events following stimulation of the sympathetic nerves were examined. When the responses to parasympathetic stimulation were reduced or abolished by trimethoprim, the response to applied acetylcholine was also reduced when compared to control values (Figure 5a). Similarly, the recovery time following exposure to noradrenaline (300–500 nM) was increased, when the recovery from sympathetic nerve stimulation was slower, following trimethoprim administration (Figure 5b).

Discussion

Until the small amounts of acetylcholine released spontaneously and during electrical stimulation from guinea-pig ileum have been assayed in the presence of antibiotics used in this study, only indirect evidence for the basis of their inhibitory effects can be presented. Attempts have been made to distinguish a depressant effect of antibiotics directly on the smooth muscle component of the tissues from their actions on neuronal elements. Since acetylcholine and histamine act directly on the longitudinal muscle of guinea-pig ileum to cause it to contract, it has been assumed that antibiotics having no effect on the dose-response curves to acetylcholine and histamine must have exerted an inhibitory effect by depressing the output of neurotransmitter from the intrinsic nerves. Further support for an action of antibiotics on cholinergic nerves is obtained in experiments in which neostigmine was used in low concentrations, since at the concentration used in this study neostigmine does not affect the release of acetylcholine but decreases its enzymatic hydrolysis (Cox & Lomas, 1972).

It was interesting to find that the inhibition produced by both gentamicin and kanamycin in transmurally stimulated guinea-pig ileum preparations was respectively reduced or prevented by raising the calcium ion concentration of the bathing medium. It could be argued that increasing the calcium ion concentration reversed partially or wholly the effects of the two aminoglycoside antibiotics simply by increasing the response of the tissue to acetylcholine (see Coville & Telford, 1970). However, such an explanation is unlikely since we have recently found that the dose-response curve to acetylcholine is not altered in 5.08 mM Ca^{2+}-Krebs in place of 2.54 mM Ca^{2+}-Krebs nor is there a change in the ratio of the maximum tensions produced by acetylcholine and electrical stimulation (unpublished observations). In the case of trimethoprim however, the inhibition appears to be predominantly post-junctional and elevating the calcium ion concentration was without effect. Since kanamycin and, to a lesser extent, gentamicin appear to have a pre-junctional action (Figures 2b,c and 3b,c), the reversal of their inhibition by increasing calcium ion concentrations would therefore seem to be due to an augmentation of acetylcholine release. The partial reversal of the inhibitory effect of gentamicin probably reflects an additional post-junctional site of action; this is further supported by the lesser potentiating effect of neostigmine.

The absence of any effect of these aminoglycoside antibiotics on the responses of the doubly-innervated rabbit colon may, therefore, have been due to the calcium ion concentration of the Krebs solution being sufficiently high to facilitate the release of acetylcholine and of noradrenaline in the presence of these drugs, thus masking any inhibitory action. The gentamicin-induced increase in recovery time following stimulation of the sympathetic nerves of this preparation may be explained in terms of a post-junctional component of the action of this drug; such an effect was not seen with kanamycin.

Complete inhibition of the peristaltic reflex of the guinea-pig ileum can be achieved with a concentration of kanamycin producing only 34% inhibition of the response to transmural electrical stimulation, and by a concentration of gentamicin that produced only 47% inhibition, presumably because the number of cholinergic neurones involved in the peristaltic reflex must be less than the total number activated by transmural stimulation; thus, lower concentrations of these drugs are required for the same degree of inhibition, in these circumstances.

The small potentiations of response to transmural stimulation seen with kanamycin and the raised resting tension sometimes induced by both gentamicin and kanamycin may be related to the increase in resting tone of isolated guinea-pig intestine produced by neomycin, which Popovici, Moisă, Negoită, Manoilă, Botez, Hafner & Gumeni (1965) attributed to a ganglion stimulating action, although under conditions of supramaximal stimulation this seems unlikely to be the underlying mechanism in this case.

Pittinger & Adamson (1972) found that penicillins were without somatic neuromuscular blocking action but, more recently, Singh, Harvey & Marshall (1978) reported a blockade by penicillin V (phenoxymethyl penicillin) using the mouse phrenic nerve-diaphragm preparation. In the present study, the predominantly post-junctional actions of pivmecillinam seem to stem from the pivaloyloxymethyl ester side-chain rather than from the 6β-amidinopenicillanic acid moiety of the molecule, since mecillinam (in the dihydrate hydrochloride form) was without effect. The mechanism of action of the ester component is unclear, although, if its inhibitory actions were related to its hydrolysis by tissue esterases to pivalic acid and formaldehyde (manufacturer's information), its lack of inhibitory effect on the doubly-innervated rabbit colon may reflect differences in the

enzymatic hydrolyzing capacity of the guinea-pig ileum and rabbit colon.

Clindamycin and trimethoprim were the only two of nine antibiotics examined that exerted effects on all four preparations used in this study. Following exposure to trimethoprim, the responses of the isolated ileum to acetylcholine and histamine were decreased, and the response to transmural stimulation depressed even when the calcium ion concentration of the bathing medium was doubled, a condition in which acetylcholine release would be expected to be augmented. These findings stongly suggest that the inhibitory actions of trimethoprim are post-junctional rather than predominantly pre-junctional because, even if ACh release were greater, the ability of the smooth muscle to respond to it is depressed. The effects of trimethoprim on the doubly-innervated rabbit colon may also be attributed to a post-junctional action because depression of the responses to stimulation of parasympathetic nerves was accompanied by decreased responses to applied acetylcholine and the times taken for the tissue to recover following sympathetic nerve stimulation or noradrenaline administration were both increased.

Clindamycin, on the other hand, appeared to have pre- and post-junctional effects depending on the concentration used. The inhibition it produced in the transmurally stimulated guinea-pig ileum and in ileal segments exposed to acetylcholine or histamine (where high concentrations were required) was post-junctional, while the inhibition it produced at lower concentrations (50 μg/ml) in the doubly-innervated rabbit colon was pre-junctional, since the responses to exogenous acetylcholine and noradrenaline were not affected at a time when the responses to nerve stimulation were abolished. The increase in spontaneous activity and resting 'tone' of the rabbit colon exposed to this concentration of clindamycin may be due to a depressant effect on the release of some inhibitory transmitter other than noradrenaline, e.g. from 'purinergic nerves' (Burnstock, 1972) since this effect was seen before the responses to parasympathetic stimulation were completely abolished and some considerable time before any effect of clindamycin was manifest in the tissue response to stimulation of the sympathetic nerves. However, whether differences in the time taken for different nervous elements of the rabbit isolated colon to be affected by clindamycin represent anatomical differences (e.g. fibre size), the presence of ganglia, or a biochemical difference (e.g. in mechanisms of transmitter release) remains to be seen. The 5 to 10 fold difference in the concentrations required to abolish the peristaltic reflex when compared to inhibition of contraction in response to transmural stimulation may also indicate that interneuronal transmission is more sensitive to the effects of clindamycin than is transmitter release from terminals of the final motor neurones.

The increases in the time taken for these antibiotics to depress the responses of the transmurally stimulated ileum in the presence of either neostigmine or 5.08 mM calcium possibly reflect alterations in the rate and ease with which the antibiotic reaches an equilibrium with the particular system on which it acts. Spontaneous recovery may therefore be due, in part, to the reversal of this 'unstable equilibrium' in association with metabolism or inactivation of the drug in question.

Pittman (1979) emphasises the increased severity with which antibiotic associated colitis (AAC) occurs when gastro-intestinal 'tone' is lost, either as a result of chronic laxative abuse, or the self administration of atropine and diphenoxylate (Lomotil) if certain antibiotics are administered to human subjects under these conditions. The antibiotics most commonly associated with AAC are clindamycin, lincomycin, ampicillin, tetracycline derivatives and aminoglycosides (Donta, 1977). A convincing relationship between *C. difficile* toxins and AAC has been established (Bartlett *et al.*, 1978), and it has been demonstrated that *C. difficile* itself is more resistant to antibiotic therapy than other clostridia species (Burdon, Brown, Youngs, Arabi, Shinagawa, Alexander-Williams & Keighley 1979). Therefore, in the light of the evidence presented in this study, it is proposed that an antibiotic e.g. clindamycin may, by decreasing gastro-intestinal motility facilitate the proliferation of *C. difficile* (which in turn would be resistant to the antibiotic), leading to the development of AAC as a result of production of its toxins. Although peak serum concentrations of a drug such as clindamycin are low when compared to the concentrations required to inhibit motility *in vitro*, the observation that the human gastro-intestinal luminal content of this antibiotic is as high as 450 μg/g faeces, following oral administration (George, Sutter & Finegold, 1978), implies that it is quite likely that following oral administration of poorly absorbed antibiotic compounds these drugs may be present in sufficiently high concentration to inhibit gastrointestinal motility, giving rise to the situation described. The AAC occurring with antibiotics that do not inhibit gastro-intestinal motility (e.g. ampicillin) would, therefore, have a different aetiology. Interestingly, the concentrations required for blockade in any of these intestinal preparations are usually less than are required to produce a similar degree of blockade in skeletal muscle preparations.

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