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A SENSITIVE METHOD FOR THE ASSAY OF ANGIOTENSIN

BY

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A method is described for the assay of angiotensin on the rat colon. Under the conditions of the assay, the preparation is sensitive to angiotensin and relatively insensitive to 5-hydroxytryptamine, to histamine and to other substances which might be present in blood. Bradykinin and catechol amines do not interfere with the assay. Evidence is given for the specificity of the angiotensin receptors.

The commonest method for the assay of angiotensin depends on the rise in blood pressure produced by injecting angiotensin intravenously into the rat (Shipley & Tilden, 1947; Skeggs, Kahn & Marsh, 1953) or the cat (Page & Helmer, 1940; Valle, Prado & Prado, 1952). Recently, Feldberg & Lewis (1964) found angiotensin to be a highly potent stimulant of the secretion of catechol amines from the adrenal glands. It is, therefore, possible that catechol amines so released may contribute to or interfere with the pressor response to angiotensin in a variable way, depending on the species from which the angiotensin was derived or on the synthetic analogue chosen as standard.

Other methods for the assay of angiotensin make use of various preparations of isolated smooth muscle. These include the guinea-pig ileum (Collins, 1948; Picarelli, Kupper, Prado, Prado & Valle, 1954); the intestines of the rabbit (Page, 1940) and the toad (Prado, Valle & Picarelli, 1954); and the uterus of rat and rabbit (Ludueña, 1940). Of these, the rat uterus is the most sensitive but is not specific. In an attempt to find an isolated tissue as sensitive as the rat uterus but more specific, we have compared the reactions of different parts of the intestine of the rat, the guinea-pig, the pigeon and the chick to a synthetic angiotensin, α-hypertensin (Ciba) which is identical with bovine angiotensin (Elliot & Peart, 1957).

METHODS

The animals were killed by stunning and bleeding through the carotid arteries. The part of the intestine required was removed, washed, and suspended either in an isolated organ-bath or in a superfusion jacket. The following tissues from the rat were used: stomach strip (Vane, 1957); duodenum (Horton, 1959); middle and terminal ileum; ascending colon (the part which has diagonal striations and is immediately adjacent to the caecum); and descending colon. Other tissues used were guinea-pig ileum and taenia coli; pigeon rectum; and chick rectum (Mann & West, 1950). The isolated organs were suspended either in baths of 5 to 10 ml. capacity or, for superfusion experiments (Gaddum, 1953), in a polypropylene jacket with the solution flowing over the tissue at a constant rate of 12 to 15 ml./min.

The compositions of the bathing solutions (in g/l. of distilled water) were as follows:

Krebs solution: NaCl 6.9, KCl 0.35, $CaCl_2.6H_2O$ 0.55, KH_2PO_4 0.16, $MgSO_4.7H_2O$ 0.29, glucose 1 and $NaHCO_3$ 2.1. This solution was gassed with a mixture of 95% oxygen and 5% carbon dioxide.

Tyrode solution: NaCl 8, KCl 0.2, $CaCl_2.6H_2O$ 0.396, $MgCl_2.6H_2O$ 0.214, NaH_2PO_4 0.05, glucose 1 and $NaHCO_3$ 1. This solution was gassed with oxygen or with 95% oxygen and 5% carbon dioxide.

Rat uterus Ringer solution: NaCl 9, KCl 0.42, $CaCl_2.6H_2O$ 0.06, glucose 0.5 and $NaHCO_3$ 0.5. This solution was gassed with oxygen.

Rat colon Ringer solution (Gaddum, Peart & Vogt, 1949): NaCl 9, KCl 0.4, $CaCl_2.6H_2O$ 0.059, glucose 1 and $NaHCO_3$ 0.15. This solution was gassed with oxygen.

The gases were bubbled vigorously through the reservoir of the salt solutions and, when used, through the organ-bath. The movements of the tissues were recorded on smoked kymograph-paper using auxotonic levers (Paton, 1957) with a magnification of ×16 and an initial load on the tissues of 1 to 4 gm.

The following substances were used: α-L-asparagine[1]-voline[5]-angiotensin II (Ciba, referred to in the text as α-hypertensin) and various analogues of α-hypertensin, as specified in the text; acetylcholine perchlorate, (−)-adrenaline bitartrate, aldosterone (Ciba), bradykinin (Parke Davis), hexamethonium bromide, histamine acid phosphate, 5-hydroxytryptamine creatinine sulphate, hyoscine hydrobromide, (±)-isoprenaline sulphate, mepyramine maleate, methysergide (UML491), morphine sulphate, nicotine acid tartrate, (−)-noradrenaline bitartrate, oxytocin (Pitocin, Parke Davis), Substance P, phenoxybenzamine hydrochloride, pronethalol (I.C.I.), renin (hog renin, Nutritional Biochemical Corporation), reserpine (ampoules, Ciba) and vasopressin (Pitressin, Parke Davis). Doses of salts are expressed as base.

RESULTS

Tissues bathed in Krebs solution. The dose ranges of 5-hydroxytryptamine, acetylcholine, α-hypertensin and bradykinin required to give a contraction of the tissues corresponding to a 3 to 4 cm trace on the kymograph are shown in Table 1 (denoted " C "). When substances caused relaxation, this is denoted by " R " and represents a trace of 0.5 to 3 cm. Of the various tissues examined, the most sensitive to α-hypertensin were the stomach strip and the ascending and descending colon of the rat. However, both the stomach strip and the descending colon were sensitive also to 5-hydroxytryptamine and to bradykinin; all were moderately sensitive to acetylcholine. The ascending rat colon was, therefore, the most specific of these tissues for the assay of angiotensin. Its sensitivity to 5-hydroxytryptamine was low; bradykinin, adrenaline and noradrenaline caused relaxation. When the rat colon was superfused, the sensitivity to bradykinin was decreased whereas the sensitivity to α-hypertensin was increased. Because of this, the following compounds were tested on the superfused rat colon: histamine (10 μg) and uridine diphosphate (100 μg) gave small contractions; renin (up to 2 U), substance P (1700 U/mg, up to 20 U) and nicotine (10 μg) had no effect; and oxytocin (0.3 U), vasopressin (0.1 U) and aldosterone (1 mg) all caused a small relaxation.

Solutions. Three other solutions were used to find out which was best for the assay of angiotensin. On changing from Krebs to Tyrode solution, the spontaneous activity of the rat colon increased and the sensitivity to α-hypertensin decreased. On changing from Krebs solution to rat uterus or rat colon Ringer solution, the colon relaxed and

TABLE 1

SENSITIVITY OF VARIOUS TISSUES TO DIFFERENT DRUGS

Sensitivity is expressed as the dose range, in nanograms per 5 ml. organ-bath, needed to produce a contraction (C) of 3 to 4 cm lever movement, or a relaxation (R) of 0·5 to 3 cm lever movement in a 5 ml. organ-bath.

Preparation	5-Hydroxy-tryptamine	Acetyl-choline	α-Hyper-tensin	Brady-kinin	Nor-adrenaline	Adren-aline
Rat colon (ascending)	100–1,000 C	10–100 C	1–10 C	100–1,000 R	100–1,000 R	100–1,000 R
Rat colon (descending)	10–100 C	10–100 C	1–10 C	10–100 R	>1,000 R	>1,000 R
Rat stomach strip	0·1–5 C	10–100 C	1–10 C	10–100 C or R	>1,000 R	>1,000 R
Rat duodenum	10–100 C	10–100 C	1,000 C	1–10 R	>1,000 R	>1,000 R
Rat ileum (18 cm before caecum)	1,000 C	100–1,000 C	100–1,000 C	100–1,000 C	>1,000 R	>1,000 R
Rat ileum (just before caecum)	100–1,000 C	100–1,000 C	100–1,000 C	10–100 C	>1,000 R	>1,000 R
Guinea-pig ileum	10–100 C	10–100 C	10–100 C	>1,000 C	>1,000 R	>1,000 R
Guinea-pig taenia coli	>1,000 C	—	10–100 C	>1,000 C or R	>1,000 R	>1,000 R
Pigeon rectum	10–100 C	10–100 C	10–100 C	—	100–1,000 R	100–1,000 R
Chick rectum	—	10–100 C	Insensitive to 10,000	—	100–1,000 R	10–100 R

thereafter had less spontaneous activity; however, the sensitivity to α-hypertensin was also reduced. For these reasons Krebs solution was chosen for use in the assay.

Effect of temperature. Lowering the temperature of the bathing fluid somewhat reduced the spontaneous activity without changing the sensitivity to α-hypertensin, but the contractions were greatly prolonged. The experiments were, therefore, performed at 37° C.

p*H effects.* The pH of Tyrode solution was changed from 8.4 (when bubbled with oxygen) to 7.4 (when bubbled with 95% oxygen and 5% CO_2). No consistent change in sensitivity to α-hypertensin was seen.

Reduction of spontaneous activity. In Krebs solution at 37° C the rat colon had considerable spontaneous activity. Other authors (Garcia de Jalon, Bayo Bayo & Garcia de Jalon, 1945; Gaddum, Peart & Vogt, 1949) succeeded in reducing the spontaneous activity of rat smooth muscle by reducing the calcium concentration in the medium and the temperature; in our hands these changes either reduced the sensitivity to α-hypertensin and /or prolonged the contraction. Hyoscine (10^{-6} g/ml.) did not reduce the spontaneous activity, but catechol amines did. Isoprenaline, noradrenaline and adrenaline, in descending order of potency, all relaxed the rat colon and reduced its spontaneous activity. Noradrenaline (10^{-6} to 10^{-7} g/ml.) stabilized the baseline, but the sensitivity to α-hypertensin was much reduced, especially during the first 20 to 30 min. The contractions to α-hypertensin were reduced to about half by noradrenaline (10^{-7} to 10^{-8}), but its presence had the following advantages (Fig. 1): first, the colon was relaxed and its spontaneous activity was reduced; secondly,

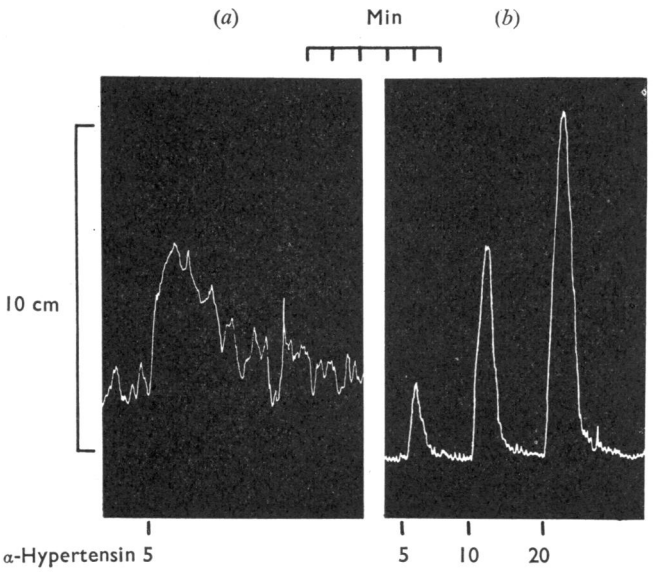

Fig. 1. Rat colon preparation superfused with Krebs solution at 37° C. (*a*) shows the response to α-hypertensin (5 ng). Note the irregular baseline and the duration of the response. (*b*) shows responses with noradrenaline (10^{-7}) in the Krebs solution. The responses to α-hypertensin (5, 10 and 20 ng) are of much shorter duration and the baseline is more stable. Time in minutes. Vertical scale, 10 cm.

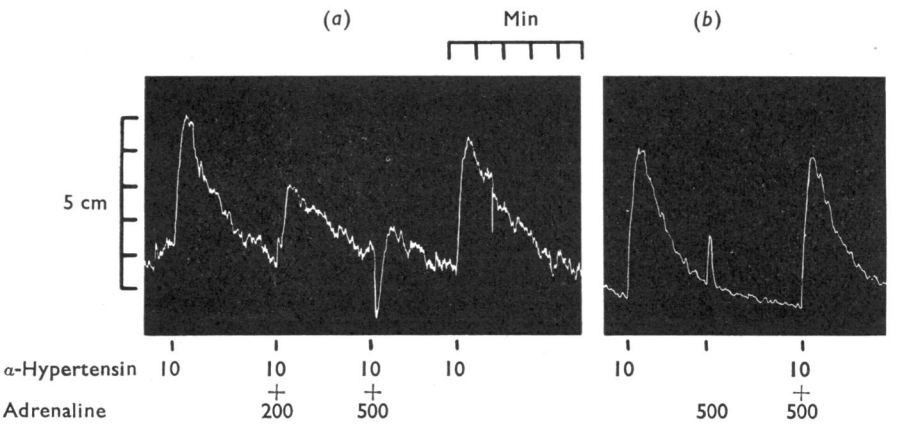

Fig. 2. Rat colon preparation superfused with Krebs solution at 37° C. (*a*) shows the response to α-hypertensin (10 ng) and the effects of adrenaline (200 and 500 ng) mixed with α-hypertensin (10 ng) on the response. (*b*) shows responses with pronethalol (10^{-5}) in the Krebs solution. Adrenaline (500 ng) now produces a small contraction, but this does not add to the response to α-hypertensin. Time in minutes. Vertical scale, 5 cm.

the contractions elicited by α-hypertensin passed off more quickly, allowing doses to be given more frequently; and thirdly, the sensitivity to substances which induced relaxation, such as bradykinin and catechol amines, was diminished, so that the presence of these substances in blood samples interfered less with the assay of angiotensin.

Two other substances were used in attempts to decrease spontaneous activity. Bradykinin (10^{-8}) reduced spontaneous activity but also reduced to a greater extent than noradrenaline the contractions produced by α-hypertensin. Pronethalol (10^{-5} to 10^{-6}) relaxed the rat colon, inhibited its spontaneous movements, and sometimes reduced its sensitivity to α-hypertensin slightly. In the presence of pronethalol, adrenaline and noradrenaline no longer caused relaxation and reduction of the response to α-hypertensin, but instead contraction; this, however, did not interfere with the response to α-hypertensin (Fig. 2).

Specificity of the receptors for α-hypertensin. Some evidence for the specificity of the receptors for α-hypertensin has already been given. Thus, α-hypertensin caused contraction whereas other polypeptides such as bradykinin, vasopressin, and pitressin, caused relaxation; so did the catechol amines. To define further the specificity of the receptors for α-hypertensin, antagonists of other contractor substances were used. Methysergide (10^{-5} to 10^{-6}) reduced the effect of 5-hydroxytryptamine but not that of α-hypertensin. Hyoscine (10^{-6}) abolished the response to acetylcholine, and mepyramine (10^{-6}) that to histamine, but neither antagonist affected the response to α-hypertensin. The response to α-hypertensin was not decreased by hexamethonium (up to 10^{-4}), by morphine (up to 10^{-6}), or by phenoxybenzamine and reserpine except in concentrations high enough (10^{-6}) to reduce the effects of acetylcholine and other contractor substances.

In some experiments, 5-hydroxytryptamine (10^{-8}), bradykinin (5×10^{-9}) or acetylcholine (10^{-8}) were added to the superfusion fluid. This diminished the responses to injections of the respective substance much more than to injections of α-hypertensin. Similarly, when α-hypertensin (5×10^{-9}) was present in the superfusion fluid, the effects of injections of α-hypertensin were reduced much more than those of 5-hydroxytryptamine, bradykinin or acetylcholine.

In other experiments, large doses of 5-hydroxytryptamine (1 to 10 μg) or acetylcholine (1 to 10 μg) were injected. During the recovery phase after its contraction, the rat colon was less sensitive to further doses of these substances whilst the sensitivity to α-hypertensin was unchanged. After a large dose of α-hypertensin (1 to 10 μg), the rat colon was desensitized to α-hypertensin for a period of 0.5 to 1 hr., but it was still sensitive to 5-hydroxytryptamine and to acetylcholine.

Effects of hypertensin analogues. The order of activities of twelve polypeptide analogues of α-hypertensin on the rat colon corresponded closely (Table 2) with their pressor activities in nephrectomized rats (Gross & Turrian, 1960; Brunner & Regoli, 1962).

Estimation of angiotensin in blood. Blood samples were taken from control rats, from rats which had been nephrectomized during ether anaesthesia on the previous day and from nephrectomized rats injected intravenously with renin (0.2 U/100 g)

TABLE 2

RELATIVE ACTIVITY ON THE RAT BLOOD PRESSURE AND THE RAT COLON OF ANALOGUES OF α-HYPERTENSIN

*Gross & Turrian (1960) ; † Brunner & Regoli (1962)

Analogue	Activity	
	Pressor	On the rat colon
H.Asp–Arg–Val–Tyr–Val–His–Pro–Phe.OH (α-L-Asp[1]-angiotensin II)	1*	1
H.Asp–Arg–Val–Tyr–Val–His–Pro–Phe.OH (β-L-Asp[1]-angiotensin II)	1·5†	1·0
H.Asp(NH_2)–Arg–Val–Tyr–Val–His–Pro–Phe.OH (α-L-Asp(NH_2)-angiotensin II)	1·0*	0·8–1·0
H.*Gly*–Arg–Val–Tyr–Val–His–Pro–Phe.OH	0·5*	0·8
H.Arg–Val–Tyr–Val–His–Pro–Phe.OH	0·5*	0·8
H.Asp(NH_2)–Arg–Val–*X*.Val–His–Pro–Phe.OH (X=NH.CH(CH_2.C_6H_4OH).*NH*.CO.)	0·3*	0·12
H.Asp(NH_2)–Arg(*NO_2*)–Val–Tyr–Val–His–Pro–Phe.OH	0·5*	0·1
H.Asp(NH_2)–Arg–Val–*Phe*–Val–His–Pro–Phe.OH	0·1*	0·05
H.Asp(NH_2)–*Orn*–Val–Tyr–Val–His–Pro–Phe.OH	0·2*	0·03
H.Val–Tyr–Val–His–Pro–Phe.OH	0·01*	0·001
H.Asp(NH_2)–Arg–Val–*Tyr*–*Tyr*–Val–His–Pro–Phe.OH	0·01*	0·0008
H.Asp(NH_2)–Arg–Val–D–*Tyr*–Val–His–Pro–Phe.OH	0·01*	0·00025
H.Asp(NH_2)–Arg–Val–Tyr–Val–His–Pro.*OH*	0·0005*	0·00002

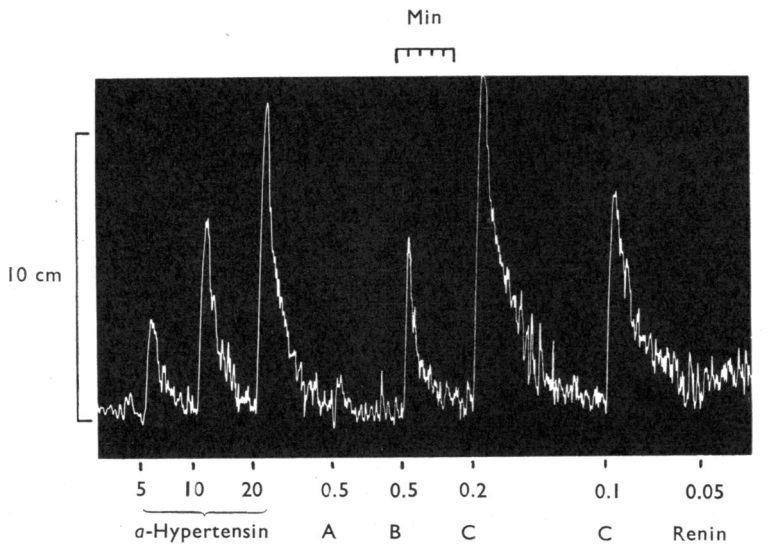

Fig. 3. Rat colon preparation superfused with Krebs solution containing pronethalol (10^{-5}) at 37°C. Responses to α-hypertensin (5, 10 and 20 ng), to renin (0·05 U) and to three rat plasmas (doses in ml.). (A) pooled plasma from three rats nephrectomized on the previous day. (B) pooled plasma from three control rats. (C) plasma from a rat nephrectomized on the previous day, but injected with renin (0·2 U/100 gm, intravenously, 3 min before bleeding). Time in minutes. Vertical scale, 10 cm.

3 min before bleeding. The rats were anaesthetized with ether and blood from the carotid artery was collected in polyethylene tubes kept at 0° C in iced water; edetic acid (0.2 M) was used as an anticoagulant. The blood samples were centrifuged at 3,000 revs/min for 10 min in a refrigerated centrifuge and the plasma was siphoned off with a polyethylene tube and kept at 0° C. Fig. 3 shows the assay of the three pooled plasma samples. No activity could be detected in the plasma from nephrectomized rats. An activity corresponding to 15 to 20 ng/ml. of angiotensin was found in plasma of control rats, and 100 to 200 ng/ml. in plasma of nephrectomized rats which had received renin.

DISCUSSION

The results show that the ascending rat colon is a sensitive and comparatively specific organ for the assay of angiotensin. The only substances which give a clear pharmacological antagonism to the response of isolated tissues to angiotensin are phenoxybenzamine and, in the guinea-pig ileum, atropine and morphine (Khairallah & Page, 1961). The antagonism of phenoxybenzamine to angiotensin is probably nonspecific and the stimulant action of angiotensin on nervous elements seems to be unique to the guinea-pig ileum. Because there is, as yet, no specific antagonist for angiotensin, it is particularly important that a method for its assay should be as selective as possible. It is for this reason that we chose the rat colon; apart from angiotensin it responds to irin but this is not present in plasma (Ambache, 1959). The rat uterus and rat stomach strip respond not only to angiotensin but also to 5-hydroxytryptamine and to bradykinin. Moreover, assays of angiotensin on the blood pressure of the rat may well suffer from interference by release of catechol amines.

As an assay organ the rat colon has two main disadvantages. The first is that it has a high spontaneous activity; other workers eliminated this by reducing the temperature or the calcium concentration of the bathing solution. The second is that catechol amines reduce the response to angiotensin. We have tried to eliminate these two disadvantages by adding noradrenaline or pronethalol to the bathing solution. Noradrenaline relaxes the rat colon, abolishes its spontaneous activity and diminishes the interference with the angiotensin assay by catechol amines and also by bradykinin. The disadvantage of noradrenaline is that it reduces the sensitivity to angiotensin. Pronethalol also relaxes the rat colon and reduces its spontaneous activity. In the assay of angiotensin, pronethalol diminishes interference by bradykinin and completely abolishes that by the catechol amines. Again, however, the sensitivity to angiotensin itself is lessened. Both substances somewhat reduced the duration of the response to angiotensin; this is an advantage for an assay technique.

There seems to be some confusion about the sensitivity of the rat colon to Substance P, 5-hydroxytryptamine and angiotensin. Dalgleish, Toh & Work (1953) used the rat colon for estimating 5-hydroxytryptamine but found that it contracted only to as much as 1 to 2 μg of 5-hydroxytryptamine creatinine sulphate in an 18 ml. organ-bath; this corresponds with the order of sensitivity that we found. They also found the rat colon contracted to 1 to 2 mg of crude Substance P. Bisset & Lewis (1962) calculated the sensitivity to be about 1 ng/ml. of pure Substance P. However, Cleugh, Gaddum, Mitchell, Smith & Whittaker (1964) found the rat colon to be

insensitive to their purer preparation; we have confirmed this. Bisset & Lewis (1962) also reported that a concentration of 500 ng/ml. of angiotensin was necessary to contract the rat colon. On re-examination of their records, Bisset & Lewis (personal communication) agree that the rat colon is sensitive to about 1 ng/ml. of angiotensin and that the figure in their paper was an error. Bisset & Lewis (1962) also agree with our results that the rat colon is relatively insensitive to bradykinin, oxytocin and vasopressin.

Without the use of specific inhibitors it is difficult to characterize receptors for a particular substance, but our experiments with desensitization and with the various analogues of angiotensin show that the receptors for angiotensin in the rat colon are specific and different from those for other contractor substances, such as 5-hydroxytryptamine, acetylcholine and histamine.

The relative potencies of the various analogues of angiotensin correspond well with their relative potencies on the rat blood pressure: this is evidence that, in the rat, the receptors for angiotensin in the colon are similar to those of vascular smooth muscle.

Our results show that plasma taken from nephrectomized rats does not contain a substance which contracts the rat colon whereas both normal plasma and plasma from nephrectomized rats injected with renin contain a contractor substance which is presumably angiotensin. However, in the control samples, the levels of angiotensin found were probably much higher than normal because of the rapid bleeding by which the samples were obtained. Therefore, for the assay of angiotensin by the rat colon, it is only necessary to take blood samples under conditions which prevent the generation of large quantities of bradykinin and do not cause gross circulatory changes.

For the assay of angiotensin we have preferred to use superfusion. If the rate of superfusion were reduced or if the colon were suspended in a bath of small capacity, its sensitivity to angiotensin might be further increased.

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