Br. J. Pharmac. (1974), **51**, 73-79

A STUDY OF THE METABOLIC REQUIREMENTS FOR THE CONTRACTILE ACTION OF ANGIOTENSIN UPON GUINEA-PIG ILEUM

ANN D. CROCKER & K.A. WILSON

The Pharmacology Laboratories, The Department of Pharmacy, University of Aston in Birmingham, Gosta Green, Birmingham B4 7ET

1 Acetylcholine and angiotensin elicit a contraction of guinea-pig ileum. The metabolic requirements for these actions have been investigated.

2 The angiotensin-induced response was far more dependent upon the presence of glucose than was the response to acetylcholine.

3 Anoxia and oxidative enzyme inhibition preferentially reduced the angiotensin-induced response.

4 The significance of these results is discussed. It is concluded that the response to angiotensin is dependent upon an ATP source distinct from that required by the contractile process. It is further shown that this energy-dependent stage cannot be identified with the indirect, cholinergic component of the angiotensin response in this tissue.

Introduction

The contractile response of many smooth muscle preparations to angiotensin is well documented and has been reviewed (Khairallah, 1971, Gross, 1971). The action of angiotensin on guinea-pig ileum has been shown to consist of two components (Khairallah & Page, 1961; Robertson & Rubin, 1962; Godfraind, Kaba & Polster, 1966a, b), a direct action upon smooth muscle cells and an indirect action through cholinergic nerves which can be blocked by atropine.

The indirect component has been the subject of many reports and appears to be primarily due to stimulation at a post-ganglionic site (Panisset, 1967). However, evidence concerning the nature of the direct response is conflicting (see review Khairallah, 1971).

In the present study we have investigated the metabolic requirements for the contractile response of guinea-pig ileum to angiotensin, in order to elucidate the mechanism of its direct action on this preparation.

A preliminary account of some of these findings has been given (Crocker & Wilson, 1972).

Methods

Preparation

Segments of terminal ileum, 2 to 3 cm in length, were taken from young male guinea-pigs, killed by a blow to the neck. Only animals in a weight range of 200 to 300 g were used and they were all starved for 12 h before the experiment. Preparations were suspended in 20 ml organ baths containing Tyrode solution bubbled with air and maintained at 28°C. The composition of the normal Tyrode solution was as follows (mM): NaCl 137, KCl 2.7, $MgSO_4.7H_2O$ 1.1, $NaH_2PO_4.2H_2O$ 0.42, $NaHCO_3$ 11.9, $CaCl_2.2H_2O$ 1.8, glucose 5.6.

In order to deprive preparations of oxygen, the Tyrode solution was placed under vacuum for 4 hours. The vacuum was replaced by oxygen-free nitrogen and the Tyrode reservoir was gassed with nitrogen under a nitrogen atmosphere. During these experiments the organ baths were gassed with nitrogen instead of air.

In the experiments involving carbon monoxide, the gas was bubbled through the organ baths in the absence of air. Coceani & Wolfe (1966) used simultaneous administration of air and carbon monoxide during experiments upon isolated rat uterus. This technique was investigated, but failed to yield consistent results. Carbon monoxide was supplied by BDH and was of 99.5% purity.

Recordings

Recordings were made isotonically with Devices 2LD01 isotonic transducers, the output of which

was fed into Devices M2 recorders. In all experiments, preparations were loaded with 2 grams. An equilibration period of 1.5 h was allowed before the start of experiments. Contractile responses were measured at the maximum sustained deviation from the baseline. A tissue contact time of 90 s was employed for both angiotensin and acetylcholine. This is sufficient to allow the recording of both components of the angiotensin response. (Godfraind *et al.*, 1966a).

In all experiments, concentrations of acetylcholine and angiotensin were selected which produced responses approximately equal to 50% of the maximum response to acetylcholine.

Electrical stimulation

In some experiments, the ileum was stimulated electrically through co-axial electrodes (Paton, 1955) with supramaximal (usually about 30 V) rectangular wave pulses. Repetitive stimuli at a frequency of 20 Hz were used to produce sustained contractions of 10 s duration. A constant pulse width of 3 ms was employed.

Drugs

The following drugs were used: angiotensin II (Val 5-angiotensin II, Asp-β-amide, hypertensin, Ciba), acetylcholine chloride (BDH), hyoscine hydrobromide (BDH) and histamine acid phosphate (BDH). Angiotensin II was stored as a sterile solution at a concentration of 10^{-5} M. Test drugs were prepared in Tyrode solution and each was added to the bath in a volume of 0.2 ml.

Calculation of results

All results have been expressed as a percentage reduction of the control response taken in normal Tyrode gassed with air. The control responses were measured when three successive exposures to the selected concentration of spasmogen had induced contractions of equal size. For each treatment, the reported results are the means of values obtained from at least six tissues. The effect of the experimental treatments has been analysed by use of Student's *t* test.

Results

Effects of altering the glucose concentration of Tyrode solution

The glucose concentration in the Tyrode solution was increased or decreased from the normal concentration of 5.6 mM and the responses to acetylcholine and angiotensin were recorded. It was found that progressively increasing the glucose concentration to 44.8 mM had no effect on either agonist, but that reducing the concentration had marked effects.

The reduction in the response to acetylcholine was 10.8 ± 3.6% and 11.8 ± 1.7% at concentrations of 2.8 mM and 1.4 mM respectively. In contrast, the angiotensin responses were reduced by 24.4 ± 12.9% at 2.8 mM and 47.9 ± 3.5% at 1.4 mM. There was a significant difference ($P < 0.001$) between the reduction in the responses to acetylcholine and angiotensin at the lower glucose concentration of 1.4 mM. It appeared that angiotensin was more dependent upon the availability of glucose for the initiation of a contraction than was acetylcholine.

Effects of removing glucose from Tyrode solution

Since a lowering of glucose concentration produced a preferential reduction of the angiotensin response, the effect of complete glucose removal was investigated with respect to time. To avoid any possible effects due to changes in osmolarity of the Tyrode solution, mannitol (5.6 mM) was substituted for glucose. It was found that during a 20 min exposure to glucose-free Tyrode, there was a consistent difference between the percentage reduction of the responses to acetylcholine compared with those to angiotensin (see Figure 1). After 10 min the angiotensin response was reduced by 27% and by 68% after 20 min, while the reductions in the acetylcholine responses were only 4% and 27% respectively. The difference in the percentage reductions of the acetylcholine compared with the angiotensin responses was significant throughout the exposure to glucose-free solution ($P < 0.01$), and also during the first 10 min of recovery.

Effects of nitrogen-induced anoxia upon responses to angiotensin and acetylcholine

Figure 2 shows the percentage reductions in responses to angiotensin and acetylcholine which occurred during a period of oxygen deprivation. The ileal preparations were suspended in oxygen-free Tyrode and gassed with nitrogen. There is a marked difference in the percentage reductions of the angiotensin- and acetylcholine-induced contractions, which was highly significant ($P < 0.001$) throughout the phase of anoxia. The sensitivity of the angiotensin response to oxygen deprivation contrasted with the absence of any consistent effect upon the acetylcholine response. These findings suggested that oxidative pathways of metabolism might be involved in the mechanisms

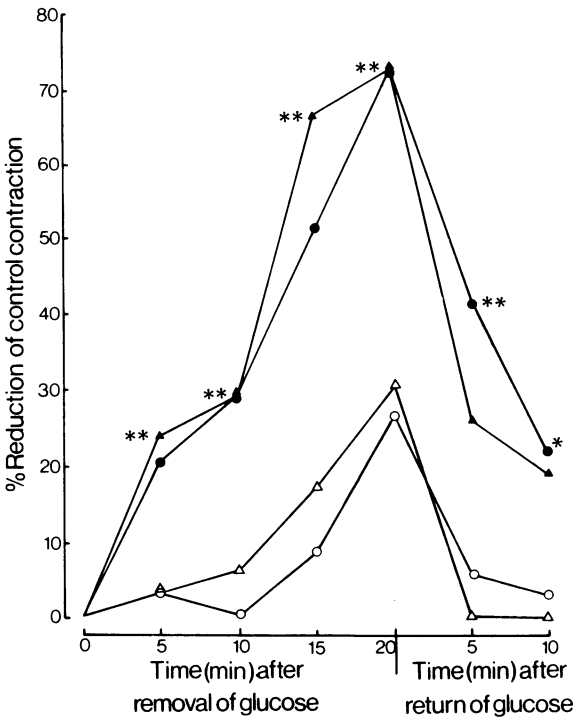

Fig. 1 The effects of the removal of glucose, or its replacement with mannitol, upon the contractile responses of guinea-pig ileum to angiotensin and acetylcholine. ○ and ● Mean % reduction of the acetylcholine (open symbols) and angiotensin (solid symbols) responses. Triangles denote the inhibition pattern produced by glucose removal and circles that produced when glucose was replaced by mannitol. ** $0.001 < P < 0.01$, * $0.01 < P < 0.05$, when comparing % reductions of angiotensin responses with acetylcholine responses obtained under the same experimental conditions.

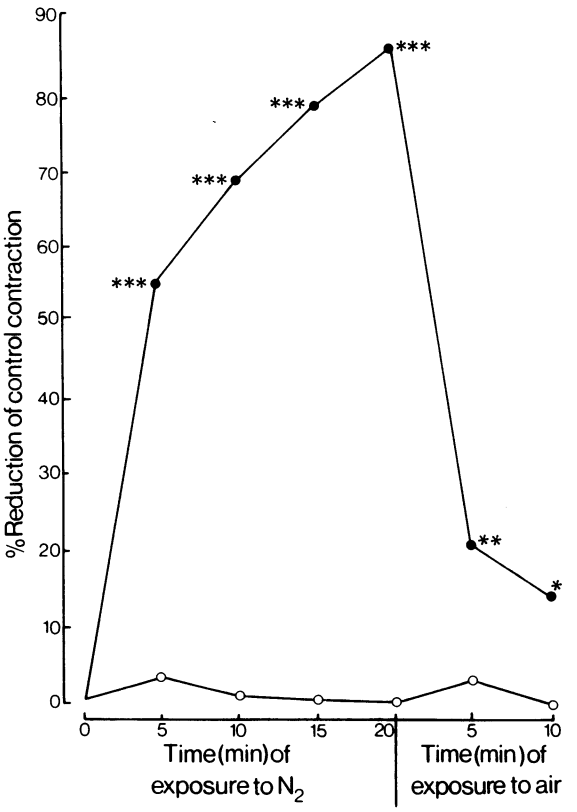

Fig. 2 The effects of nitrogen-induced anoxia upon the contractile responses of guinea-pig ileum to angiotensin and acetylcholine. Mean % reduction of the responses to (○) acetylcholine (ACh) and (●) angiotensin. *** $P < 0.001$; ** $0.001 < P < 0.01$; * $0.01 < P < 0.05$.

by which angiotensin elicited a contraction of smooth muscle cells.

Effects of carbon monoxide

Carbon monoxide, a specific inhibitor of the electron transport chain, was used to investigate further the angiotensin response. Figure 3 shows the percentage reductions of the responses to angiotensin and acetylcholine which occurred during a 16 min exposure to carbon monoxide. The angiotensin response was reduced by 47.6% after 9 min and 66.6% after 16 minutes. Small but consistent percentage increases were observed in the responses to acetylcholine throughout the 16 min exposure to carbon monoxide. The difference between the percentage reductions of the two agonists was highly significant ($P < 0.001$).

Effects of hyoscine

In all the preceding experiments there was a marked difference in the percentage reductions of the angiotensin- and acetylcholine-evoked contractions. However, the response to angiotensin of guinea-pig ileum is known to consist of two components. Therefore, hyoscine hydrobromide was added to the Tyrode solution in order to block the indirect, cholinergic component of the response. The effect of glucose lack upon the remaining direct component was then investigated. Figure 4 shows the percentage reductions of

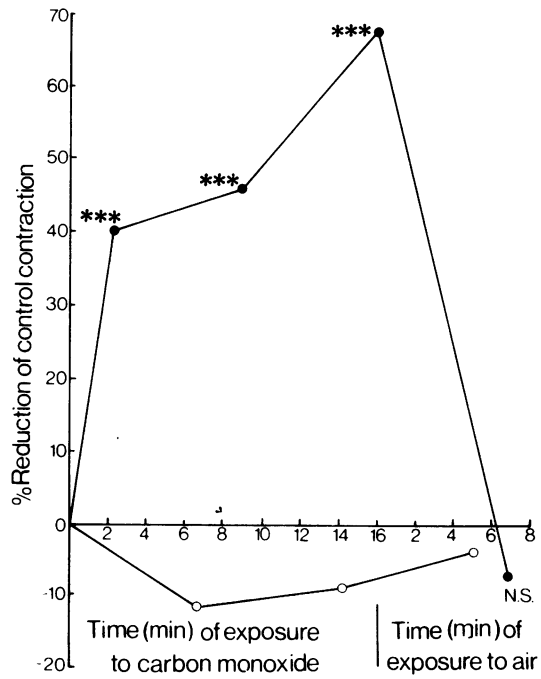

Fig. 3 The effects of carbon monoxide upon the contractile responses of guinea-pig ileum to angiotensin and acetylcholine. Mean % reduction of the responses to (○) acetylcholine and (●) angiotensin. *** $P < 0.001$; N.S. $P > 0.05$, when comparing the percentage reduction of the acetylcholine response with that of the immediately preceding angiotensin response.

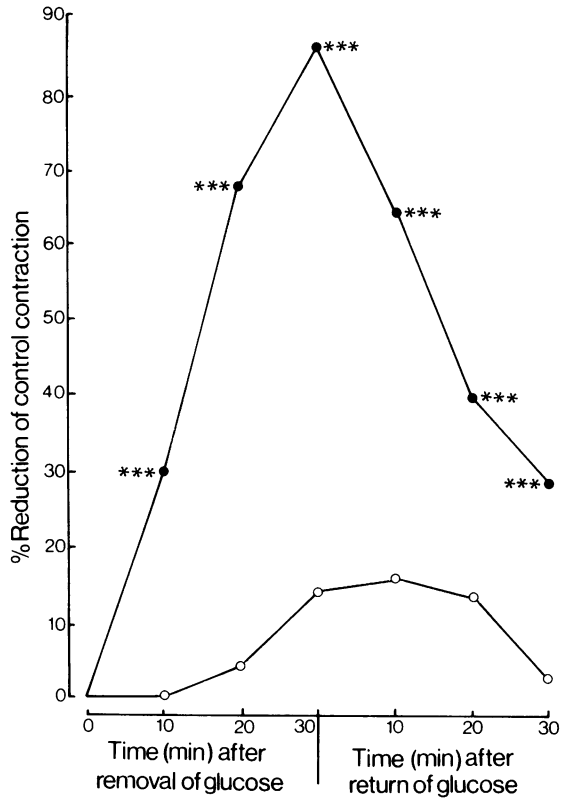

Fig. 4 The effects of the complete removal of glucose from the Tyrode solution upon contraction of guinea-pig ileum to angiotensin and histamine in the presence of 2×10^{-7} M hyoscine. Mean % reduction of responses to (○) histamine and (●) angiotensin respectively. *** $P < 0.001$, when comparing the percentage reduction of the histamine response with that of the immediately preceding angiotensin response.

angiotensin responses during a 30 min exposure to glucose-free Tyrode containing hyoscine hydrobromide, 2×10^{-7} M. Histamine was used as a control in these experiments, and the corresponding percentage reductions in these responses are also shown in Figure 4. It may be seen that the direct component of the angiotensin response was very sensitive to glucose lack, and was reduced 30% after 10 min and 86% after 30 minutes. Responses to histamine were only slightly reduced, and decreased by 14.2% after 30 minutes. The difference in the percentage reductions of the two agonists was highly significant ($P < 0.001$) throughout the phase of glucose absence and also during the first 30 min of recovery.

Transmural electrical stimulation and glucose-lack

Table 1 shows the percentage reductions of responses to sustained electrical stimuli when ileal preparations were exposed to glucose-free Tyrode solution for 30 minutes. Contractions were elicited every 10 minutes. The percentage reduction was 7% after 10 min, increasing to 20.5% after 30 minutes. Co-axial electrodes of the type used by Paton (1955) were employed and the responses elicited are reported to be due to stimulation of post-ganglionic, parasympathetic nerve fibres (Paton, 1955, Day & Vane, 1963). This was confirmed by the use of hyoscine hydrobromide. At a concentration of 2×10^{-7} M, responses to stimulation were reduced by 80 to 90%. The difference between the percentage reductions of the electrical responses and those of the direct component of the angiotensin response (see previous section) were highly significant ($P < 0.001$) throughout the phase of glucose absence (Table 1).

Table 1 Percentage reduction of responses of guinea-pig ileum to co-axial electrical stimulation due to absence of glucose from the Tyrode solution, compared to the percentage reduction of the direct component of the angiotensin response

Time of absence of glucose	*% Reduction of electrical response*	*% Reduction of angiotensin response (direct component)*	P
10	7.0 ± 1.9 (7)	29.5 ± 3.6 (8)	<0.001
20	8.1 ± 2.8 (7)	68.0 ± 4.4 (8)	<0.001
30	20.3 ± 4.2 (7)	86.7 ± 3.0 (8)	<0.001

Number of observations in parentheses.
Results are mean ± s.e.

Discussion

The effect of withdrawing glucose and oxygen from *in vitro* smooth muscle preparations has been extensively investigated (Gross & Clark, 1923; Garry, 1928; Prasad, 1935; Feldberg & Solandt, 1942). The resulting progressive decline in mechanical activity, both spontaneous and induced, has been attributed to an inhibition of the energy processes within the muscle. In the present study, the effect of substrate depletion and anoxia were used primarily to compare the modes of action of two spasmogens.

The contractile action of angiotensin upon guinea-pig ileum has been shown to consist of two components, a direct one and an indirect one via parasympathetic nervous elements (Khairallah & Page, 1961; Robertson & Rubin, 1962; Godfraind *et al.*, 1966a, b). In the first series of experiments we made no attempt to separate these components, either by the use of chemical inhibitors (Khairallah & Page, 1961; Robertson & Rubin, 1962) or by the selection of recording techniques (Godfraind, *et al.*, 1966). Therefore, the angiotensin response that was recorded and measured, was the combination of the individual contributions of these two components and was compared with the response to acetylcholine under the same experimental conditions.

The primary action of acetylcholine on intestinal smooth muscle cells has been reported to involve an increase in the passive conductance of the membrane to sodium ions (Bolton, 1972), and therefore the energy requirements of an acetylcholine-induced contraction are those of the muscle contraction process itself. In our experiments, responses to acetylcholine were used as an indication of the ability of the muscle to contract under conditions where certain energy yielding processes were impaired. If, under these conditions, there was a greater reduction of the angiotensin response than of the acetylcholine response, then this is an indication that there is an impairment of an energy-dependent stage in the angiotensin response which is distinct from the actual contraction process.

Removal of glucose from the Tyrode solution was found to cause a preferential reduction of the angiotensin response compared with the acetylcholine response which indicated that glucose was involved in certain stages of the angiotensin response that have no parallel in the acetylcholine response. The possibility that glucose was required as a source of energy for part of the angiotensin interaction with smooth muscle cells was investigated by the use of nitrogen. Although there was a marked reduction in the angiotensin response, the responses to acetylcholine were unaffected by anoxia, confirming the reports of Day & Vane (1963). Since glucose was present, anaerobic glycolysis would be expected to function, but the reduction of the angiotensin response indicated that this was not an adequate energy source. Evidently, aerobic metabolism was necessary to support the angiotensin response and hence the significance of glucose lack in the previous experiments.

These findings were confirmed by the use of carbon monoxide, which is an inhibitor of cytochrome oxidase and therefore blocks the production of adenosine 5′-triphosphate (ATP) by oxidative phosphorylation. Exposure of tissues to carbon monoxide resulted in a large reduction of the response to angiotensin. However, this was not due to an impairment of the ability of the muscle to contract, since responses to acetylcholine were unaffected, an observation which has also been reported for rat uterus. (Coceani & Wolfe, 1966). It was therefore concluded that ATP was involved in the angiotensin response at some stage prior to the contraction process.

In this, and in all the other experiments, the recovery of the responses to acetylcholine and angiotensin were investigated to ensure that there was no permanent damage to the tissue which might lead to a non-specific reduction of induced

contractions. No treatment produced any indication of tissue damage during the exposure times used, although all forms of metabolic inhibition have been shown to do so when exposures are extended. (Gross & Clark, 1923; Garry, 1928; Prasad, 1935; Feldberg & Solandt, 1942; West, Hadden & Farah, 1951). The differences between the rates of recovery for acetylcholine and angiotensin appeared to be a reflection of the initial reduction in the response produced by the experimental treatment.

All these experiments involved a study of both components of the angiotensin response, i.e. direct and indirect, although the final mediator of the indirect response is acetylcholine, which in our experiments was little reduced by any of the treatments. Nevertheless the nervous transmission giving rise to its release could well be affected; and inactivation of nervous tissues has been reported both under conditions of anoxia (Gross & Clark, 1923; Garry, 1928; West *et al.*, 1951; Day & Vane, 1963), and during cooling (Blair & Clark, 1956; Innes, Kosterlitz & Robinson, 1957). Therefore, before attempting to interpret the previous experiments, it was necessary to ensure that the treatments were indeed affecting the direct component of the angiotensin response.

This was done by the use of hyoscine and in these experiments the ability of the muscle to contract was controlled with histamine. Removal of glucose from the Tyrode solution in the presence of 2×10^{-7} M hyoscine resulted in an 80% reduction of the angiotensin response and an 11% reduction of the control response to histamine after 30 minutes. This was confirmation that the experimental treatments did affect the direct component of the angiotensin response.

A series of experiments was also performed where ileal segments were electrically stimulated through co-axial electrodes. Removal of glucose from the Tyrode solution for 30 min produced only a 17% reduction in the contractions. It is known that the contractions of co-axially stimulated guinea-pig ileum are largely due to activation of parasympathetic nervous elements (Paton, 1955; Day & Vane, 1963). These experiments further support the conclusion that the reductions in the angiotensin responses produced by the various treatments are not solely due to an effect on the indirect, cholinergic mechanism. In addition, it was shown that hyoscine 2×10^{-7} M is effective in inhibiting electrically induced contractions. It may therefore be presumed that the same concentration would also be effective in inhibiting the indirect component of the angiotensin response.

It has been shown that the energy-sensitive stage of the angiotensin response is separate from the indirect cholinergic component of its action on guinea-pig ileum. In fact it would appear that it is the direct component which is energy dependent, although comcomitant effects of these treatments upon the other component cannot be excluded. Our results, therefore, indicate that the action of angiotensin upon guinea-pig ileum is fundamentally different from that of acetylcholine. It appears that the angiotensin-induced contraction is dependent upon the presence of an ATP source distinct from that required by the contractile process. It further appears that the ATP involved in the angiotensin interaction must be supplied by oxidative phosphorylation. The nature of the ATP-dependent mechanism is as yet unresolved.

K.A. Wilson acknowledges a training grant from the Medical Research Council.

References

BLAIR, M.R. & CLARK, B.B. (1956). An evaluation of the action of substance P on the jejenum of the rabbit. *J. Pharmac. exp. Ther.*, **117**, 467-477.

BOLTON, T.B. (1972). The depolarizing action of acetylcholine or carbachol in intestinal smooth muscle. *J. Physiol., Lond.*, **220**, 647-671.

COCEANI, F. & WOLFE, L.S. (1966). On the action of prostaglandin E and prostaglandin from the brain on the isolated rat stomach. *Can. J. Physiol. Pharmac.*, **44**, 933-950.

CROCKER, A.D. & WILSON, K.A. (1972). A preliminary investigation into the metabolic requirements of the response of guinea-pig ileum to angiotensin. *Br. J. Pharmac.*, **45**, 150-151P.

DAY, M. & VANE, J.R. (1963). An analysis of the direct and indirect actions of drugs on the isolated guinea-pig ileum. *Br. J. Pharmac. Chemother.*, **20**, 150.

FELDBERG, W. & SOLANDT, O.M. (1942). The effects of drugs, sugars and allied substances on the isolated small intestine of the rabbit. *J. Physiol., Lond.*, **101**, 137-171.

GARRY, R.C. (1928). The effect of oxygen lack on surviving smooth muscle. *J. Physiol., Lond.*, **66**, 235-248.

GODFRAIND, T., KABA, A. & POLSTER, P. (1966a). Dissociation into two contractile components of the isolated guinea pig ileum response to angiotensin. *Arch. Int. Pharmacodyn.*, **163**, 226.

GODFRAIND, T., KABA, A. & POLSTER, P. (1966b). Specific antagonism to the direct and indirect action of angiotensin on isolated guinea pig ileum. *Br. J. Pharmac. Chemother.*, **28**, 93-104.

GROSS, L. & CLARK, A.J. (1923). The influence of oxygen supply on the response of the isolated intestine to drugs. *J. Physiol., Lond.*, **57**, 457-460.

GROSS, F. (1971). Angiotensin. In: *International*

Encyclopedia of Pharmacology and Therapeutics, Section 72, Volume 1. Pharmacology of naturally occurring polypeptides and lipid-soluble acids, pp. 73-286. Oxford and New York: Pergamon Press.

INNES, I.R., KOSTERLITZ, H.W. & ROBINSON, J.A. (1957). The effects of lowering the bath temperature on the responses of the isolated guinea pig ileum. *J. Physiol., Lond.*, **137**, 396-409.

KHAIRALLAH, P.A. (1971). The Pharmacology of Angiotensin. In: *Kidney Hormones*, ed. Fisher, J.W., pp. 129-171. London, New York: Academic Press.

KHAIRALLAH, P.A. & PAGE, I.H. (1961). The mechanisms of action of angiotensin and bradykinin on smooth muscle *in situ*. *Am. J. Physiol.*, **200**, 51-57.

PANISSET, J.C. (1967). Effect of angiotensin on the release of acetylcholine from preganglionic and postganglionic nerve endings. *Can. J. Physiol. Pharmac.*, **45**, 313-317.

PATON, W.D.M. (1955). The response of the guinea pig ileum to electrical stimulation by coaxial electrodes. *J. Physiol., Lond.*, **127**, 40-41P.

PRASAD, B.N. (1935). The mechanical activity of gut muscle under anaerobic conditions. *J. Physiol., Lond.*, **85**, 249-266.

ROBERTSON, P.A. & RUBIN, D. (1962). Stimulation of intestinal nervous elements by angiotensin. *Br. J. Pharmac. Chemother.*, **19**, 5-12.

WEST, T.C., HADDEN, G. & FARAH, A. (1951). Effect of anoxia on responses of the isolated intestine to various drugs and enzyme inhibitors. *Am. J. Physiol.*, **164**, 565-572.

(Received June 25, 1973)