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Pharmacology of Angiotensin[1]

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I. Introduction

Angiotensin is a natural octapeptide which is presumed to act both as local tissue hormone, within the kidney (32, 171, 242, 278, 319) and as true hormone, on the peripheral vessels and the adrenal glands (42, 228, 330).

Systematic pharmacological studies of this peptide started with the identification of its chemical structure by Skeggs *et al.* (303) and by Elliott and Peart (71) and the subsequent synthesis by Schwyzer *et al.* (289) and by Schwarz *et al.* (287).

Publications are available on about 200 analogues of angiotensin, which have been studied to explore the structure-activity relationship and to find specific inhibitors. A review of this size cannot give a precise and detailed description of all these compounds: an account of the work done before 1966 will be found in the review by Schröder and Lübke (283) and in the book, *Renal Hypertension*, edited by Page and McCubbin (202).

This review was completed in May 1973. With few exceptions it covers papers published before March 1973. The rules of the IUPAC-IUB Commission on Biochemical Nomenclature (125) have been followed as far as possible for the designation of synthetic peptides.

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Here, we will consider groups of angiotensin analogues in which minor structural changes have been made in order to study separately the steric or the chemical functional aspects of single amino acids. Results of conformational analyses and the models proposed for the secondary structure of angiotensin in solution will also be reviewed. Recent developments in the basic pharmacology of this octapeptide will receive major emphasis as well as the study of its mechanism of action at the tissue and cellular level. Beyond the scope of this review are the multiple actions of angiotensin *in vivo*, recently reviewed by Khairallah (156) and by Gross (106).

II. General Pharmacology of Angiotensin

Although peptides related to angiotensin affect a large number of smooth muscles, the majority of the compounds described to date have been tested only *in vivo* (37, 136, 202, 218, 283). According to Ahlquist (1), "one of the most important factors that influences conclusions about drug receptor interactions in the intact organism, is the measurement of drug-induced responses against a variable background of physical and humoral influences."

To interpret the effects of chemical agents in terms of drug action at the receptor level, isolated organs are preferable to the tests *in vivo*. The advantages of *in vitro* preparations have been clearly underlined by Furchgott (88). No systematic pharmacological studies have yet been made with angiotensin on isolated organs to establish whether the concepts and equations of receptor theory are applicable to this peptide. Such an attempt is now possible, because appropriate analogues of angiotensin, acting as agonists, partial agonists, and antagonists (see section IV, tables 2 and 4) are now available. The utility of such studies was pointed out by Ariens (11) when he wrote: "receptor theory can contribute to our understanding of the relationship between structure and action, while the study of structure-activity relationship in its turn can contribute to our insight into receptor mechanisms."

A. The Action of Angiotensin on Smooth Muscle

Angiotensin induces contraction of several isolated organs, but relaxation has not yet been described. The smooth muscle preparations most frequently used for the assay of angiotensin and analogues are: the guinea-pig ileum (50, 96, 97, 105, 148–150, 230, 263), the rat uterus (105, 148, 150, 174, 176, 177, 192, 203), the rat colon (90, 239, 243), and the rabbit aorta strip (2, 113, 114, 211, 324). Rabbit intestine (200), rabbit uterus (174), and isolated veins (307) have been used occasionally.

In general, these isolated organs have been used to test and compare activities of angiotensin and derivatives without considering that some of these tissues have possibly more than one receptor for angiotensin. Application of receptor theory to a group of chemicals is much easier if the pharmacological preparation possesses only one type of receptor for the agents under study. How can the validity of this assumption be demonstrated? Convincing evidence may be derived from pharmacological experiments in which the agent under study is tested in the presence of specific antagonists for cholinergic or adrenergic neurotransmitters, as well as antagonists for endogenous autacoids such as 5-hydroxytryptamine, histamine, and prostaglandins. The intrinsic cholinergic nervous system can be blocked at several points: at ganglia by hexamethonium, at nerve endings by morphine, which prevents the release of acetylcholine, (222, 272) and of the effector cell by atropine. Moreover, the effects of an "indirect" cholinomimetic should be increased in the presence of inhibitors of acetylcholinesterase. These criteria have been applied by Khairallah and Page (148, 150) to study the action of angiotensin on the guinea-pig ileum. Contractions elicited by angiotensin were reduced (60–70 %) by atropine or morphine,

and abolished by depolarizing doses of nicotine, but not modified by hexamethonium. It was suggested that angiotensin acts: a) by liberating acetylcholine from the parasympathetic nerve terminals through a non-nicotinic effect on the intramural ganglia and b) by stimulating the smooth muscle directly. These results were confirmed by Robertson and Rubin (263) who also showed a potentiation of angiotensin by a specific inhibitor of acetylcholinesterase (1,5 - di -(*p* - N - allyl - N - methylaminophenyl)-pentane 3-one dibromide) (51, 84) and a block by botulinum toxin. Strong evidence for an indirect action of angiotensin on the guinea-pig ileum was also provided by Godfraind *et al.* (96, 97), who demonstrated that the fast indirect acetylcholine-mediated action of angiotensin is blocked by atropine, while the slow direct myotropic effect is inhibited by lidoflazine, a noncompetitive antagonist (259).

Recently, Khairallah *et al.* (155) have attempted to differentiate the two types of receptors for angiotensin on the guinea-pig ileum: they have shown that the replacement of 8-Phe with Tyr does not affect the fast acetylcholine-mediated action, while reducing considerably the direct myotropic effect. This indicates that the receptors for angiotensin in the muscle and in the nervous elements of the guinea-pig ileum may be different: further studies are needed to substantiate these findings.

Attempts to demonstrate an acetylcholine-mediated effect of angiotensin on the rat colon (239), rat uterus (150), rat stomach strip, and rabbit aorta strip (260) were unsuccessful. It seems that the indirect action of angiotensin is special to the guinea-pig ileum. The presence of two different receptors would make the analysis of the structure-activity relationship on the guinea-pig ileum more complicated than on other smooth muscles.

In the few years since the two major reviews by Khairallah (156) and Gross (106), the actions of angiotensin on the sympathetic nervous system has been intensively studied. This topic will be discussed in detail in section VII. We will consider at this point only the evidence in favour or against a possible indirect myotropic action of angiotensin, mediated through the release of noradrenaline from the endogenous stores (tyramine-like effect) or through interference with noradrenaline uptake (cocaine-like effect). The experiments of Bell (21), on isolated guinea-pig vas deferens, a smooth muscle insensitive to angiotensin (21), have shown that this peptide does not induce noradrenaline release from the nerve terminals in the absence of nervous stimulation. Moreover, cocaine, but not angiotensin, potentiates the effect of exogenous noradrenaline on the same preparation (20). Contrary reports that angiotensin may stimulate the release or inhibit uptake of noradrenaline *in the absence of nervous stimulation* are based on experiments with isolated organs (segments of veins, arteries, heart, intestine) in which angiotensin has either a myotropic action or an initial effect followed by tachyphylaxis (22, 64, 124, 154, 224). From these results, it is difficult to decide whether the frequently inconsistent noradrenaline release (163) observed with high doses of angiotensin plays any part in the contractile action of the peptide. Careful experiments by Hughes and Roth (124) on vascular smooth muscle (rabbit portal vein and celiac artery) have shown that only high doses of angiotensin, eliciting strong contractions, release small amounts of noradrenaline. On the contrary, during nerve stimulation, the release of noradrenaline is significantly increased by relatively small doses of the octapeptide. They concluded that facilitation of noradrenaline released by angiotensin depends on sympathetic nerve activity. They found that this effect of angiotensin was not inhibited by cocaine (123, 124) thus suggesting that it cannot be attributed to inhibition of noradrenaline uptake. Cocaine does not contract the rat stomach strip or rabbit aorta strip (86, 260). Phentolamine, at doses sufficient to block

catecholamines, does not influence the action of angiotensin on the rabbit aorta strip (see table 1). In addition, angiotensin is equally effective on vascular smooth muscle, both arterial and venous, taken from normal and reserpine-pretreated animals (270, 322).

These results, indicate that the myotropic action of angiotensin is independent of the release of noradrenaline from nerve endings.

Other local tissue hormones such as histamine and 5-hydroxytryptamine are not involved in the myotropic action of angiotensin on vascular or intestinal smooth muscle, because mepyramine or diphenhydramine and methysergide, at high doses, do not affect the responses to the peptide on rat colon (90, 239), rat stomach strip, and rabbit aorta strip (260) (see table 1).

According to Vane (329), the presence of other smooth muscle contracting substances in isolated organs has to be considered, because these substances can interfere with the myotropic effect of angiotensin and affect its response on a second and a third tissue in a cascade superfusion system. Recently, Eckenfels and Vane (69) have shown that prostaglandins may contribute to the maintenance of the intrinsic tone of the rat stomach strip, because this tissue is gradually relaxed after treatment with indomethacin, an anti-inflammatory agent which blocks the synthesis of prostaglandins (331). By using the same agent in dog isolated mesenteric arteries, Aiken (4) was able to show an increase of the myotropic effect of angiotensin. Indomethacin (up to 2.8×10^{-5} M) was used in our laboratory to establish the possible interference by prostaglandins on the myotropic effect of angiotensin in rat stomach and rabbit aorta strips (260). It was found that pretreatment with indomethacin slightly increases the effect of prostaglandin E_2 on the rat stomach, but does not affect the contractions by angiotensin on either isolated smooth muscle. These results contradict the findings by Chong and Downing (47) that relatively high doses of indomethacin ($5.6–11.2 \times 10^{-5}$ M) reduce the myotropic effect of angiotensin on rat stomach and rabbit aorta strips. Summarized in table 1 is the effect of several drugs, used to establish the specificity of the myotropic effect of angiotensin in the two preparations.

The release of myotropic agents by vasoactive drugs from the spleen, the kidney, and the lung has been reported by several authors (3, 55, 179, 231, 232, 268). In most cases, these factors have been identified as prostaglandins. Recent experiments in our laboratory have shown that angiotensin releases prostaglandins from isolated rabbit kidneys perfused with Krebs solution (93), but not from isolated renal papillas (301). These results raise the question whether angiotensin stimulates the release of prostaglandins directly (chemical action) or indirectly (through vasoconstriction). Release of prostaglandins by angiotensin occurs only in perfused organs, where the fluid runs through the vessels, but not in strips of tissues superfused in a cascade system (333). In such a system, three segments of the same tissue are set in series so that the fluid superfusing the first segment runs over the other two. If the drug under investigation promotes the liberation of active myotropic agents such as prostaglandins, the response of the second and third segment will be affected: this has been suggested by Türker *et al.* (323) for angiotensin.

In order to exclude any interference by endogenous myotropic agents with the contractions of rat stomach and rabbit aorta strips to angiotensin, this agent was infused in different parts of the cascade system to reach one or all the segments (248). Our results confirm the findings of Vane (333), indicating that angiotensin does not release any endogenous myotropic agent which affects the response of a second or a third tissue in a cascade system.

Rat uterus and colon are as sensitive to angiotensin as the rabbit aorta strip. Rat uterus has been used frequently in the past to compare the oxytocic activity of angiotensin and analogues (202, 283). However, this preparation responds to angiotensin with repeated contractions of increasing intensity

TABLE 1

Effects of various inhibitors on the myotropic action of angiotensin and of other stimulating agents on the rat stomach and rabbit aorta strips

Concentrations are given in M/1 of the base. All antagonists were left in contact with the tissues for 15 min before applying the agonist (Rioux *et al.*, Can. J. Physiol. Pharmacol., 51: 665–672, 1973).

Rat Stomach Strip					Rabbit Aorta Strip				
Antagonist	Agonist	Dose	Contraction (mm)[a]		Antagonist	Agonist	Dose	Contraction (mm)[a]	
			Before	After				Before	After
Atropine	ACh[b]	6.5×10^{-8}	26.3 ± 6.1	0**[c]	Phentolamine	NA	3.0×10^{-6}	51.0 ± 6.0	0***
1.7×10^{-7}	AT_{II}	2.5×10^{-8}	34.6 ± 5.4	36.3 ± 7.5	3.6×10^{-6}	AT_{II}	2.0×10^{-9}	59.7 ± 7.6	59.0 ± 6.7
1.7×10^{-6}	ACh	6.5×10^{-7}	60.3 ± 3.2	0***	Methysergide	5-HT	2.8×10^{-7}	48.0 ± 7.0	2.1 ± 0.7***
	AT_{II}	1.0×10^{-7}	48.8 ± 8.8	43.3 ± 8.8	1.4×10^{-6}	AT_{II}	2.0×10^{-9}	38.6 ± 4.0	40.6± 4.0
Morphine	ACh	6.5×10^{-8}	29.5 ± 3.3	29.8 ± 4.7	Diphenhydramine	Hist.	2.2×10^{-6}	32.8 ± 6.1	0***
1.7×10^{-6}	5-HT	5.7×10^{-9}	41.0 ± 2.0	46.3 ± 5.4	4.6×10^{-6}	AT_{II}	2.0×10^{-9}	39.8 ± 10.0	38.6 ± 10.7
	AT_{II}	2.5×10^{-8}	42.7 ± 3.8	42.3 ± 4.9					
Methysergide	5-HT	2.8×10^{-8}	38.6 ± 9.0	0**	Cocaine	NA	3.0×10^{-8}	51.5 ± 4.6	75.0 ± 10.6*
2.8×10^{-6}	AT_{II}	2.5×10^{-8}	48.3 ± 8.1	45.1 ± 9.9	3.3×10^{-5}	AT_{II}	2.0×10^{-9}	28.0 ± 5.2	26.0 ± 4.8
Diphenhydramine	Hist.	2.2×10^{-5}	31.5 ± 5.9	3.3 ± 3.7***	Tetrodotoxin	NA	3.0×10^{-8}	53.2 ± 8.0	53.2 ± 8.7
4.6×10^{-6}	AT_{II}	2.5×10^{-8}	56.0 ± 7.4	62.1 ± 8.0	1.6×10^{-6}	AT_{II}	2.0×10^{-9}	49.5 ± 7.7	43.5 ± 7.4
Indomethacin	PGE_2	1.4×10^{-8}	58.8 ± 5.9	62.0 ± 6.2	Indomethacin	NA	3.0×10^{-8}	66.0 ± 2.1	68.5 ± 1.0
2.8×10^{-6}	AT_{II}	2.5×10^{-8}	43.8 ± 2.6	41.8 ± 4.1	7.0×10^{-6}	5-HT	2.8×10^{-7}	52.5 ± 1.7	55.0 ± 2.1
7.0×10^{-6}	PGE_2	1.4×10^{-8}	54.2 ± 4.5	65.0 ± 7.8		AT_{II}	2.0×10^{-9}	35.7 ± 5.2	32.1 ± 5.0
	AT_{II}	2.5×10^{-8}	40.0 ± 5.7	41.6 ± 5.8		PGE_2	1.1×10^{-6}	19.2 ± 4.3	7.8 ± 2.0*

[a] Mean ± standard error of the mean of at least 6 experiments.

[b] The abbreviations used are ACh, acetylcholine; 5-HT, 5-hydroxytryptamine; AT_{II} = 1-Asp-angiotensin II; Hist., histamine; PGE_2, prostaglandin E_2; NA, noradrenaline.

[c] Statistical significance is indicated by *, $P < .05$; **, $P < .01$; ***, $P < .01$.

both when suspended in an organ bath (203) or superfused in a cascade system (248). This represents a limitation to the use of this tissue for precise pharmacological studies on structure-activity relations of angiotensins.

The rat colon, suspended in a bath or superfused in a cascade system with Krebs solution, is extremely sensitive to angiotensin, but shows a marked spontaneous activity, which makes the evaluation of the responses very difficult. This can be effectively reduced by the use of a mixture of antagonists including pronethalol, atropine, and phentolamine (332). When bathed in blood, the spontaneous activity is reduced and the tissue is a suitable preparation in estimating changes of angiotensin concentration in the circulation (240). The rat colon also has the disadvantage that it cannot be conveniently used for isometric recordings and that high doses of angiotensin produce variable effects and do not maintain stable plateaux of contraction (248).

From these results, we conclude that thin strips of rat stomach and rabbit aorta are more suitable for accurate pharmacological studies on angiotensin than segments of intestine or uterus. The addition of 50 to 100 times the minimum effective dose of angiotensin elicits a contraction of the rat stomach in 5 to 10 seconds and the contraction is complete in less than 3 minutes: rabbit aorta responds after 5 to 20 seconds, but the contraction is complete only after 5 to 7 min. Stable plateaux are maintained both in stomach and aorta strips for at least 5 minutes even during application of a relatively high dose of angiotensin. Time required to develop complete contraction to angiotensin is similar to that of 5-hydroxytryptamine for the stomach (248), and to that of noradrenaline for the aorta (86). These results are in accord with the findings of Aiken and Vane (2), while Khairallah *et al.* (156) have reported that contraction of the aorta to angiotensin reaches a maximum in 1 to 2 minutes. The discrepancy may be due to the high doses of angiotensin used by these authors to measure the time of development of the contraction. Responses of rat stomach and rabbit aorta strips to small doses of angiotensin are reduced during the period immediately following the effect of a large dose of the peptide: however, full sensitivity is restored if enough time (50 to 60 min) is allowed for recovery after a large dose.

We have used rat stomach and rabbit aorta strips for the study of structure-activity relationships because a) the response to angiotensin consists of a reasonably strong contraction, b) the receptors of angiotensin in the two tissues are very similar (219) and they are specific for the octapeptide (see data of table 1), c) the two preparations are thin strips 0.5 to 1 mm thick, in which drugs can rapidly diffuse, d) they usually do not show any spontaneous activity which could interfere with the recording of the contractions, and e) they can be used equally well in the classical bath or in a cascade superfusion system, with isotonic or isometric recording.

B. Application of Receptor Theory to Angiotensin

It is still not established whether peptides containing several charged groups such as angiotensin are confined to the extracellular fluid or pass across the cell membrane. The natural peptide derives from the degradation of a group of proteins (angiotensinogen) under the action of an enzyme (renin), originating from the kidney (303). Renin releases a poorly active precursor, the decapeptide angiotensin I, which is converted to angiotensin II by the lung (193, 195) and peripheral tissues (15, 197, 198, 242).

Renin has been found in extrarenal tissues [see Gross (106), for a review on this topic] and "pseudorenin" (304) or "isorenin" has been extracted from several organs and tissues, including arteries (94, 104). However, the enzyme is inactive on isolated smooth muscle preparations. It has not yet been established whether angiotensin is synthesized or stored in cells.

The recent report by Johnston (131) on the presence of angiotensin I (AT_I) and angiotensin II (AT_{II}) in juxtaglomerular

granules constitutes a single and preliminary observation which needs further experimental support. Autoradiographic studies by Richardson and Beaulnes (250, 251) on the rabbit aorta have shown that angiotensin accumulates on the surface of endothelial cells and apparently does not enter the cells. When the octapeptide is coupled to a large molecule, for example poly-D,L-alanine (247), poly-O-acetyl-serine (9) or horseradish peroxidase (196, 251), the complex maintains most of the activity of the free peptide: in addition, the onset of action as well as the rate of development of contractions are similar to those of the free peptide (248). These findings favour the hypothesis that angiotensin acts on the cell membrane and there is no evidence that the receptors on smooth muscle are concentrated in some regions (*e.g.*, near the nerve terminals) as is the case for neurotransmitters. This is a rather important point, because if the receptors are on the cell surface and major diffusion barriers can be excluded, then the study of the interaction between angiotensin and its receptors can be attempted by applying the simple mass action principles. The most interesting analogues of angiotensin, both those showing greater potency than the native hormone and the antagonists, have been obtained by replacing one or both the terminal amino acids (1-Asp alone, 1-Asp and 8-Phe, or only 8-Phe). The physicochemical properties of these compounds are similar to those of angiotensin (221) and their affinity for the receptors is fairly strong. It seems that the peptides related to angiotensin constitute a group of agonists and antagonists more homogeneous than other series of compounds (cholinomimetics, cholinolytics; adrenergic, antiadrenergic drugs) reviewed by Ariens (12). In fact, they are all octapeptides of similar length and size as the natural hormone and they presumably act on the same receptors as AT_{II} (246, 248). These considerations seem appropriate before attempting a general interpretation of the action of angiotensin and analogues according to the theory of Clark (48) as modified by Ariens (11).

The critical appraisal will be pursued by answering the following questions: a) Do contractile responses of rat stomach and rabbit aorta strips to increasing doses of angiotensin fall closely along a theoretical curve given by Clark's equation? b) Do the two systems show a direct proportionality between stimulus and effect, in order to allow the utilization of the intrinsic activity (α^E), as suggested by Ariens (11)? c) Is there any evidence of threshold phenomena or of spare receptors (316) for angiotensin in the two tissues?

Dose-response curves and relationship between stimulus and effect. We have recently shown that contractile responses of rat stomach and rabbit aorta strips, over a wide range of angiotensin concentration, fall closely along the theoretical curves given by Clark's equation (48). Ratio between doses of AT_{II} producing 16% and 84% of maximum response is $1/25$ for the rat stomach and $1/20$ for the rabbit aorta. According to Clark, these values indicate that the interaction between angiotensin and its receptors follows the mass-action law (fig. 1).

Data from the experimental curves at the upper part of figure 1 have been used to establish the relationship between the stimulus (as a percentage of the maximum concentration) and the effect (as a percentage of the maximum contraction). The plot is linear and of unit slope for the two tissues: this suggests that there is a direct proportionality between the dose of angiotensin and the effect, particularly in the range of 20 to 80%. On the rat stomach strip, the low and high points deviated from linearity, but this may be considered within the experimental error of a biological system (48).

The existence of a direct proportionality between stimulus and effect and the fact that the straight lines (fig. 1) cross the intersection, suggest that threshold phenomena and spare receptors are absent (316, 334).

It is therefore assumed that a) the contraction is proportional to the concentration of

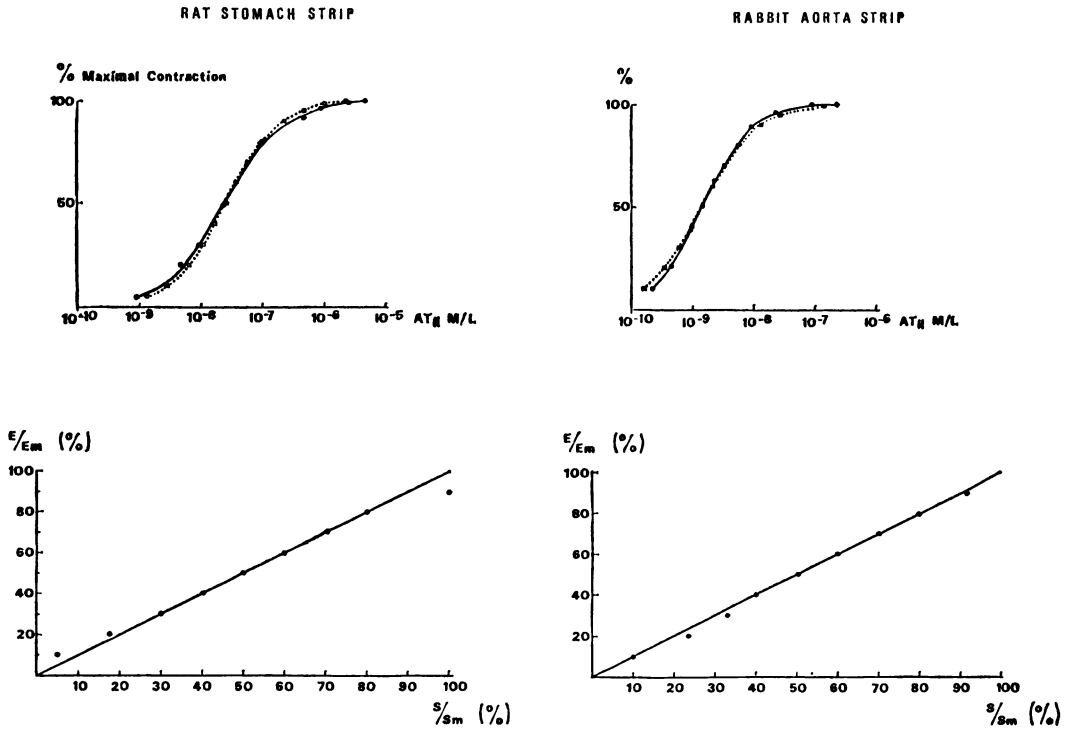

FIG. 1. Top, dose-response curves obtained with 1-Asp-AT_{II} (AT_{II}) on rat stomach strip (left) and rabbit aorta strip (right) suspended in a 10-ml organ bath with oxygenated (95%-O_2, 5%-CO_2) Krebs solution at 37°C. ●——●, experimental curves; O- - -O, theoretical curves. Theoretical curves were made by using ED50 experimental values of 2.5×10^{-8} on the stomach and 1.4×10^{-9} on the aorta in the equation of Clark (48): $C/C_M = \{[A]/[A] + K_A\}$, where C/C_M represents the fraction of the maximal response, [A] the concentration of agonist into the bath, and K_A the apparent dissociation constant of the drug-receptor complex. Ordinates, percentage maximum contraction; abscissae, molar concentration of angiotensin.

Bottom, relationship between stimulus (as a percentage of maximum stimulus, abscissae) and effect (as a percentage of maximum effect, ordinates) on stomach (left) and aorta (right). Points of experimental dose-response curves indicate mean values of at least 15 determinations (Rioux *et al.*, Can. J. Physiol. Pharmacol. 51: 665-672, 1973).

drug-receptor complex, and b) the maximum contraction obtainable with angiotensin in the two tissues is an actual measure of the intrinsic activity.

Maximum effects of a large number of angiotensin derivatives have been measured on rat stomach and rabbit aorta strips. The results are shown in tables 2, 3 in section III. It was found that analogues more potent than the native hormone (*e.g.*, 1-Sar-AT_{II}, 1-D-Ala-AT_{II}) or derivatives with prolonged duration of action *in vivo* (1-β-Asp-AT_{II}, 1-β-D-Asp-AT_{II}) as well as compounds showing variable degrees of reduction in affinity maintain full intrinsic activity. The affinity of some compounds (*e.g.*, 4-Ala-AT_{II}, 6-Ala-AT_{II}) is so low that it cannot be measured in one pharmacological preparation (the rat stomach strip). In these cases, α^E has been measured in the rabbit aorta and found to be close to unity.

The possibility that angiotensin may produce maximal contractions by stimulating only one fraction of the receptors has been excluded for the rabbit aorta strip by using a long-acting angiotensin inhibitor (1-Sar,8-Leu-AT_{II}) to occupy irreversibly a fraction of receptors. This compound is specific for angiotensin (246, 259) and apparently acts on the same receptor as the

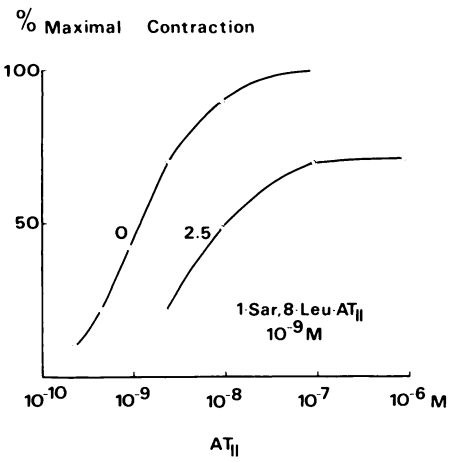

FIG. 2. Dose-response curves of 1-Asp-angiotensin II (AT_{II}) on rabbit aorta strips in the absence (0) and in the presence of 1-Sar,8-Leu-AT_{II} (2.5×10^{-9} M). The antagonist was left in contact with the tissue for 10 min before measuring the effect of the agonist. Points are means of at least eight individual determinations (Rioux *et al.*, Can. J. Physiol. Pharmacol. **51**: 665–672, 1973).

native hormone, as suggested from the results of protection experiments (246). The dose-response curve of AT_{II} in the presence of a small dose of 1-Sar,8-Leu-AT_{II} (fig. 2) is displaced to the right, loses parallelism with the control curve, and maximum contractions are no more obtained even with doses of AT_{II} 10 times higher than the maximum used for control.

These results suggest that spare receptors for angiotensin in this tissue are absent. Similar displacement of the dose-response curve of AT_{II} to the right and depression of maximum effect were observed in experiments with rat stomach strips. However, the dose-response curve of AT_{II} in presence of the antagonist remained parallel to the control (260). This point has been recently discussed by Rioux *et al.* (260).

As it emerges in the course of this section, the occupation theory of Clark (48) provides an acceptable basis for the analysis of the interactions between this group of peptides and their specific receptors on smooth muscle. Linear relations between the observed effects of AT_{II} and the actual stimulus make highly tenable the assumption that the extent of contraction is a function of the concentration of drug-receptor complex. Concentration-effect curves are therefore useful for the evaluation of pharmacological parameters such as pD_2 and α^E (11).

III. Structure-Activity Relationship

In the last 6 or 7 years, since the major reviews by Schröder and Lübke (283) and by Page and McCubbin (202), considerable progress has been made in the chemistry and the pharmacology of angiotensin. One of the most important contributions has been the introduction of the solid-phase method for peptide synthesis (175, 180) and the improvements elaborated by various workers for the purification and the analysis of angiotensins obtained with this method. An account of that can be found in the review by Merrifield (182) and in the proceedings of several symposia dedicated to peptides (45, 181, 182, 235, 236, 317).

A few investigators have also explored the conformational aspects of angiotensin by applying most modern physicochemical techniques, such as thin film dialysis (52), tritium hydrogen exchange (234), optical rotatory dispersion and circular dichroism (76), and analyses with Raman spectra and nuclear magnetic resonance (27, 77, 345). Several reports have recently been published, dealing with structure-activity relationships for angiotensin and analogues (37, 136, 243, 245, 247).

The primary intention of this section is to recapitulate and summarize the information emerging from two major approaches used in exploring the structure-activity relationships of angiotensin:

1. The conformational analyses and the models proposed for the three-dimensional structure of the octapeptide.

2. The biological analyses performed with a large number of angiotensin analogues, with various pharmacological tests.

A. Three-dimensional Structures of Angiotensin

The ideal situation for the study of the relations between chemical structure and biological activities of a large molecule, such as angiotensin, would be to know the conformation which it assumes when it binds to the receptors. With this knowledge, the pharmacologist could draw conclusions about the receptor topography and be able to understand the relative importance of the backbone and of the groups scattered along the peptide chains, for the binding to or the stimulation of the receptors. This explains why numerous workers have attempted to elucidate the spatial conformation of angiotensin.

Interest in this area began with the observation by Bumpus *et al.* (36, 152) that the myotropic effect of angiotensin on the rat uterus is inhibited in the presence of urea and arginine, agents which have been demonstrated to disrupt protein secondary and tertiary structure. This finding led Smeby *et al.* (305) to propose a helical model for angiotensin which consists of one and a half turns of an *alpha*-helix stabilized by three hydrogen bonds, involving the polypeptide backbone. Simultaneously, Paiva and Paiva (204) and Paiva *et al.* (205), on the basis of titration studies, established that side chains of 4-Tyr, 6-His, and 8-Phe are free of interactions. The helical model was first challenged by Craig *et al.* (52), by measuring the escape rate of angiotensin with thin film dialysis (addition of urea to solutions of angiotensin did not induce any important change in the rate of dialysis) and thereafter, by Printz *et al.* (234), with the tritium hydrogen exchange technique. Printz *et al.* (233) argued that: "... it is unlikely that the stable conformation of small peptides ($n \leqq 10$) is an α-helix. Such small peptides would form structures similar to β pleated sheets, that is, the chain would fold back on itself and allow for internal hydrogen bonding."

Experiments on the conformation of angiotensin were carried out by Printz *et al.* (234) with the tritium hydrogen exchange technique. It was shown that the rate of exchange of two peptide bond hydrogens were abnormally slow. Subsequent N.M.R. measurements by Bleich *et al.* (27) assigned these slow hydrogens as those belonging to the 3-Val and 5-Val amides in the center of the molecule. At least one and possibly both of these two hydrogens were proposed as being involved in intramolecular hydrogen bonds. Although it has been claimed that intrachain hydrogens may exchange at a slower rate than the peripheral ones, particularly if they are placed in the vicinity of a hydrophobic pocket, Printz *et al.* (234) presented evidence that the reduction of the exchange rate is superior to that found in model unstructured peptides larger than angiotensin. Based on these data, Printz *et al.* proposed two models for angiotensin II in aqueous solution: the *gamma* turn model and the *beta* turn model. In both models, the side chains of 2-Arg, 4-Tyr, and 6-His are apparently free of interaction: the peptide bond between 6-His and 7-Pro is towards a "*cis*"-conformation in the *gamma* turn model and towards a *trans*-conformation in the *beta* turn model. The *gamma* turn model is preferred on the basis of conformation energy computations and has been substantiated most recently with nuclear magnetic resonance studies by Bleich *et al.* (27). The model is shown in figure 3.

Fermandjian *et al.* (78) studied the conformation of angiotensin with several methods: circular dichroism spectra of angiotensin dissolved in water or in organic solvents and on dry films of the peptides obtained from trifluoroethanol solutions (76); infrared and Raman spectra with concentrated aqueous solutions and nuclear magnetic resonance spectra with angiotensin dissolved in deuterated trifluoroethanol or D_2O. Fermandjian *et al.* (78) concluded that angiotensin adopts a preferential antiparallel *beta* conformation with the first turn at the level of 3-Val, 4-Tyr and a second turn, at 6-His, 7-Pro: His and Pro carbonyl are pointing to the same direction ("*cis*"-conformation). This conformation seems to favour the formation of a hydrophobic pocket by the side chains of

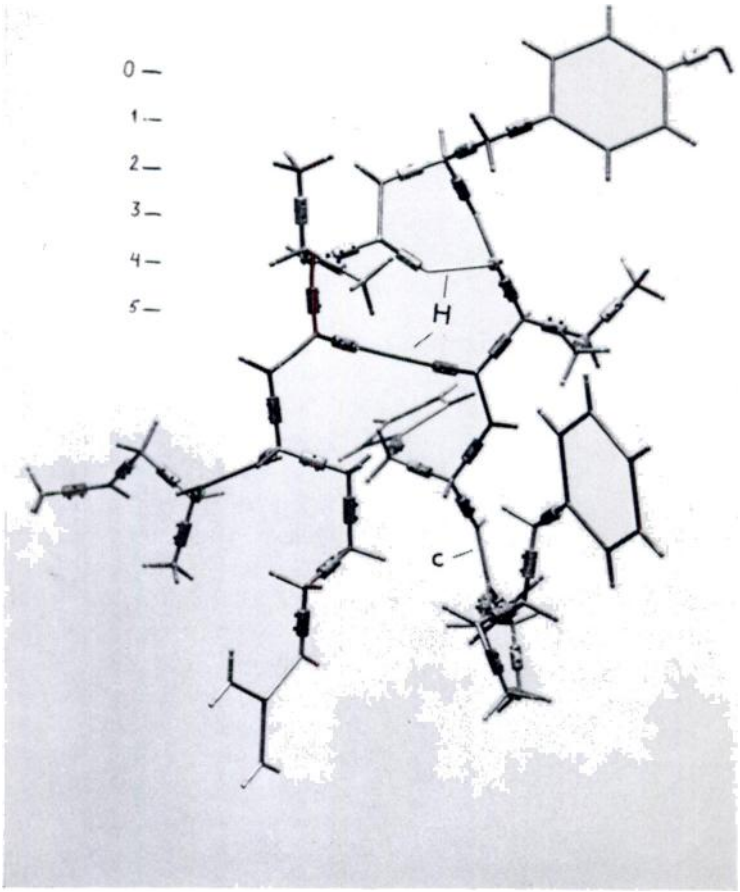

FIG. 3. Skeletal molecular model of the proposed *gamma*-turn structure of angiotensin II, constructed with Kendrew atomic models. The N-terminus of the molecule is at lower left, the *gamma*-turn is on the top. The two hydrogen bonds are indicated by H. The His-Pro peptide bond around which a *cis-trans* isomerization is possible, is shown in the *cis* form and is indicated by c. The scale is given in Å (Printz *et al.*, Nature, New Biol. 237: 136, 1973).

3-Val, 5-Val, and 8-Phe, contributing to stabilize the preferred conformation. Further stabilization is probably obtained with hydrogen bonds between peptide groups of the backbone, still unidentified. The side chains of 2-Arg, 4-Tyr, and 6-His are apparently free of interaction. On the other hand, Fermandjian *et al.* (78) maintained that one and possibly two hydrogen bonds involving NH are present in angiotensin: however, their nuclear magnetic resonance spectra did not allow the assignment of the peaks to individual NH, because of the overlapping C_α proton peaks.

The conformation proposed by Weinkam and Jorgensen (339, 340) namely that the stable conformer of angiotensin in solution includes an intramolecular ion-dipole bond between the Phe carboxylate ion and the imidazole ring of histidine is not supported

by the finding of Printz *et al.* (233) and Bleich *et al.* (27).

These results show that physicochemical methods can contribute important data on the conformation of angiotensin. However, the models proposed may reveal the capacity of the molecule to adopt a preferential conformation in concentrated solution and/or inorganic solvents, rather than the actual one, prevailing at the receptor sites. Attempts have been made by Printz *et al.* (233) and by Fermandjian *et al.* (78) to correlate the results of their physicochemical analyses with biological activities.

B. Angiotensin-receptor Interaction

In reviewing the recent developments from a critical standpoint, special emphasis will be placed on structure-activity relationships as derived from experiments on isolated smooth muscle. Some of the compounds discussed in this section have been described before (36, 202), but for the sake of clarity, it seems desirable to bring them into focus with the more recent developments. As pointed out before, most of the conclusions on the structure-activity relationships of angiotensin and analogues reported before 1967 (202) and even more recently were derived from studies *in vivo*. A few comparisons *in vitro* were performed on the rat uterus or the guinea-pig ileum (202, 283): we have mentioned in Section II the limitations of these isolated organs for the assay of angiotensin.

The relationships between structure and activity of angiotensin cannot be studied by using only a bioassay *in vivo*. When applied intravenously, this peptide does not bind strongly to plasma proteins (7) and consequently, it diffuses rapidly into the tissues [50 to 60% in one single circulation, according to Hodge *et al.* (117)]. The rate of degradation by plasma and particularly by tissue peptidases is rapid, as indicated by the disappearance of the pressor effect. The increase of blood pressure, produced by angiotensin in living animals, results from several physiological actions, such as the stimulation of the heart (19, 83, 164), the constriction of the arterioles and probably of the capacitance vessels (201, 211), and the stimulation of the sympathetic nervous system at different sites (see section VII). It is still unknown whether angiotensin exerts these multiple actions by stimulating one single type of receptor in the various organs and systems (see section V): therefore, the multiplicity of the effect may be further complicated by the summation or the interference of actions qualitatively different from each other. Even if the multiple actions contribute to the main effect (increase of blood pressure), the change of this physiological parameter in its turn, initiates a series of compensatory reflexes which counteract the action of the drug: it can be therefore concluded that bioassays of angiotensins *in vivo* do not allow either the evaluation of maximum responses or of potencies, because maximum effects cannot be measured and steady state may be difficult to attain because of the rapid metabolism. In spite of these limitations, the test *in vivo* should be constantly used in parallel with a reliable test *in vitro*. In fact the evaluation of the activities of analogues *in vivo* may give important indications of the metabolism or the therapeutic value of a specific analogue. In the present study, the test *in vivo* is needed also because it enables the newly synthesized compounds to be compared with those previously reported in the literature (202, 283).

Bioassays on isolated organs are devoid of most of the bias mentioned above. They generally allow the measurement of at least two fundamental pharmacological parameters, the affinity and the intrinsic activity (11). With these parameters, the chemical groups responsible for binding to (affinity) or for stimulating the receptors (intrinsic activity) may be identified. This differentiation seems feasible for large molecules such as polypeptides, because only a limited number of amino acids side groups are apparently involved in the stimulation, while a large portion of the peptide molecule is concerned with binding to receptors (118). Interaction between drug and receptor implies two events: the binding of the drug to the recep-

tor site(s) and some changes in the distribution of charges and/or in the conformation of both the drug and the receptor. In the case of an agonist both events occur and the physicochemical changes that subserve the biological effect (stimulation) originate from the interaction. Antagonists bind, but do not stimulate. When a series of compounds of similar chemical structure, acting on the same receptors, are tested in a pharmacological preparation which responds with contractions linearly related to the concentration of the drug, the intrinsic activity (α^E) measures the ability of each compound to contribute to the stimulus and to the effect (11). Affinity is estimated from the negative logarithm of the drug concentration that gives half maximum response (pD_2) (11). By using such a system, one can attempt to make correlations between the primary structure of the peptide and the conformation that it assumes at the receptor site.

It is assumed that: a) Analogues showing strong affinity have the specific conformation required for occupation of receptors. b) Analogues showing full intrinsic activity possess the group(s) essential for stimulation of receptors. c) If an analogue loses affinity, but maintains full intrinsic activity, this can be attributed either to the elimination of a group directly involved in binding or to a conformational change that reduces the binding capacity of the molecule as a whole. d) If an analogue shows a decrease of intrinsic activity (partial agonist or antagonist), but maintains affinity, the substitution has eliminated the stimulating group and/or changed the critical conformation required for stimulation of receptors. e) Simultaneous loss of intrinsic activity and affinity without the elimination of the essential group for stimulation (*e.g.*, pentapeptide 4–8, nonapeptide 1–9 (see table 2), 4-Gly-AT_{II}, 6-Gly-AT_{II} (219) may result from major conformational changes.

C. Relationships between Chemical Structure and Biological Activities

A large number of synthetic analogues of angiotensin have been prepared and tested in various laboratories to find a) compounds more potent and with longer duration of action than AT_{II}, and b) antagonists. The data to be found in the literature contain in most instances only scant information about the pharmacological properties of several compounds. We will attempt to analyze the structure-activity relationships of angiotensin with a rational and systematic approach, recently proposed by Rudinger (267). This will permit our analyses to be restricted to a limited number of angiotensin analogues which have been characterized on strictly standardized pharmacological assays and have been checked for purity with several chemical methods (220, 221). The first series of compounds is presented in table 2.

This includes: a) chain extension at the carboxyl end (no. 1, 2, 3); b) standard angiotensins (no. 4, 5 and 6); c) shortening of peptide chain at the amino end (no. 7, 8, 9, 10); d) shortening of peptide chain at the carboxyl end (no. 11 and 12); e) extension of the chain at the amino end (no. 13, 14 and 15); and f) omission of the side chains by replacing the normal constituents of AT_{II} with alanine. Ala was chosen instead of Gly or Acpc (1-aminocyclopentane carboxylic acid) (219, 245) because the steric properties of Gly and Acpc may be different from those of all other natural amino acids, while Ala should maintain the original conformation of the peptide backbone. The main limitation of this approach (replacement of the normal components of AT_{II} with Ala) is that it gives equal weight to all positions in the peptide chain, without considering their unequal significance for the biological activity of angiotensin as a whole. Because of this limitation, the analysis will be extended to a large group of analogues, in which the natural components have been replaced with "*isosteric*" or "*isofunctional*" residues (see table 3).

The results of biological assays of the compounds shown in table 2 are presented in figures 4 and 5. The angiotensin I used in these experiments has the structure of the natural decapeptide found in the horse (168),

TABLE 2

Primary structure, biological activities and pharmacological parameters of angiotensin analogues

No.	Sub.[a]	Name	Primary Structure 1 2 3 4 5 6 7 8 9 10	Blood Pressure %	Rat Stomach Strip[b]			Rabbit Aorta Strip[b]			Reference(s)
					α^E	pD_2	Rel. aff.	α^E	pD_2	Rel. aff.	
1		1-Asp-AT_I	H·Asp -Arg -Val -Tyr -Ile -His-Pro-Phe-His-Leu·OH	50–55	1.0	7.10	22	1.0	7.53	5.1	
				50							(318)*[c]
2		Nonapeptide 1–9	H·Asp -Arg -Val -Tyr -Ile -His-Pro-Phe His·OH	0.08	0.64	5.52	0.6	0.52	5.72	<0.1	*
3		Nonapeptide 1–9-Leu·OH	H·Asp -Arg -Val -Tyr -Ile -His-Pro-Phe Leu·OH	0.24	1.0	5.5	0.6	0.8	6.74	0.74	*
4		1-Asp-AT_{II}	H·Asp -Arg -Val -Tyr -Ile -His-Pro-Phe·OH	100	1.0	7.75	100	1.0	8.86	100	
				100							(291)*
				100							(287)
5		1-Asn[2],5-Val-AT_{II} Hypertensin Ciba	H·Asn[2]-Arg -Val -Tyr -Val-His-Pro-Phe·OH	93	1.0	7.80	105	1.0	8.11	18	(262, 248)
				100							(290, 292)
6		1-Asp,5-Val-AT_{II} A.R.S. "A"[d]	H·Asp -Arg -Val -Tyr -Val-His-Pro-Phe·OH	100	1.0	7.75	100	1.0	8.85	100	
				100							(289)
7		Heptapeptide 2–8	— H·Arg -Val -Tyr -Ile -His-Pro-Phe·OH	38	1.0	7.50	56	1.0	7.62	5.6	
				21.6							(36)*
8		Hexapeptide 3–8	— — H·Val -Tyr -Ile -His-Pro-Phe·OH	0.25	1.0	5.2	0.3	0.95	4.82	<0.1	
				2.30							(36)*
9		Pentapeptide 4–8	— — — H·Tyr -Ile -His-Pro-Phe·OH	0.04				0.5–0.6	~4.00	<0.1	
				0							(36)*
10		Tetrapeptide 5–8	— — — — H·Ile -His-Pro-Phe·OH	0.0007	0			0			
				0							(36)*
11		Heptapeptide 1–7	H·Asp -Arg -Val -Tyr -Ile -His-Pro·OH —		0.05			0			(36)*
12		Tetrapeptide 1–4	H·Asp -Arg -Val -Tyr OH — — — —	0	0			0			*
13		Acpc-AT_{II}	H·Acpc Asp -Arg -Val -Tyr -Ile -His-Pro-Phe·OH	60	1.0	7.7	90	1.0	7.84	10	*
14		Poly-D,L-Ala-AT_{II} (P)	(*P*) Asp -Arg -Val -Tyr -Ile -His-Pro-Phe·OH	50	1.0	7.8	110	1.0	7.90	12	*
15		[Ser-(Ac)]$_n$-AT_{II} (S-A)$_n$	(S-A)$_n$ Asp -Arg -Val -Tyr -Ile -His-Pro-Phe·OH	40							(9)
16	1	1-Ala-AT_{II}	H·Ala -Arg -Val -Tyr -Ile -His-Pro-Phe·OH	37	1.0	7.66	80	1.0	7.78	8.4	*
17	2	2-Ala-AT_{II}	H·Asp -*Ala* -Val -Tyr -Ile -His-Pro-Phe·OH	7.7	1.0	5.85	1.2	1.0	5.90	0.1	*

18	3	3-Ala-AT_{II}	H·Asp -Arg	*-Ala*	-Tyr	-Ile -His-Pro-Phe·OH	80	1.0	7.33	38	1.0	7.73	7.4	(159)*
19	4	4-Ala-AT_{II}	H·Asp -Arg	-Val	*-Ala*	-Ile -His-Pro-Phe·OH	0.37				0.8–0.9	4.14	<0.1	
							0.31							(296)*
20	5	5-Ala-AT_{II}	H·Asp -Arg	-Val	-Tyr	*-Ala*-His-Pro-Phe·OH	5.0	1.0	5.50	0.6	1.0	6.42	0.4	
							7.5							(160)*
21	6	6-Ala-AT_{II}	H·Asp -Arg	-Val	-Tyr	-Ile -*Ala*-Pro-Phe·OH	0.86	0.8	5.50	0.6	1.0	6.10	0.17	
							1.0							(297)*
22	7	7-Ala-AT_{II}	H·Asp -Arg	-Val	-Tyr	-Ile -His-*Ala*-Phe·OH	1.5	1.0	5.83	1.1	0.93	6.73	0.74	
							1.0							(295)*
23	8	8-Ala-AT_{II}	H·Asp -Arg	-Val	-Tyr	-Ile -His-Pro-*Ala*·OH	<0.1	0			0			
							1.0							(216)*

[a] Number of the position in which the natural component has been replaced.

[b] α^E, Intrinsic activity: pD_2, −log of the concentration of peptide producing 50% of maximal response (used as a measure of affinity). Rel. aff., Relative affinity in %: 1-Asp-AT_{II} = 100%.

[c*] These compounds were synthesized in our laboratory with the solid-phase method of Merrifield (180). Results of chemical analyses have been reported (220, 221).

[d] Angiotensin Research Standard "A" supplied by National Institute for Medical Research, Mill Hill, London.

TABLE 3

Primary structure, biological activities and pharmacological parameters of angiotensin analogues

No.	Sub.[a]	Name	Primary Structure 1	2	3	4	5	6	7	8	Blood Pressure %	Rat Stomach Strip α^{Ex}	Rat Stomach Strip pD_2	Rat Stomach Strip Rel. aff.	Other Isolated Organs (Rel. Aff.)	Reference(s)[b]
1		1-Asp-AT_{II}	H·Asp	-Arg	-Val	-Tyr	-Ile	-His	-Pro	-Phe·OH	100	1.0	7.75	100	100[c]	(287, 260)
2	1	1-Suc-AT_{II}	*-Suc*	-Arg	-Val	-Tyr	-Ile	-His	-Pro	-Phe·OH	60				50[d]	(9)
3	1	1-Me_2But-AT_{II}	*-Me_2But*	-Arg	-Val	-Tyr	-Ile	-His	-Pro	-Phe·OH	2.5	1.0	6.41	45		(221)
4	1	1-Sar-AT_{II}	*Sar*	-Arg	-Val	-Tyr	-Ile	-His	-Pro	-Phe·OH	70	1.0	8.21	280	175[c]	(246, 260)
5	1	1-Me_2Gly-AT_{II}	*Me_2Gly*	-Arg	-Val	-Tyr	-Ile	-His	-Pro	-Phe·OH	88	1.0	7.64	79		(221, 247)
6	1	1-Bet-AT_{II}	*Bet*	-Arg	-Val	-Tyr	-Ile	-His	-Pro	-Phe·OH	10	1.0	6.9	14		(221, 247)
7	1	1-Gly-AT_{II}	*H·Gly*	-Arg	-Val	-Tyr	-Val	-His	-Pro	-Phe·OH	50				27[d]	(253)
8	1	1 < Glu-AT_{II}	*<Glu*	-Arg	-Val	-Tyr	-Val	-His	-Pro	-Phe·OH	100	1.0	7.38	42		(282, 247)
9	1	1-β-Asp-AT_{II}	H·β-Asp	-Arg	-Val	-Tyr	-Val	-His	-Pro	-Phe·OH	150	1.0	7.96	160	27.5[c]	(255, 247)
10	1	1-β-D-Asp-AT_{II}	H·β-D-Asp	-Arg	-Val	-Tyr	-Val	-His	-Pro	-Phe·OH	200	1.0	8.26	322	87[c]	(255, 247)
11	1	1-D-Ala-AT_{II}	H·D-Ala	-Arg	-Val	-Tyr	-Ile	-His	-Pro	-Phe·OH	100	1.0	8.03	190	220[c]	(221, 247)
12	2	2-Arg(NO_2)-AT_{II}	H·Asn[2]	*-Arg(NO_2)*	-Val	-Tyr	-Val	-His	-Pro	-Phe·OH	50				3.1[d]	(252, 291)
13	2	2-Cit-AT_{II}	H·Asn[2]	*-Cit*	-Val	-Tyr	-Val	-His	-Pro	-Phe·OH	2					(271)
14	2	2-Lys-AT_{II}	H·Asn[2]	*-Lys*	-Val	-Tyr	-Val	-His	-Pro	-Phe·OH	10					(279)
15	2	2-Orn-AT_{II}	H·Asn[2]	*-Orn*	-Val	-Tyr	-Val	-His	-Pro	-Phe·OH	20				1.6[d]	(253, 291)
16	2	2-Val-AT_{II}	H·Asn[2]	*-Val*	-Val	-Tyr	-Val	-His	-Pro	-Phe·OH	5					(279)
17	2	2-Gly-AT_{II}	H·Asp	*-Gly*	-Val	-Tyr	-Ile	-His	-Pro	-Phe·OH	10					(137)
18	2	2-D-Arg-AT_{II}	H·Asn[2]	*-D-Arg*	-Val	-Tyr	-Val	-His	-Pro	-Phe·OH	5					(257)
19	2	2-Pro-AT_{II}	H·Asp	*-Pro*	-Val	-Tyr	-Ile	-His	-Pro	-Phe·OH	1.3	1.0	5.64	0.7		(221, 248)
20	2	2-D-Abu-AT_{II}	H·Asp	*-D-Abu*	-Val	-Tyr	-Ile	-His	-Pro	-Phe·OH	0.9	1.0	5.47	0.53		(221, 248)
21	3	1-Asp-3-Aib-AT_{II}	H·Asp	-Arg	*-Aib*	-Tyr	-Val	-His	-Pro	-Phe·OH	1				1[d]	(177)
22	3	1-Asn[2]-3-Leu-AT_{II}	H·Asn[2]	-Arg	*-Leu*	-Tyr	-Ile	-His	-Pro	-Phe·OH	100				100[d]	(288, 291)
23	3	3-Pro-AT_{II}	H·Asn[2]	-Arg	*-Pro*	-Tyr	-Ile	-His	-Pro	-Phe·OH	40				40[d]	(177)
24	3	3-Acpc-AT_{II}	H·Asp	-Arg	*-Acpc*	-Tyr	-Ile	-His	-Pro	-Phe·OH	4	1.0	6.9	14		(218, 243)
25	4	4-Tyr(Me)-AT_{II}	H·Asn[2]	-Arg	-Val	*-Tyr(Me)*	-Val	-His	-Pro	-Phe·OH	0.2				0.1[d]	(281)
26	4	4-Phe-AT_{II}	H·Asp	-Arg	-Val	*-Phe*	-Ile	-His	-Pro	-Phe·OH	22	0.8	6.2	2.8	0.54[c]	(221, 260)
27	4	4-Leu-AT_{II}	H·Asp	-Arg	-Val	-Leu	-Ile	-His	-Pro	-Phe·OH	0.2					(221, 248)
28	4	4-Acpc-AT_{II}	H·Asp	-Arg	-Val	-Acpc	-Ile	-His	-Pro	-Phe·OH	0.04	0.8	4.7	<0.1		(243)
29	4	4-Gly-AT_{II}	H·Asp	-Arg	-Val	*-Gly*	-Ile	-His	-Pro	-Phe·OH	0.7	0.7	4.8	0.11		(221, 245)
30	5	1-Asn[2]-5-Abu-AT_{II}	H·Asn[2]	-Arg	-Val	-Tyr	*-Abu*	-His	-Pro	-Phe·OH	16					(138)
31	5	1-Asn[2]-5-Nle-AT_{II}	H·Asn[2]	-Arg	-Val	-Tyr	*-Nle*	-His	-Pro	-Phe·OH	21					(138)

32	5	1-Asn^1-5-aIle-AT$_{II}$	H·Asn^1	-Arg	-Val	-Tyr	*-aIle*	-His	-Pro	-Phe·OH	100					(138)
33	5	1-Asn^1-5-Aev-AT$_{II}$	H·Asn^1	-Arg	-Val	-Tyr	*-Aev*	-His	-Pro	-Phe·OH	75					(138)
34	5	1-Asn^1-5-Cpg-AT$_{II}$	H·Asn^1	-Arg	-Val	-Tyr	*-Cpg*	-His	-Pro	-Phe·OH	89					(138)
35	5	1-Asn^1-5-Chg-AT$_{II}$	H·Asn^1	-Arg	-Val	-Tyr	*-Chg*	-His	-Pro	-Phe·OH	114					(138)
36	5	1-Asn^1-5-Aib-AT$_{II}$	H·Asn^1	-Arg	-Val	-Tyr	*-Aib*	-His	-Pro	-Phe·OH	0.9					(139)
37	5	1-Asn^1-5-Sar-AT$_{II}$	H·Asn^1	-Arg	-Val	-Tyr	*-Sar*	-His	-Pro	-Phe·OH	1					(139)
38	5	1-Asn^1-5-Pro-AT$_{II}$	H·Asn^1	-Arg	-Val	-Tyr	*-Pro*	-His	-Pro	-Phe·OH	10					(139)
39	5	1-Asn^1-5-D-Pro-AT$_{II}$	H·Asn^1	-Arg	-Val	-Tyr	*-D-Pro*	-His	-Pro	-Phe·OH	0.05					(139)
40	6	6-D-His-AT$_{II}$	H·Asn^1	-Arg	-Val	-Tyr	-Val	*-D-His*	-Pro	-Phe·OH	1					(257)
41	6	6-Lys-AT$_{II}$	H·Asn^1	-Arg	-Val	-Tyr	-Val	*-Lys*	-Pro	-Phe·OH	0.1				0.02[d]	(281)
42	6	6-Phe-AT$_{II}$	H·Asn^1	-Arg	-Val	-Tyr	-Val	*-Phe*	-Pro	-Phe·OH	1				0.04[d]	(280)
43	6	6-Pyr(3)-AT$_{II}$	H·Asn^1	-Arg	-Val	-Tyr	-Val	*-Pyr(3)*	-Pro	-Phe·OH	57–79				52[e]	(108)
44	6	6-Gly-AT$_{II}$	H·Asp	-Arg	-Val	-Tyr	-Ile	*-Gly*	-Pro	-Phe·OH	0.03	0.5	4.9	0.14		(221, 245)
45	6	6-Acpc-AT$_{II}$	H·Asp	-Arg	-Val	-Tyr	-Ile	*-Acpc*	-Pro	-Phe·OH	<0.01	0.3				(218, 243)
46	7	7-Hyp-AT$_{II}$	H·Asp	-Arg	-Val	-Tyr	-Ile	-His	*-Hyp*	-Phe·OH	10				10[d]	(302)
47	7	7-Pip-AT$_{II}$	H·Asp	-Arg	-Val	-Tyr	-Ile	-His	*-Pip*	-Phe·OH	0.2					(37)
48	7	7-Acpc-AT$_{II}$	H·Asp	-Arg	-Val	-Tyr	-Ile	-His	*-Acpc*	-Phe·OH	1.2	0.9	5.8	1.1		(218, 245)
49	8	8-Phe-OMe-AT$_{II}$	H·Asp	-Arg	-Val	-Tyr	-Val	-His	-Pro	*-Phe·OMe*	10					(290)
50	8	8-Phe(NH$_2$)-AT$_{II}$	H·Asp	-Arg	-Val	-Tyr	-Val	-His	-Pro	*-Phe·(NH$_2$)*	3					(252)
51	8	8-Apb-AT$_{II}$	H·Asp	-Arg	-Val	-Tyr	-Ile	-His	-Pro	*-Apb*·OH	10					(44)
52	8	8-Apib-AT$_{II}$	H·Asp	-Arg	-Val	-Tyr	-Ile	-His	-Pro	*-Apib*·OH	0.1					(44)
53	8	8-Glu-AT$_{II}$	H·Asp	-Arg	-Val	-Tyr	-Ile	-His	-Pro	*-Glu*·OH	0.08	0				(221, 245)
54	8	8-Lys-AT$_{II}$	H·Asp	-Arg	-Val	-Tyr	-Ile	-His	-Pro	*-Lys*·OH	0	0				(221, 245)
55	8	8-Cha-AT$_{II}$	H·Asp	-Arg	-Val	-Tyr	-Ile	-His	-Pro	*-Cha*·OH	20				20[c]	(162)

[a] Sub., α^E, pD$_2$, Rel. aff., same explanations as in table 2.

[b] The reference cited first contains data on synthesis and pressor activity of the corresponding compound; the second contains data on myotropic activity in rat stomach strip or other isolated organs.

[c] Rabbit aorta strip.

[d] Rat uterus.

[e] Guinea-pig ileum.

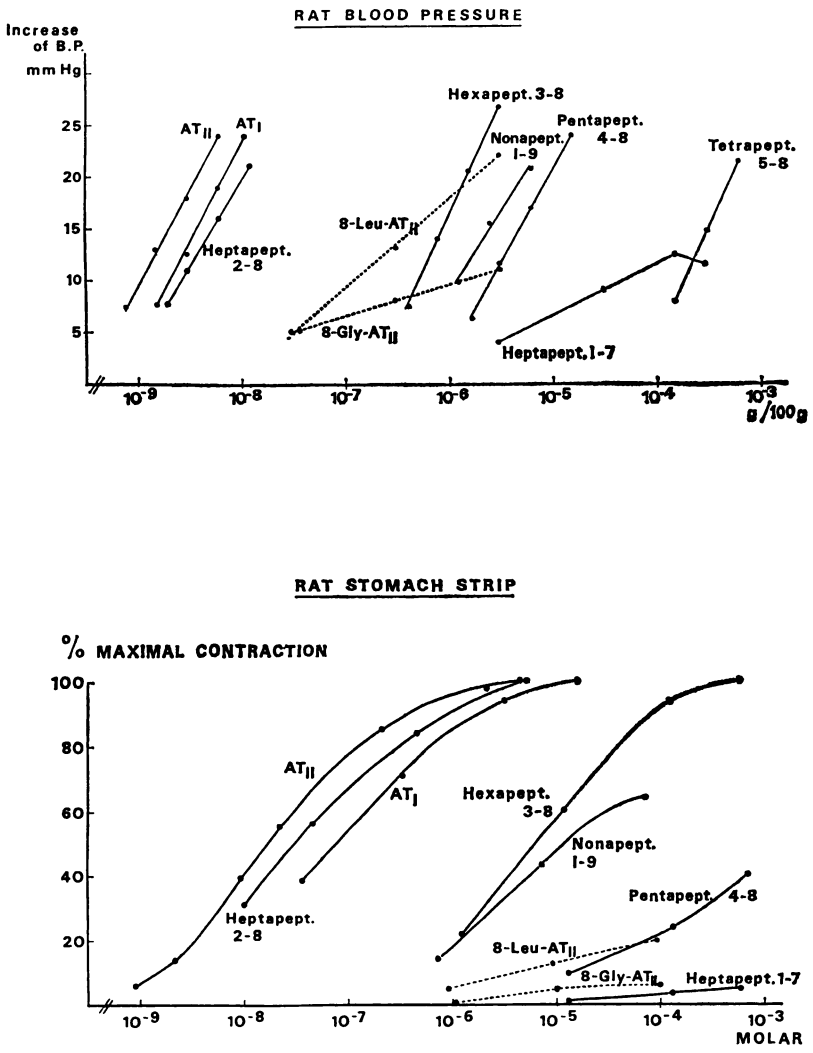

FIG. 4. Dose-response curves of angiotensin and derivatives on the rat blood pressure (upper graph) and the rat stomach strip (lower graph), suspended in a cascade superfusion system, according to Vane (329), and superfused with oxygenated (95%–O_2, 5%–CO_2) Krebs solution at 37°C. Each point indicates the mean of at least 10 determinations: increasing concentrations of peptides dissolved in physiological saline were applied by intravenous injections *in vivo* and into the fluid (10 ml/min) superfusing the isolated rat stomach strip. ●——●, indicates agonists; ●- - -●, antagonists (D. Regoli, W. K. Park and F. Rioux, unpublished data).

pig (35), man (10), and rat (189). It differs from bovine AT_I (71), only by the presence of 5-Ile instead of 5-Val. The first point that emerges from the results presented in figure 4 and table 2 is that AT_I is more active *in vivo* than *in vitro*.

This result has been reported several times since 1958 (105, 113, 201, 202, 248) and has been explained by Ng and Vane (193–195) who demonstrated that the decapeptide is converted in the lung to AT_{II}. Depending on the content or on the activity of the intramural converting enzyme, segments of arteries (2), or isolated organs show different sensitivity to AT_I (122). This may explain the different relative affinities (see table 2) found in the present experiment on rat stomach and rabbit aorta strips.

AT_I is more potent than nonapeptide 1–9 (no. 2) both *in vivo* and *in vitro*. Nonapeptide 1–9 is not converted by lung or plasma converting enzyme (198). It is still unknown whether the residual myotropic and pressor actions of compound 2 are direct or derive from an activation by carboxypeptidase, through splitting of the 8-Phe-9-His bond. The important decrease of intrinsic activity of compound 2 supports the hypothesis that complementarity between the drug and the receptor is lost, probably because 9-His hinders the access of 8-Phe to the receptor site. A significant increase of potency is observed *in vivo* and *in vitro* when 9-His is replaced by Leu (no. 3). This finding raises the question whether carboxypeptidase may activate these nonapeptides intramurally. In fact, Leu is a better substrate for carboxypeptidase than His (109).

In conclusion, the lengthening of the chain at the carboxyl terminal reduces the biological action of angiotensin: however, if the chain is prolonged by two residues (as in AT_I), the octapeptide AT_{II} can be released in the proximity of the receptors by the converting enzyme. If this enzyme is blocked, as by the peptides from *Bothrops jararaca* (79, 333), the action of AT_I is proportionally reduced (2). It can be concluded that AT_I has very little if any direct stimulant effect (2) and that its action is the result of two components: a) activation by the converting enzyme; and b) occupation of the receptors by the octapeptide released intramurally. In this connection, it is worth mentioning that the generation of the contraction induced by AT_I on rabbit aorta strip, is slower than that by AT_{II} (2). This result has been confirmed in our laboratory (248).

Compounds 4, 5, and 6 have been compared to establish whether Asn^2 or Asp in position 1 and Ile or Val in position 5 influence the pharmacological spectrum. The three angiotensins (no. 4, 5, and 6) are equally active *in vivo* and on the rat stomach strip: 1-Asn^2-AT_{II} is however less potent than the other two on the rabbit aorta strip. This difference must be taken into account when 1-Asn^2-AT_{II} is used as reference standard to compare the activity of 1-Asp derivatives of AT_{II} on the rabbit aorta strip, as suggested by Helmer (114) several years ago. 5-Val or 5-Ile angiotensins are about equally active.

The effect of reducing progressively the chain from the amino end has been studied with numbers 7, 8, 9, and 10 (table 2): it should be noted that these fragments represent some of the metabolic products of the degradation of AT_{II} by plasma and tissue homogenates (41, 68, 151, 238). Potency *in vivo* decreases progressively from number 7 to 10, but the dose-response curves remain parallel to that of AT_{II} (fig. 4). The test *in vitro* on the other hand, reveals that dose-response curves parallel to AT_{II} and maximum effects (intrinsic activity, 1.0) are given only by numbers 7 and 8: the pentapeptide 4–8 (no. 9) is almost inactive and the dose response curve is no longer parallel to that of AT_{II} (fig. 4). Number 10 is completely inactive *in vitro* (see table 2). None of these compounds shows any antagonistic effect. From these results, it can be concluded that: a) the minimum structure needed for full intrinsic activity is *Val-Tyr-Ile-His-Pro-Phe*; b) 2-Arg contributes substantially to the binding and is not involved in the stimulation. These conclusions are in accord with the conformation model proposed by Printz *et al.* (233) and by Fermandjian *et al.* (78) in which 3-Val is a determinant for the secondary structure of AT_{II}. The results obtained with numbers 7 to 10 reveal some of the limitations of the test *in vivo* and point again to the necessity of using at least one pharmacological assay *in vitro* for studying structure-activity relationships.

Heptapeptide 1–7 as well as tetrapeptide 1–4 (the two compounds used to establish the influence of shortening the side chain from the carboxyl end) are inactive *in vivo* and *in vitro*. Number 11 slightly antagonizes AT_{II} on the rat stomach strip, only when applied at very high concentrations (ratio of doses for AT_{II}/heptapeptide 1–7 = $\frac{1}{2000}$). These results indicate the essential role of 8-Phe for

the biological action of AT_{II}. In the absence of 8-Phe neither occupation nor stimulation of AT_{II} receptors occur, although most of the side chains involved in binding AT_{II} to receptors are still present in the molecule. It seems that 8-Phe is the side chain that can trigger the conformational changes of the receptors needed to contract smooth muscles. Additional evidence for the essential role of 8-Phe derives from substitution of aliphatic amino acids in position 8. 8-Gly-AT_{II} and 8-Leu-AT_{II} have been included in figure 5 to show the different activity *in vivo* and *in vitro* and the slope of the dose-response curves *in vivo*. The two compounds are specific and competitive antagonists of AT_{II} with different affinities for the receptors (8-Gly-AT_{II}:pA_2 = 7.42, 8-Leu-AT_{II}:pA_2 = 8.78, on the rabbit aorta strip). They are totally inactive *in vitro*, but they maintain a stimulant effect *in vivo* which is higher for 8-Leu-AT_{II} than for 8-Gly-AT_{II}. This effect is probably due to the residual stimulating action of the antagonists on some still unknown receptors, which contribute to the overall increase of blood pressure produced by angiotensin *in vivo*. The different affinities between antagonists and the heptapeptide 1–7 indicate that 8-Phe not only contributes to stimulating the receptors, but might also be involved in binding or in determining the critical conformation of angiotensin II, as suggested by Fermandjian *et al.* (76, 77).

Elongation of the peptide chain at the amino end with Acpc (no. 12) or with polymers such as poly-D,L-Ala and poly-O-acetyl-serine (no. 13 and 14) is compatible with 50% of biological activity. The actions of Acpc-AT_{II} and poly-D,L-Ala-AT_{II} are slightly prolonged *in vivo* and *in vitro*. When applied to rabbit aorta strips, with the oil immersion technique (144), a significant increase of the duration of action is observed with the two analogues (247). From these results, it can be concluded that the terminal $-NH_2$ contributes to the binding of AT_{II} to receptors: moreover, the elongation of the chain at the amino end probably protects the octapeptide from the inactivation by aminopeptidases or may allow for firm binding to accessory areas in the proximity of the receptors.

To provide information on the role of a single amino acid for the biological activity of AT_{II}, numbers 16 to 23 were prepared and tested in our laboratory. Results of assays *in vivo* and *in vitro* are presented in figure 5. Some of these compounds have been described and tested before (see references in table 2).

Like the preceding group of compounds, the Ala derivatives of AT_{II} have been tested in three pharmacological preparations to establish their vasoconstrictor and myotropic action as well as the potential inhibitory properties and the duration of action *in vivo* and *in vitro*.

Figure 5 reveals that replacing in turn one amino acid residue with Ala brings about variable decreases of potency: all the dose-response curves lie on the right of that of AT_{II} and the compounds distribute in three major groups: a) 1 and 3-Ala maintain a good affinity and full intrinsic activity with a decrease in potency less than one log unit; b) 2,5,7-Ala show a marked decrease of affinity (about two log units) but full intrinsic activity; and c) 6 and 4-Ala have a marked loss of affinity so that their intrinsic activity cannot be measured on the rat stomach strip: it has however been measured on the rabbit aorta strips (see table 2). 8-Ala is an antagonist and consequently, pD_2 and α^E cannot be measured. The other Ala derivatives do not show any antagonistic effect when administered intravenously *in vivo* or added to the fluid perfusing the stomach strips at inactive doses or at doses producing approximately 5 to 10% of the maximum response. The duration of action of all Ala substituted agonists *in vivo* were similar to that of AT_{II} (248). With the exception of 4-Ala-AT_{II} (α^E = 0.8–0.9) and 8-Ala-AT_{II} (α^E = 0) all the other derivatives have intrinsic activity of 1.0. When these findings are interpreted in terms of receptor theory as discussed earlier, it seems that side chains in positions 1, 2, 3, 5, 6, and 7 contribute to affinity,

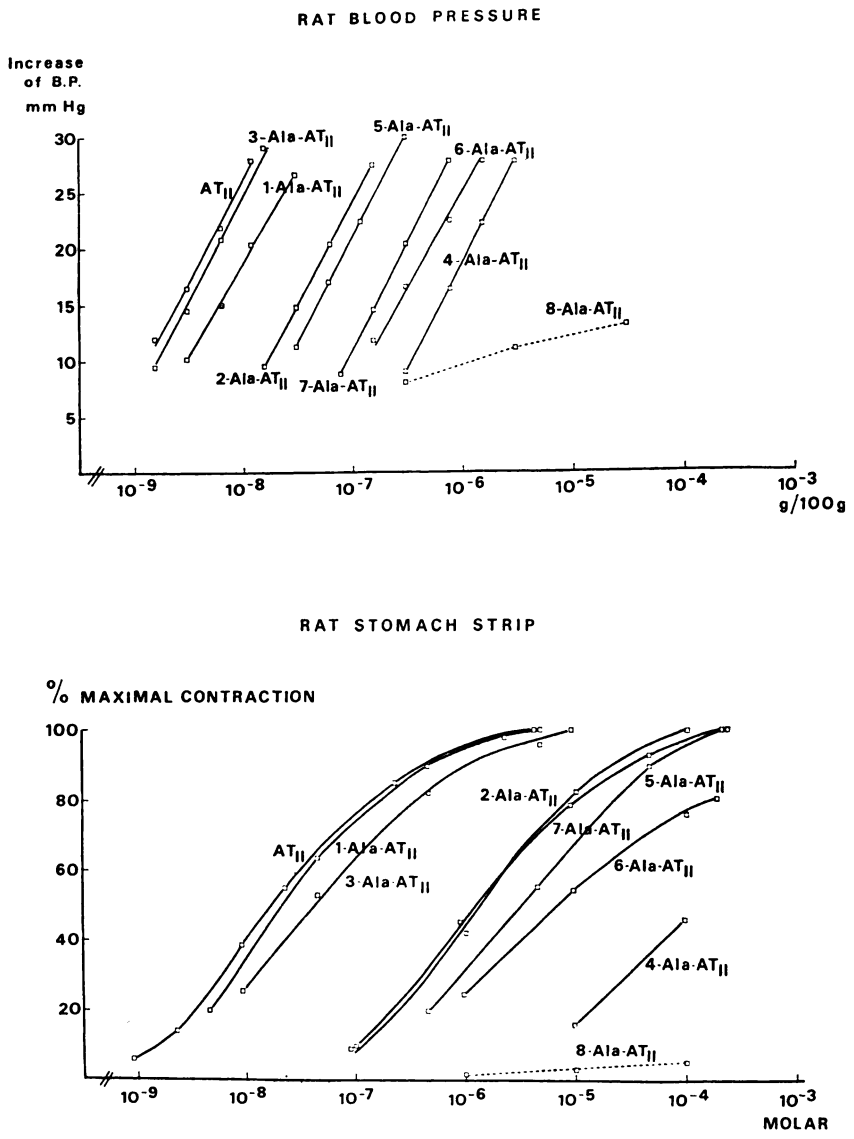

FIG. 5. Dose-response curves of angiotensin and Ala derivatives on the rat blood pressure (upper) and the rat stomach strip (lower). Each point indicates the mean of at least 10 experiments. Same experimental conditions and same explanations as in figure 4 (D. Regoli, W. K. Park and F. Rioux, unpublished data).

while 4-Tyr (to some extent) but primarily 8-Phe are essential for intrinsic activity. The contribution of the individual side chains to affinity can be evaluated from the extent of the displacement of the dose-response curve to the right, because the intrinsic activity is equal to 1.0. A tentative interpretation of the results, presented in table 2, figures 4 and 5, is given in figure 6. The following conclusions may be drawn from the structure-activity analysis presented above.

1. The minimum structure required for full stimulation is the hexapeptide H·Val-Tyr-Ile-His-Pro-Phe·OH.

2. 8-Phe is essential for stimulation and is important for affinity: a hydrophobic group in position 8 may be required to assure optimum conformation.

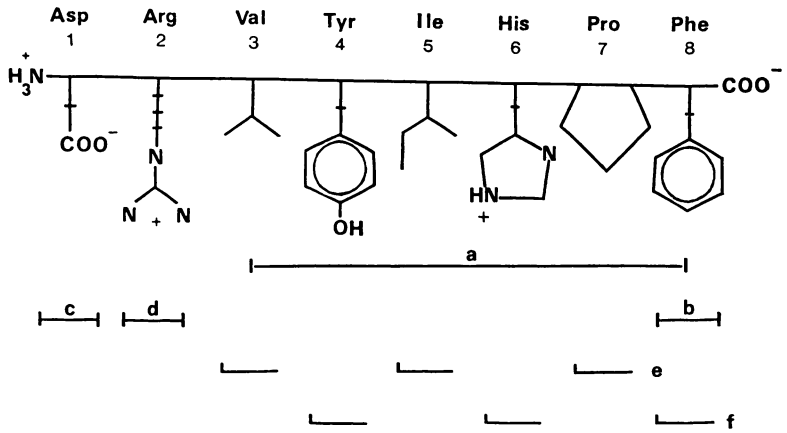

FIG. 6. Abbreviated chemical structure of 1-Asp, 5-Ile-AT_{II}. a, Hexapeptide 3–8: minimum molecular length required for full biological activity. b, 8-Phe: hydrophobic ring essential for stimulation of receptors. c, N-terminal Asp: contributes to binding and influences duration of action. d, Arg residue: contributes to binding. e, Hydrophobic side chains: stabilize secondary structure. f, Functional groups essential for binding to receptors (4-Tyr,6-His) and for stimulation (8-Phe).

3. The N-terminal amino acid plays a definite role for the binding to receptors and is a determinant for the duration of action: this is inversely related to the ability of aminopeptidases to split the first peptide bond.

4. The guanido group of 2-Arg contributes to the binding of AT_{II} to receptors, but not to stimulation.

5. The neutral side chains of 3-Val, 5-Ille (Val), and 7-Pro are probably involved in maintaining the distance between active groups of 4-Tyr, 6-His, and 8-Phe; moreover, 7-Pro is undoubtedly contributing to maintain the critical orientation of 8-Phe. The hydrophobic chains may also play an important role on the whole conformation of the peptide as suggested by Printz *et al.* (233) or by contributing to the hypothetical hydrophobic pocket (3-Val, 5-Val, and 8-Phe) proposed by Fermandjian *et al.* (78).

6. Finally, the side chains of 4-Tyr and 6-His are determinant for binding angiotensin to receptors. In addition, 4-Tyr is apparently involved in the stimulation. However, the most important contribution to stimulation of receptors comes from 8-Phe.

This interpretation is supported by the results obtained with other analogues of angiotensin II, in which the natural components have been replaced with *"isosteric"* or *"isofunctional"* groups. From a large series of compounds, described since 1958, a group was selected (see table 3), with the purpose of defining more precisely the functional contribution of every side chain and of the terminal groups to the action of angiotensin.

Position 1. The residue in position 1 has been shown to contribute primarily to the duration of action of AT_{II}, through two possible mechanisms: a) it makes the molecule more or less resistant to aminopeptidases; or b) it changes the binding affinity of the peptides for the receptors (247). The existence of these two mechanisms is illustrated in figure 7. The oil immersion technique of Kalsner and Nickerson (144) was used to measure the rate of relaxation of rabbit aorta strips in Krebs solution and in oil, after contracting the tissues with heptapeptide 2–8 and analogues of AT_{II} substituted in position 1. We assumed that the rate of relaxation of rabbit aorta in oil is a valid index of the rate of inactivation of these peptides by intramural proteolytic enzymes. With compounds which differ only with respect to the first amino acid, the eventual differences between their rate of relaxation may reflect the rate of cleavage

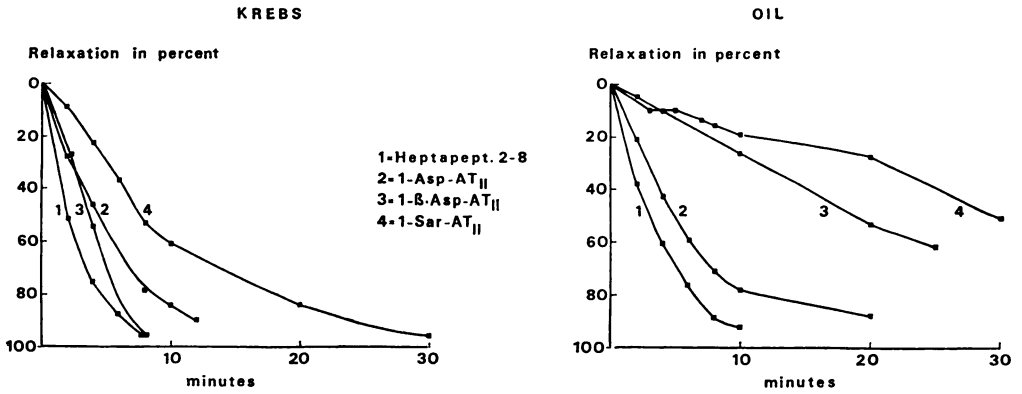

FIG. 7. Relaxation curves of equally effective doses of angiotensin and derivatives on rabbit aorta strips, suspended in a 10-ml organ bath at 37°C. The tissues were suspended in Krebs solution to obtain 60 to 70% of the maximum contractions. Thereafter, the tissues were washed several times with fresh Krebs (on the left) or the Krebs was replaced with mineral oil (on the right). Each point indicates the means of at least six determinations. Ordinates, percentage relaxation; abscissae, time in minutes. (Regoli *et al.*, Can. J. Physiol. Pharmacol. 52: 39–49, 1974).

by aminopeptidase. Thus the sustained response of 1-β-Asp-AT_{II} in oil was attributed to a slow inactivation by aminopeptidase, because this compound is rapidly removed by washing the tissue with Krebs (fig. 7 on the left). This confirms the results of other studies (68, 151, 238). On the other hand, the slow disposition of 1-Sar-AT_{II} was probably the result of an increased binding strength as well as of a reduced metabolism (compare relaxations in Krebs and in oil).

Two other compounds, 1-Asn^2-AT_{II} and heptapeptide 2–8, were so rapidly inactivated by the aorta, that pD_2 values may be underestimated on this tissue.

A slow rate of degradation is the mechanism that can be invoked to explain the observation of various authors, in particular: a) the high potency (150%) and prolonged duration of action of 1<Glu-AT_{II} and 1-Glu-AT_{II}, observed by Jorgensen *et al.* (136); b) the prolonged action of polymers of angiotensin, such as poly-D,L-Ala-AT_{II} (247) and horseradish peroxidase AT_{II} (196); and c) a different potency and duration of action reported by Havinga *et al.* (110–112) for des-Asp angiotensins of varied structures, particularly D-(N-terminal) heptapeptides.

Replacement of 1-Asp with a simple fatty acid residue (1-Suc-AT_{II} and 1-Me_2But-AT_{II}) (9, 221) or with different types of nitrogen groups (1-Sar-AT_{II}, 1-Me_2Gly-AT_{II}, 1-Bet-AT_{II}) (245, 247) may change the physicochemical property of the peptide and consequently, its binding affinity for the receptors. A fatty acid decreases the activity by 50% or more: a secondary nitrogen increases the affinity and the duration of action *in vitro*, but not *in vivo*, while a tertiary and a quaternary nitrogen reduce the potency significantly (247). This suggests that the terminal N-group requires a free hydrogen.

Position 2. There is unanimous agreement that the guanido group of 2-Arg plays an important role for the binding of AT_{II} to receptors. This emerges from biological analyses (136, 137) and is supported by conformational studies showing that the side chain of 2-Arg is free of interaction (78). Results obtained by Jorgensen *et al.* (136, 137) and more recently in our laboratory require a re-assessment of the criteria which, some years ago, formed the basis of a comprehensive analysis by Havinga and Schattenkerk (112). With heptapeptides instead of octapeptides, these authors conclude that the contribution of 2-Arg to the overall action of angiotensin "is not connected with the positive charge of the guanidinium group, but rather with its capacity for hy-

drogen donation to form a hydrogen bond" (112). This conclusion is based on the potency of heptapeptides *in vivo*, which may largely depend on the rate of splitting of the N-terminal residues, rather than by the activity of the side chain. Indeed, replacement of Arg with its D-isomer increases the activity of the heptapeptide (112), whereas it decreases the activity of the octapeptide (see table 3). This finding indicates that a high degree of orientation specificity is required for Arg in position 2. On the other side, the marked decrease of activity shown by 2-Ala-AT_{II} (see table 2) and the gradual decrease from 2-Arg (NO_2)-AT_{II} to 2-Orn-AT_{II} and 2-Cit-AT_{II} indicates that the length of the side chain and the functional guanido group of 2-Arg are both important for binding AT_{II} to receptor sites. This presumably occurs through electrostatic interaction or hydrogen bonding, as suggested by Printz (233), because the side chain of Arg is protonated at physiological pH. The involvement of Arg in the binding is substantiated by the fact that substitution of Arg with Ala affects affinity, but not intrinsic activity.

Position 3. Val in position 3 of AT_{II} has been considered in the past to play a minor role for the activity (136, 202). Conformational analyses have however shown that 3-Val may be involved in the formation of linear or nonlinear hydrogen bonds with 5-Val [in the *gamma* turn model (233)], or with 6-His (in the *beta* turn model): moreover, in the *beta* antiparallel structure suggested by Fermandjian *et al.* (78), 3-Val participates in the first turn. The persistence of 100% of activity with replacement of 3-Val with Leu, indicates that *beta* and *gamma* branching in position 3 are equally active in contrast to position 5 (139). Moreover, the residual 40% activity of 3-Pro-AT_{II} suggests that one hydrogen bond may be sufficient to maintain a relatively stable conformation, while the methylation of the *alpha* carbon (3-Aib-AT_{II}) or the absence of H in the *alpha* carbon (3-Acpc-AT_{II}) seems to have a greater degree of interference.

Position 4. Conformational analyses have suggested that side chains of 3-Val, 4-Tyr, and 5-Val (and presumably Ile) contribute to the hydrophobic cluster that is essential for occupation and stimulation of receptors by angiotensin. 4-Tyr, in addition, may form a hydrogen bond with the receptor site, capable of accepting a hydrogen from the phenolic hydroxyl (233). This might explain the drastic decrease of potency observed with 4-TyrOMe-AT_{II} and several other derivatives in which 4-Tyr has been replaced with an aliphatic chain (4-Leu-AT_{II}, 4-Acpc-AT_{II}, 4-Ala-AT_{II}), but not with aza-α-homo-Tyr (256). When Tyr is replaced by Phe, the compounds still maintain $\frac{1}{5}$ of the potency *in vivo* and $\frac{1}{30}$ *in vitro*. 4-Phe-AT_{II} has been found to behave as a partial agonist, showing that the hydroxyl group probably participates in stimulation of the receptors. Whether this is due to the acidic property of the hydroxyl group of Tyr, to its possible effect on the electron density of the aromatic ring or to its capacity to form a hydrogen bond, is still unknown. The decreased potency of 4-Tyr(I)-AT_{II} and 4-Tyr(I_2)-AT_{II} (165, 172) would suggest that ionization of the phenol group influences affinity, because the pK of this group is 10 in the native hormone, 8 in monoiodinated, and 6 in diiodinated AT_{II} (165).

Position 5. This position has been extensively studied by Jorgensen *et al.* (138, 139) with an interesting series of analogues, included in table 3. The results indicate clearly that a *beta* aliphatic or alicyclic chain is required to maintain the activity of the native hormone. Moreover, the conformational preference at the 5 position is favoured by rotation angles $\phi = -120$ and $\psi = -120$ (139). More recently, Jorgensen *et al.* (141) have explored the importance of the physicochemical characters (in particular, the lipophilic character) of the amino acid in position 5, with 5-ThrOMe-AT_{II} and 5-Thr-AT_{II}. The first compound shows higher potency (118%), while the second one is weaker (10%) than the native hormone. This is probably because 5-Thr-AT_{II} forms

an intramolecular hydrogen bond which may change the optimum conformation for receptor occupation. In conclusion, the results of Jorgensen *et al.* (138) point to the importance of *beta* branching and the lipophilic character of the side chain in position 5. A broad range of lipophilicity is tolerated (141).

Position 6. The unique properties of the imidazole ring of histidine, such as its ionization at physiological pH, its ability to act as a proton donor or acceptor, and its aromatic character are the most important features to be taken into account for evaluating the role of 6-His of angiotensin. Conformational models suggest that 6-His may participate in hydrogen bond formation [*beta* model, proposed by Printz *et al.* (233)] or in the second turn of the antiparallel structure by influencing the orientation of 7-Pro (78). The replacement of 6-His with D-His decreases the activity (202) and changes the conformation (78). 6-His has been replaced by Lys (281) to maintain the basicity of the side chain, and with Phe (280) probably to explore the role of an aromatic ring in position 6 of AT_{II}. Both substitutions bring about a marked loss of activity, indicating that the chemical attributes of the imidazole ring are important. Indeed, the acid-base properties of the imidazole ring seem not to be determinant, because 6-Pyr(3)Ala-AT_{II} (108) maintains 60% to 80% of the activity of AT_{II}. Hofmann *et al.* (119) conclude that the stereostructure of the five-membered heterocyclic ring of histidine and not its charge is of crucial significance for high-level of angiotensin activity. Elimination of the ring (6-Gly-AT_{II}) or substitution with a saturated five-membered ring (6-Acpc-AT_{II}) brings about important loss of affinity and of intrinsic activity (219). 6-Ala, on the contrary, does not show any decrease of intrinsic activity: these observations indicate that 6-His has probably a conformational role, and substitution with Gly and Acpc produces changes qualitatively different from Ala (219, 245).

Position 7. Proline is believed to be essential for the secondary structure and, in particular, for providing the critical orientation of 8-Phe. This has been suggested by Weinkam and Jorgensen (339, 340) and is an essential feature of the models proposed by Printz *et al.* (233) and by Fermandjian *et al.* (78). Nuclear magnetic resonance and circular dichroism spectra indicate that the 6-His, 7-Pro bond is towards a "*cis*"-conformation (78). Replacement of Pro with Ala changes the intrinsic activity very little, while decreasing affinity: major changes of affinity and intrinsic activity are however observed when 7-Pro is replaced by Acpc and Gly (245). Replacement with Sar, which should preserve the orientation specificity of Pro (267) has not been tried. These results indicate the possible conformational role of 7-Pro in angiotensin. The group in position 7 has to be hydrophobic, because hydroxyl proline shows only 10% of the activity of angiotensin. This is confirmed by the low activity of 7-Pip(pipecolic acid)-AT_{II} (37).

Position 8. The effect of eliminating 8-Phe has been analyzed before: heptapeptide 1–7 is completely inactive. A large number of analogues of AT_{II} substituted in position 8 have been prepared and tested recently: it is therefore possible at present to analyze separately the steric and functional effect of the active group of Phe. Both the hydrophobic phenyl ring and the anionic carboxyl terminal of 8-Phe appear to be important for binding angiotensin to receptors. The contribution of the carboxyl terminal will be analyzed first. When the ring is eliminated (*e.g.*, 8-Ala-AT_{II}), the analogue is an antagonist with a relatively good affinity for the receptors (see pA_2 values in table 6): 8-Phe-(OMe)-AT_{II} and 8-Phe-(NH_2)-AT_{II} have respectively 10 and 3% of the activity of AT_{II}, but do not exert any antagonism (202, 291). It seems therefore that the carboxyl terminal group subserves part, but not all, of the binding capacity of 8-Phe. In addition, the distance between the phenyl ring and the carboxyl terminal group, as well as the distance between 8-Phe and the

other side chains of AT_{II}, appear to be critical for the biological activity, because 8-Apb(1-amino-phenyl-butyric acid)-AT_{II} and 8-Apib(1-amino-phenyl-isobutyric acid)-AT_{II} are respectively 10 and 1000 times less active than AT_{II} (44). Unfortunately, the intrinsic activity of these compounds has not been measured: it will be interesting to know whether these analogues behave as partial agonists. Recently, 8-Apib-AT_{II}, but not 8-Apb-AT_{II}, has been reported to exert a weak antagonism against AT_{II} on smooth muscle (38). This compound seems to be interesting in view of the development of antagonists to block the action of angiotensin on receptors different from those of smooth muscle (*e.g.*, nervous elements of the guinea-pig ileum).

The phenyl ring of AT_{II} is essential for stimulation of receptors. In fact, when 8-Phe is replaced with a cationic or an anionic residue (*e.g.*, 8-Glu-AT_{II} and 8-Lys-AT_{II}), the compounds are almost inactive, or exert a weak antagonism (*e.g.*, 8-Glu-AT_{II}) (245). Moreover, the size and the length of the aromatic group in position 8 appear to be determinant for stimulation, because if the ring is saturated (*e.g.*, 8-Cha(cyclohexylalanine)-AT_{II}), there is a marked decrease of potency and a weak antagonism (162). Replacement of 8-Phe with an aliphatic derivative possessing side chains of different length changes the characteristic of the compound from agonist to anatgonist. The results of recent experiments (248) are summarized in table 4.

When 8-Phe is replaced a) with Tyr, the analogue shows a reduction of affinity, but maintains full intrinsic activity; b) with an aliphatic straight chain (*e.g.*, 8-Nle-AT_{II}), the analogue assumes the property of a typical partial agonist (261); c) with a shorter aliphatic unbranched (*e.g.*, 8-Nva-AT_{II}, 8-Abu-AT_{II}) or with a *gamma* or *beta* branched chain (*e.g.*, 8-Leu-AT_{II}, 8-Ile-AT_{II} and 8-Val-AT_{II}), the compounds lose intrinsic activity on smooth muscle and become potent antagonists (261): however, they maintain some stimulating action *in vivo*, as mentioned above; d) elimination of the ring or of the entire side chain (*e.g.*, 8-Ala-AT_{II} and 8-Gly-AT_{II}) gives antagonists with less affinity than 8-Val-AT_{II} and 8-Leu-AT_{II}, but without any stimulant effect *in vitro* and *in vivo* (241, 259). From these results, it seems that stimulation of receptors for angiotensin can be brought about either by *pi* bonding of the aromatic ring to the receptors or by hydrophobic interaction of the ring with a specific part of the receptors (80).

TABLE 4

Intrinsic activities (α^E) and affinities (pD_2 and pA_2) of AT_{II} and analogues substituted in position 8 on two smooth muscle preparations

Compound	Rat Stomach Strip[a]			Rabbit Aorta Strip[a]		
	α^E	pD_2	pA_2	α^E	pD_2	pA_2
AT_{II}	1.0	7.75		1.0	8.86	
8-Tyr-AT_{II}	1.0	7.16		1.0	7.94	
8-Nle-AT_{II}	0.50	7.55	8.44	0.55	8.60	8.66
8-Leu-AT_{II}	0.10		8.60	0		8.78
8-Nva-AT_{II}	0.05		8.50	0		8.62
8-Val-AT_{II}	0.05		7.94	0		8.34
8-Abu-AT_{II}	0.02		8.04	0		8.42
8-Ala-AT_{II}	0		7.22	0		7.70
8-Gly-AT_{II}	0		6.90	0		7.42

[a] α^E, pD_2, same explanations as in table 2; pA_2, the negative logarithm of the concentration of antagonist that reduces the effect of a double dose of agonist to that of a single dose [(273); D. Regoli, W. K. Park and F. Rioux, unpublished data.]

A few words should be said before ending this section on compounds that have been synthesized to determine the extent to which topochemistry of side chains, alteration of the secondary structure, and changes of the peptide backbone influence the biological activity of AT_{II}.

The all D-AT_{II} (366) as well as the retro-enantio isomer (H·Phe-Pro-His-Ile-Tyr-Val-Arg-Asp·OH) (248) are totally inactive. Simplified angiotensins (obtained by replacing one or more components with Gly), analyzed by Jorgensen *et al.* (132–135) are inactive as well as cyclic hexapeptide 3–8.

The following analogue (H·Ala-Ala-Ala-

Tyr-Ala-His-Ala-Phe·OH) was synthesized in our laboratory to see whether the presence of the groups (4-Tyr, 6-His, 8-Phe) most important for binding and stimulation would retain some activity in a peptide which is expected to have a conformation different from that of AT_{II}. No direct stimulating effect or inhibition could be demonstrated either *in vivo* or *in vitro* (248). Several constituents of AT_{II} have been replaced with D-isomers to improve the duration of action by preventing the degradation by proteolytic enzymes. This happened to be true for position 1 (255), but all other [*e.g.*, 2-D-Arg-AT_{II} (335), 4-D-Tyr-AT_{II} (257), 6-D-His-AT_{II} (257) and 8-D-Phe-AT_{II} (243)] analogues were almost inactive. None of these compounds exerted any antagonism, except for 8-D-Phe-AT_{II} (243). These results indicate that angiotensin requires a critical conformation to bind to the receptor. Several changes in the peptide backbone have been tried for the same purpose (to increase the biological half-life of the peptide). This includes a) *alpha*methylation in positions 3 and 5, which causes a marked loss of potency (177), and b) intercalation of a methylene group or extension of the chain in position 8 (see 8-Apb-AT_{II} and 8-Apib-AT_{II}, in table 3) (44). Finally, the replacement of the amide with an ester bond (—CO—O—CH—CO— instead of —CO—NH—CH—CO—) has been reported for the linkages 3–4, 4–5 and 7–8 of angiotensin: variable decreases of potency were observed *in vivo* [0.06, 3.0, and 10% respectively (294)].

IV. Antagonists of Angiotensin

A. Pharmacological Characterization of Angiotensin Antagonists

Application of the principles described by Gaddum (89) and Schild (274–276), to the study of antagonists requires the measurement of maximum effects of agonists (A) both in the absence and in the presence of an antagonist (B). The effect of any dose of A can then be calculated in percent of the maximum response and plotted against log dose. It follows that if the antagonist is competitive: a) the log dose-response curves of A in the presence of B remain parallel to the control; and b) the maximum response to A is obtainable in the presence of any concentration of B. In the case of long acting inhibitors, such as 1-Sar,8-Leu-AT_{II} (see fig 2), the slope of the log dose-response curve of the agonist is lower than that of the control curve, and the maximum effect is below 100%. These changes are similar to those expected by the theory for a noncompetitive antagonist. 1-Sar,8-Leu-AT_{II} is however specific for AT_{II}, and it is acting on the same receptors, or receptor sites, as AT_{II}. This is demonstrated by the finding that protection of receptors with AT_{II} completely prevents the action of 1-Sar,8-Leu-AT_{II} (246).

The protocol suggested by Arunlakshana and Schild (13) was recently applied in our laboratory to study the antagonism of 8-Gly-AT_{II} against AT_{II} and the partial agonist 4-Phe-AT_{II} in three tissues (rat stomach strip, rabbit aorta strip, and rat colon) suspended in a cascade superfusion system. Results are summarized in figure 8 and have been published elsewhere with more detail (219).

Dose-response curves of AT_{II} and 4-Phe-AT_{II} in the presence of 8-Gly-AT_{II} remain parallel to the control over a large range of concentrations of antagonists and the maximum effect of AT_{II} is not depressed. Plots of log (DR − 1) against −log [B] gives linear relationships with slope close to unity on the rat stomach and rabbit aorta strips. These results indicate that 8-Gly-AT_{II} is a competitive antagonist of AT_{II} and 4-Phe-AT_{II} on these two tissues. Intersection of the curves with the abscissa gives an estimated of the pA_2 (13).

A similar analysis has been carried out with 8-Leu-AT_{II} and 1-Sar,8-Leu-AT_{II} on the rat stomach strip (246, 259). It has been shown that the first two compounds behave as competitive antagonists of AT_{II}, while 1-Sar,8-Leu-AT_{II} acts as a long acting inhibitor (244, 246).

1-Sar,8-Leu-AT_{II} acts on the same receptors as AT_{II} (246), but it is slowly reversi-

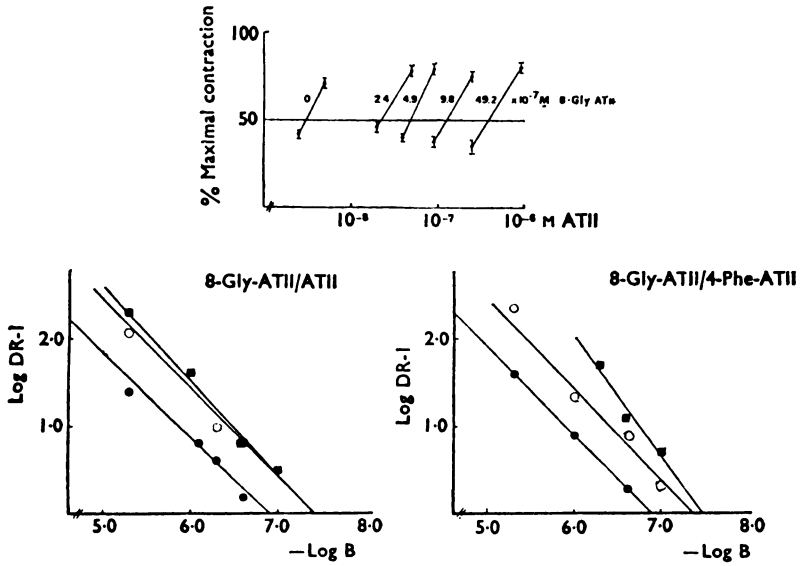

FIG. 8. Top, effect of angiotensin II (AT_{II}) in absence and in presence of 8-Gly-AT_{II} on rabbit aorta. Means ± S.E. of six experiments. Ordinate, maximal contraction (%). Abscissa, molar (M) concentration of AT_{II}. (This is an example of the experimental protocol used to obtain the data for the log-log plots shown in the two sections below.) Bottom, plots of log dose-ratio-1 (Log DR-1) (ordinates) against antagonist concentration (−Log B) (abscissae), showing competitive antagonism of AT_{II}/8-Gly-AT_{II} (left) on the three tissues and of 4-Phe-AT_{II}/8-Gly-AT_{II} (right) on rat stomach strip and rabbit aorta. In each instance, except 4-Phe-AT_{II}/8-Gly-AT_{II} on the rat colon, the slope is close to unity. ●——●, Rat stomach strip; ○——○, rabbit aorta; ■——■, rat colon (Park *et al.*, Brit. J. Pharmacol. **48**: 288–301, 1973).

ble, because it presumably forms a firm and prolonged binding with the receptors. Therefore, the slope of the curve obtained by plotting log (DR − 1) and −log [B] is greater than 1.0. This prolonged binding to receptors would explain why even small doses of this compound not only depress the maximum effect of AT_{II}, but displace the dose-response curves of AT_{II} to the right and depress the slope of the curve as an irreversible antagonist (see example in fig. 2). For this and similar compounds (the Sarcosyl analogues at AT_{II} reported in table 5) pA_2 values may be overestimated (13).

8-Abu-AT_{II}, 8-Val-AT_{II}, 8-Ile-AT_{II} and other analogues substituted in position 8 (see table 4) were evaluated by measuring pA_2 and pA_{10}, according to Schild (273). Differences between pA_2 and pA_{10} after 5 or 15 minutes of contact of antagonist with the tissue fell close to 0.95, the theoretical value of competitive inhibitors (259).

The octapeptide inhibitors of AT_{II} are specific for angiotensin: they block the contractions of rat stomach strips elicited by AT_{II} and AT_I, but do not influence at all those produced by 5-hydroxytryptamine and bradykinin (259), prostaglandin E_2, and acetylcholine (248).

Most of the inhibitors synthesized and tested in our laboratory (see table 5) are active against angiotensins on the rabbit aorta strip: responses of aorta to noradrenaline, 5-hydroxytryptamine, and histamine are not modified (248) by these compounds.

Onset and duration of action of both competitive and long acting antagonists were evaluated by testing the effect of AT_{II} before and at selected time intervals (2.5, 5, and 15 min) after contact of the antagonist with the tissue (rabbit aorta strip).

pA_2 values were measured for 8-Leu-AT_{II}, 8-Gly-AT_{II}, and other antagonists. No significant difference between pA_2 values measured after 5 and 15 minutes was seen, while the values after 2.5 minutes were signifi-

cantly lower. The inhibitory action of octapeptide antagonists of AT_{II} was rapidly reversible (25–30 min on the rat stomach, 40–60 min on the rabbit aorta strip) for all compounds substituted only in position 8. On the contrary, double substituted analogues (with Sar in position 1 and on aliphatic residue in position 8, see table 5) had a much longer duration of action (60–120 min), although their onset of action was as rapid as for the monosubstituted (only in position 8) inhibitors (248).

B. Structure-activity Relationships of Angiotensin Antagonists

The essential elements for the design of angiotensin inhibitors are contained in the analysis presented in section III. Most of the side chains of AT_{II} are largely responsible for binding the peptide to its specific receptors or for maintaining the critical secondary structure, while 8-Phe and, to a minor extent, 4-Tyr appear to be involved in initiating the processes that subserve contraction of smooth muscle. Moreover, the duration of action of angiotensin analogues can be improved by replacing Asp with Sar or other derivatives in position 1. It follows that specific inhibitors of AT_{II} should be obtained by replacing 8-Phe and leaving the other side chains unchanged in order to maintain optimum conformation and full affinity. Single substitution in position 4 should not give rise to an antagonist because of the minor role played by 4-Tyr in stimulation. Combination of 2 substitutions (in positions 4 and 8) may be disadvantageous because the inhibitor will lose affinity. On the contrary, replacement of Asp in position 1 can improve the potency and the duration of action. These assumptions are substantiated by the observations reported from various laboratories during the last 3 years. Most of the data published have been summarized in table 5. The analysis of this table reveals that 8-Phe has been replaced in all compounds except one (no. 37). The substitution in position 8 is the only one for the first 19 compounds and is associated to a substitution in position 4 or/and in position 1, for the others. All compounds of table 5 have been tested *in vivo* (on the blood pressure of nephrectomized rats or of rats treated with pentolinium) and *in vitro* on various isolated smooth muscles.

The comparison of potencies of monosubstituted (position 8) derivatives of AT_{II} reveals that:

1. Elimination of the side chain in position 8 (*e.g.*, 8-Gly-AT_{II}) brings about an antagonist which is competitive and active *in vivo* and *in vitro*. Systematic studies of this compound have been reported recently (219, 246, 259).

2. Affinity for the receptors increases in parallel with the length of the aliphatic chain in positions 8 (241, 259). This is demonstrated by the increase of the pA_2 values from 8-Gly-AT_{II} to 8-Leu-AT_{II} and has been analyzed in detail before (see section III).

3. Maximum affinity within the groups 1–19 is observed with 8-Nva-AT_{II}, 8-Leu-AT_{II}, and 8-Nle-AT_{II}.

4. Lengthening of the side chain in position 8 increases the affinity, but at the same time, restores some of the angiotensin-like activity, especially at high doses and *in vivo* (see tables 4 and 5).

5. The claim that *beta* branching (8-Ile-AT_{II}) (344) is more favorable than *gamma* branching (8-Leu-AT_{II}) has not been confirmed in our laboratory (see table 4). The two compounds are equally potent in several pharmacological tests *in vivo* and *in vitro* (rat blood pressure, rabbit isolated aorta, rat stomach) (248).

6. Hydrophobicity of the side chain are important for optimum affinity: 8-Glu-AT_{II} and 8-Lys-AT_{II} are almost inactive.

7. The potency and specificity of antagonists are not influenced by Ile or Val in position 5. On the contrary, the presence of Asn^2 instead of Asp in position 1 may decrease the potencies of antagonists on rabbit aorta strips, because the potency of 1-Asn^2-AT_{II} in this preparation is only 25% of that of 1-Asp-AT_{II}. This may in fact explain the differences between pA_2 values estimated by

TABLE 5

Primary structure and pharmacological parameters of angiotensin antagonists

No.	Sub.	Name	Primary Structure 1 2 3 4 5 6 7 8	Rat Blood Pressure			Isolated Organs			Reference(s)[a]
				ID50[b] μg/kg/min	Rel. pot.[c]	Res. activ.[d] %	pA2[e] Isometric	pA2[e] Isotonic	Res. Activ. %	
1	8	8-Gly-AT_{II}	H·Asp -Arg-Val-Tyr-Ile -His-Pro-*Gly*·OH	0.72	24	<0.1	7.17, 7.2*[f]	6.9, 7.42*[f]	0	(259, 219)
2	8	8-Ala-AT_{II}	H·Asp -Arg-Val-Tyr-Ile -His-Pro-*Ala*·OH	0.47	36	<0.1	6.86	7.22, 7.7*	0	(241, 258)
				Inactive			8.32*			(324)
3	8	8-Abu-AT_{II}	H·Asp -Arg-Val-Tyr-Ile -His-Pro-*Abu*·OH	0.34	50	0.2	7.48	8.04, 8.42*	<5.0	(241, 259)
4	8	8-Val-AT_{II}	H·Asp -Arg-Val-Tyr-Ile -His-Pro-*Val*·OH	0.47	36	0.2	7.51	7.94, 8.34*	<6.0	(241, 258)
5	8	8-Nva-AT_{II}	H·Asp -Arg-Val-Tyr-Ile -His-Pro-*Nva*·OH	0.36	47	0.3		8.50, 8.62*		(221, 248)
6	8	8-Ile-AT_{II}	H·Asp -Arg-Val-Tyr-Ile -His-Pro-*Ile*·OH	0.21	81	0.4	7.9	8.36	<10	(241, 259)
							9.21*			(161, 344)
7	8	8-Leu-AT_{II}	H·Asp -Arg-Val-Tyr-Ile -His-Pro-*Leu*·OH	0.17	*100*	0.4	8.03, 8.21*	8.60, 8.78*	10	(241, 219)
8	8	8-Nle-AT_{II}	H·Asp -Arg-Val-Tyr-Ile -His-Pro-*Nle*·OH	0.32	51	1.0		8.44, 8.66*		(221, 248)
9	8	8-Acpc-AT_{II}	H·Asp -Arg-Val-Tyr-Ile -His-Pro-*Acpc*·OH	1.05	16	0.2	5.69		<5.0	(243, 258)
10	8	8-Cha-AT_{II}	H·Asp -Arg-Val-Tyr-Ile -His-Pro-*Cha*·OH	1.0†[g]	20	20.0			20*	(162)
11	8	8-D-Phe-AT_{II}	H·Asp -Arg-Val-Tyr-Ile -His-Pro-*D-Phe*·OH	0.6	28	0.1	5.37		5.0	(243, 258)
12	8	8-D-Leu-AT_{II}	H·Asp -Arg-Val-Tyr-Ile -His-Pro-*D-Leu*·OH	1.7	10	<0.1		6.43	0	(221, 248)
13	8	1-Asn^2,8-Gly-AT_{II}	H·Asn^2 -Arg-Val-Tyr-Val-His-Pro-*Gly*·OH					6.54*		(80)
14	8	1-Asn^2,8-Ala-AT_{II}	H·Asn^2 -Arg-Val-Tyr-Val-His-Pro-*Ala*·OH	10†		‡‡[h]		6.84*		(211)
15	8	1-Asn^2,8-β-Ala-AT_{II}	H·Asn^2 -Arg-Val-Tyr-Val-His-Pro-*β-Ala*·OH					5.08*		(80)
16	8	1-Asn^2,8-Abu-AT_{II}	H·Asn^2 -Arg-Val-Tyr-Val-His-Pro-*Abu*·OH					7.93*		(80)
17	8	1-Asn^2,8-Val-AT_{II}	H·Asn^2 -Arg-Val-Tyr-Val-His-Pro-*Val*·OH					8.31*		(80)
18	8	1-Asn^2,8-Leu-AT_{II}	H·Asn^2 -Arg-Val-Tyr-Val-His-Pro-*Leu*·OH					8.26*		(80)
19	8	8-Cys-AT_{II}	H·Asp -Arg-Val-Tyr- His-Pro-*Cys*·OH	0.1†				8.3**†		(191)
20	4–8	4-Phe,8-Tyr-AT_{II}	H·Asp -Arg-Val-*Phe*-Val-His-Pro-*Tyr*·OH			<0.1		6.7†	0	(176)
21	4–8	4-Phe,8-Tyr-AT_{II}	H·Asp -Arg-Val-*Phe*-Ile -His-Pro-*Tyr*·OH	1.7	10	2				(243)
22	4–8	4-Phe,8-Ala-AT_{II}	H·Asp -Arg-Val-*Phe*-Ile -His-Pro-*Ala*·OH			<1.0	5.5*		0	(162)
23	1–4–8	1-Suc,4-Phe,8-Tyr-AT_{II}	-Suc- -Arg-Val-*Phe*-Ile -His-Pro-*Tyr*·OH	Negligible			4.5–5.0*†			(162)
24	1–8	1-Sar,8-Gly-AT_{II}	H·*Sar* -Arg-Val-Tyr-Ile -His-Pro-*Gly*·OH	0.16	106	<1.0	8.28*		0	(246, 247)
25	1–8	1-Sar,8-Ala-AT_{II}	H·*Sar* -Arg-Val-Tyr-Val-His-Pro-*Ala*·OH	<1.0		<1.0		8.61*	0	(212)
26	1–8	1-Sar,8-Ala-AT_{II}	H·*Sar* -Arg-Val-Tyr-Ile -His-Pro-*Ala*·OH	0.15	113	<0.1	8.38		0	(221, 248)
27	1–8	1-Sar,8-Abu-AT_{II}	H·*Sar* -Arg-Val-Tyr-Ile -His-Pro-*Abu*·OH	0.16	106	††		8.76		(221, 248)
28	1–8	1-Sar,8-Val-AT_{II}	H·*Sar* -Arg-Val-Tyr-Ile -His-Pro-*Val*·OH	0.21	81	<0.1		8.84		(221, 248)

29	1–8	1-Sar,8-Ile-AT_{II}	H·*Sar* -Arg-Val-Tyr-Ile -His-Pro-*Ile*·OH	0.03 Cat. B.P.			9.33*		0	(162)
30	1–8	1-Sar,8-Leu-AT_{II}	H·*Sar* -Arg-Val-Tyr-Ile -His-Pro-*Leu*·OH	0.04	425	0.2	8.6, 8.71*	9.17, 9.06*	10–20	(244, 246)
31	1–8	1-β-Asp,8-Leu-AT_{II}	H·β-*Asp*-Arg-Val-Tyr-Ile -His-Pro-*Leu*·OH	0.15	113	0.7	8.23, 8.21*		10	(221, 247)
32	1–8	1-Pro,8-Gly-AT_{II}	H·Pro -Arg-Val-Tyr-Ile -His-Pro-*Gly*·OH	1.05	16	<0.1	7.1*		0	(221, 247)
33	1–8	1-Pro,8-Leu-AT_{II}	H·Pro -Arg-Val-Tyr-Ile -His-Pro-*Leu*·OH	0.18	94	0.5	8.04*		10	(221, 247)
34	1–8	1<Glu,8-Gly-AT_{II}	<*Glu* -Arg-Val-Tyr-Ile -His-Pro-*Gly*·OH	1.0	17	<0.1	6.13*		0	(221, 247)
35	1–8	1<Glu,8-Leu-AT_{II}	<*Glu* -Arg-Val-Tyr-Ile -His-Pro-*Leu*·OH	0.32	51	0.2	7.64*		5–10	(221, 247)
36	2–8	2-Pro,8-Abu-AT_{II}	H·Asp -*Pro*-Val-Tyr-Ile -His-Pro-*Abu*·OH	20	0.8	<0.1				(221)
37	1	1-Chl-des-Asp^1-AT_{II}	(H·*Chl*) -Arg-Val-Tyr-Ile -His-Pro-*Phe*·OH	4 mg/kg/min prolonged block		1.0		5.0**†	10**	(206)

[a] Reference(s), same explanation as in table 3.

[b] ID50, the dose of antagonist which requires a two fold increase of the dose of angiotensin, to produce the same elevation of blood pressure, as occurred in the control period.

[c] Rel. pot., relative potency in percent. 8-Leu-AT_{II} = 100%.

[d] Res. activ., indicates residual pressor or myotropic (rat stomach) activities in percent. 1-Asp-AT_{II} = 100%.

[e] pA_2, Same as in table 4. The differences of pA_2 values obtained for the same compound with the isometric and the isotonic technique may be due to technical artifacts (volume of the organ bath, presence of glass surfaces, different conditions of oxygenation and temperature) (248).

[f] *, Values obtained on rabbit aorta; ** rat uterus; values without any mark have been obtained on rat stomach.

[g] †, Indicates data which have been calculated from curves or tables published by the authors, quoted in the corresponding reference(s).

[h] ††, Indicate that residual pressor activity is present after high doses of the compound, but has been not quantified.

Fessler *et al.* (80) and our group. Another important difference is on the time of contact of the antagonist with the tissue before applying the standard agonist. Both Fessler *et al.* (80) and Türker *et al.* (324, 325) used 2.5 to 3.0 minutes of contact. We have shown before that this time is too short for obtaining equilibrium conditions.

8. Replacement of 8-Phe a) with Acpc or Cha (to study the contribution of aromaticity or ring size) gives very weak antagonists which maintain some angiotensin-like activity (162, 243), b) with the D-enantiomer (which should be functionally identical) leads presumably to substantial steric changes and consequently to a drastic loss of affinity (see 8-D-Phe-AT_{II}) (243), and c) with "Cys" to allow strong binding at the receptors site through the active SH group, gives a potent antagonist, but apparently without prolonged duration of action (192). All monosubstituted inhibitors in position 8 presumably act as pure competitive antagonists, according to the results published by several authors (140, 162, 243, 246, 259).

The second group of analogues substituted in positions 4 and 8 (no. 20–23 in table 5) have a much lower potency than the member of first group. Moreover, the angiotensin-like activity *in vivo* is consistently higher than those of 8-Gly-AT_{II} and 8-Leu-AT_{II} (241, 243). Residual angiotensin-like activity *in vitro* varies from one tissue to another and is practically absent on the rat uterus (176) and on the rat stomach strip (248), but some residual activity is observed on the rat colon (243). When the effect of the two substitutions is analyzed separately (*e.g.*, with 4-Phe-AT_{II} and 8-Tyr-AT_{II}), it is evident that replacement of 4-Tyr by Phe gives a partial agonist with low affinity and replacement of 8-Phe with Tyr decreases the potency, but does not confer on the molecule any antagonistic properties. The combination of the two brings about weak antagonists which block the effect of AT_{II} *in vitro*, but have too much angiotensin-like activity to be used as antagonists *in vivo* (243).

The third series of analogues substituted in positions 1 and 8 (no. 24–36 in table 5) contain the most potent antagonists. The observation by Pals *et al.* (212) that Sar in position 1 increases the affinity of the antagonist has been used by us and other groups successfully to improve the potency of 8-Leu-AT_{II} (244, 245) and to some extent of 8-Ile-AT_{II} (162). pA_2 values of 1-Sar,8-Ala-AT_{II}, 1-Sar,8-Leu-AT_{II} *in vitro* (rat stomach strip) are 5 to 15 times higher than those of the corresponding monosubstituted compound. Sar in position 1 seems to improve the potency of the antagonist more than β-Asp or <Glu, two derivatives that should prevent the degradation by aminopeptidases. The duration of action of Sar derivatives is longer than those of 1-β-Asp and 1<Glu congeners. The possible mechanism responsible for prolonging the duration of action of Sar analogues has been discussed before. Replacement of 1-Asp and 2-Arg with Pro was performed in our laboratory in order to test the hypothesis of Riniker (254) that an imino group in a peptide bond prevents the action of aminopeptidases. However, the potency of the 1-Pro or 2-Pro derivatives was similar to those of the monosubstituted antagonists.

Finally, Paiva *et al.* (206) have attempted to develop angiotensin antagonists by replacing Asp with chlorambucil in position 1 or adding chlorambucil to angiotensin at the amino end. This has led to compounds which exert some antagonistic effect *in vivo* and *in vitro:* the effect is prolonged for several hours, when the antagonist is applied *in vivo* at very high doses (2 to 4 mg/kg) but the angiotensin-like activity is still so strong that the compounds should be considered as agonists rather than antagonists. The addition of chlorambucil to angiotensin could be a useful approach when combined with substitution in position 8: if the addition of chlorambucil to the amino end of angiotensin II does not modify the secondary structure of the peptide substantially, antagonists with long duration of action will theoretically be obtained, because chlorambucil would probably bind firmly to accessory areas of the recep-

tors for angiotensin. This approach has not yet been explored for angiotensin II: however, Paiva *et al.* (207) have recently reported that AT_I decapeptide substituted in position 1 with chlorambucil and in position 8 with Val, antagonizes the action of angiotensin I and II *in vivo*, but has a short duration of action.

In spite of the considerable progress accomplished in the field of angiotensin antagonists, the compounds reported in table 5 are only useful for pharmacological experiments and for exploring the physiological role of the renin-angiotensin system: they are too rapidly inactivated for any therapeutic application.

All angiotensin antagonists are active *in vivo* at small doses when given by intravenous infusion, or at high doses, when administered by injection (241). The effects of exogenous angiotensin I and II are reduced or suppressed, while the actions of noradrenaline and Lys-vasopressin are not influenced or very little (243, and unpublished results from our laboratory). The claims that 8-Ala-AT_{II} is inactive *in vivo* (324) have not been confirmed either by us (217, 243) or by Fessler *et al.* (80).

In view of the possible involvement of the renin-angiotensin system in the pathogenesis of hypertension of renal origin, 1-Sar,8-Ala-AT_{II} has been studied extensively in renal hypertensive rats. It reduces significantly the blood pressure of rats with unilateral ischemia when the other (unclamped) kidney is present, but does not change the blood pressure of unilaterally nephrectomized rats (33, 212, 269). 8-Cys-AT_{II} is also effective in reducing the blood pressure of hypertensive rats (191). The finding reported by Smeby *et al.* (306) that angiotensin antagonists are equally effective in both types of experimental hypertension (with one or both kidneys) is in contrast with the results of other laboratories (212, 269). 1-Sar,8-Ala-AT_{II} is more potent in reducing blood pressure in the acute than in the chronic phase of hypertension (212). In addition, the antagonists of angiotensin are more effective in anesthetized (33) than in conscious hypertensive rats (269).

Inhibitors of angiotensin seem to counteract the action of the native hormone on other physiological functions. Johnson and Davis (129, 130) have shown that the stimulant effect of angiotensin on aldosterone secretion in dogs is prevented by pretreatment with 1-Sar,8-Ala-AT_{II} and more recently, the same group of workers have found that this inhibitor is able to prevent the increase of aldosterone secretion produced in dogs by low sodium diet or by vena cava constriction (58). Several angiotensin inhibitors have been used by Peach (227) to block the catecholamines released by angiotensin on the isolated retrograde perfused adrenal glands of the cat. 8-Leu-AT_{II}, 8-Val-AT_{II}, 8-Ile-AT_{II} and 8-Cha-AT_{II} produce an effective block, the extent of which depends on the dose of inhibitor, and the antagonism is apparently of the competitive type (227).

Inhibitors of angiotensin are also effective in preventing the action of the natural hormone on the heart. Peach (227) has recently reported that a relatively low concentration of 8-Ile-AT_{II} prevents the positive inotropic effect of angiotensin on the cat papillary muscle. Recent results from our laboratory, with 8-Gly-AT_{II}, confirm these findings (249): in addition, Bonnardeaux and Regoli (30) have observed recently that the inotropic effect of both angiotensin and heptapeptide 2–8 on perfused rabbit heart spontaneously beating or electrically stimulated is blocked by 8-Gly-AT_{II} and 1-Sar,8-Gly-AT_{II}.

From these results, it may be concluded that analogues of AT_{II} substituted with an aliphatic derivative in position 8 are able to prevent most if not all the physiological actions of angiotensin. Some compounds possess a relatively long duration of action *in vivo* (2–3 hours) and can possibly be used for diagnostic purposes. However, the biological half-life is too short for therapeutic application. Moreover, some inhibitors still retain a relatively high angiotensin-like activity, particularly *in vivo* (244): this represents a disad-

vantage and limits the use of high doses of potent inhibitors (i.e., 1-Sar,8-Leu-AT_{II}) for the complete blockade of the action of endogenous angiotensin in living animals.

Inhibitors of AT_{II} with prolonged action *in vivo* would be valuable tools both in pharmacology and therapeutics. However, this goal will not be reached until chemical modifications can be made to the molecule, which will prevent degradation by peptidases. For further suggestions on the design of peptide inhibitors, the reader is referred to the article by Rudinger (267).

C. Other Antagonists of Angiotensin

The multiple pharmacological actions of angiotensin are reduced by drugs with opposite effects and acting on different receptor sites (physiological antagonists). This topic has been reviewed in the book *Renal Hypertension* in 1968 (202). Since then, no further studies or critical new results have been reported for compounds such as hydralazine, bretylium and *beta* adrenergic blocking agents.

A few publications have appeared on guancydine, an antihypertensive agent, and on lidoflazine, a piperazine derivative which acts as coronary vasodilator (202). Guancydine has been recently characterized as noncompetitive inhibitor of AT_{II}: Worcel *et al.* (343), with smooth muscle preparations (rabbit aorta strip, rat uterus) showed that guancydine depresses the action of AT_{II} as well as those of other myotropic agents (oxytocin, 5-hydroxytryptamine (5-HT), noradrenaline (NA)) and therefore, is nonspecific for AT_{II}. Rioux *et al.* (259) have arrived at the same conclusion, by estimating pA_2 and pA_{10}, according to Schild (273), on the rat stomach strip. They have established that guancydine antagonizes the effect of AT_{II} in a noncompetitive way.

These results contradict the findings of Cummings *et al.* (53), who claimed that guancydine is a "semi-selective" inhibitor of AT_{II} *in vivo*, because it depresses the pressor action of AT_{II} more than that of adrenaline or NA.

Lidoflazine has been shown by Godfraind *et al.* (97) to antagonize the slow component (direct myotropic action) of the contraction induced by AT_{II} in the guinea-pig ileum. Because of the presence of two receptors for AT_{II} in this tissue, Godfraind *et al.* were not able to establish the type of antagonism of AT_{II} by lidoflazine. They claimed that lidoflazine is highly specific for AT_{II}, although it interferes with the myotropic effects of histamine (H), acetylcholine (ACh) and bradykinin (Bk). Ellis and Reit (72) on the rat colon and, more recently, Rioux *et al.* (259) on the rat stomach strip have clearly established that lidoflazine is not specific for AT_{II}, because it blocks the actions of other drugs (5-HT and Bk). The type of antagonism exerted by lidoflazine on AT_{II} has been defined by Rioux *et al.* (259) as noncompetitive since pA_2–pA_{10} differences averaged 0.75 both for lidoflazine/AT_{II} and lidoflazine/5-HT on the rat stomach strip. To our knowledge, no other inhibitors of angiotensin have been described during the last 3 years.

V. Receptors for Angiotensin

A. Pharmacological Characterization of Receptors for Angiotensin

The ability of several smooth muscles to respond selectively to small doses (10^{-10} M–10^{-9} M) of AT_{II} and the fact that this peptide is totally inactive on other smooth muscle [cat nictitating membrane, guinea-pig vas deferens (21, 75)] suggest the existence of receptors for this hormone. We have shown in sections II and IV that the receptors of AT_{II} are specific because a) several drugs that affect the actions of 5-HT, ACh, or NA do not influence the myotropic effect of AT_{II}, and b) 8-substituted analogues of AT_{II} which reduce or block contractions of smooth muscle to AT_{II}, do not influence the effects of other myotropic agents.

In section II, we have shown that the relationships between doses of AT_{II} and contractions of smooth muscle can be analyzed quantitatively with the equation of the receptor theory. The concepts of affinity and intrinsic activity have been applied success-

fully to the classification of several analogues of AT_{II} into three groups: full agonists, partial agonists, and antagonists. These compounds provide useful tools for the characterization of receptor for AT_{II}. In fact, most progress in this field can be achieved with antagonists, largely because only one parameter, the affinity of the antagonist (pA_2 value) for receptors, needs to be established (275, 276). Comparison between agonists (order of potency) is also useful for classification of receptors, provided that both affinity (pD_2) and intrinsic activity (α^E) of all compounds can be measured.

These considerations seem appropriate before analyzing in a critical way several studies reported in recent years on the receptors for angiotensin (183, 219, 227).

With several angiotensin derivatives (1-Asn^2-AT_{II}, 1-Asn^2,2-Orn-AT_{II}, 1-Asn^2, 4-Phe-AT_{II}, 1-Asp,4-Phe-AT_{II}, 1-Asn^2,5-Tyr-AT_{II}, and 9-Pro,10-Phe-AT_I), Meyer *et al.* (183) explored the receptors for angiotensin in rat colon, rat uterus, and rat aorta. The order of potency was used as the single criterion, to affirm that the receptors in the three tissues are different. Further work with antagonists should be carried on to substantiate these findings.

Peach (227) has extensively explored receptors of angiotensin in cat papillary muscle *in vitro*, with several analogues and fragments of AT_{II} (AT_{II}, 3-Ala-AT_{II}, 4-Tyr-(OMe)-AT_{II}, 5-Ala-AT_{II}, 6-Ala-AT_{II}, 7-Ala-AT_{II}, 8-Ile-AT_{II}, heptapeptide 2–8 and hexapeptide 3–8). He concluded that receptors for angiotensin in the heart are different from those found in smooth muscle (*e.g.*, rat uterus), because a) the minimum length of the peptide chain required for stimulation of the heart is the heptapeptide 2–8 instead of the hexapeptide 3–8 as in the rat uterus, and b) the replacement of 7-Pro with Ala or Hyp (hydroxyproline) affects activity on the heart very little, in contrast with what has been found on smooth muscle. The inotropic contractions of cat papillary muscle to angiotensin were not influenced by *alpha* or *beta* adrenergic blocking agents (227), indicating that receptors for AT_{II} in this preparation have a good degree of specificity.

There are two main objections to the conclusion of Peach: a) the use of the order of potency as the single criterion for characterization of AT_{II} receptors is insufficient; and b) the existence of different receptors in cat heart and rat uterus does not necessarily imply that receptors for AT_{II} in the heart and in smooth muscle in general are different. Limitations of the rat uterus for the studies on structure-activity relationships of angiotensin have been underlined in section II. In addition, Meyer *et al.* (183) have suggested that receptors for AT_{II} in the rat uterus may be different from those of other smooth muscles of the rat.[3] Such a difference was confirmed in our laboratory (248), with various smooth muscle preparations. The order of potency reported by Peach (227) on the uterus (1-Asn^2-AT_{II} > 1-Asp-AT_{II} > Hepta 2–8 > 3-Ala-AT_{II} > AT_I > 5-Ala-AT_{II} > 8-Ile-AT_{II} > 7-Ala-AT_{II} and 6-Ala-AT_{II}) differs from the order of potencies in rabbit aorta strips (1-Asp-AT_{II} > 1-Asn^2-AT_{II} > 3-Ala-AT_{II} > Hepta 2–8 > AT_I > 7-Ala-AT_{II} > 5-Ala-AT_{II} > 6-Ala-AT_{II}), rat stomach strip (1-Asn^2-AT_{II} > 1-Asp-AT_{II} > Hepta 2–8 > 3-Ala-AT_{II} > AT_I > 7-Ala-AT_{II} > 5-Ala-AT_{II} > 6-Ala-AT_{II}), and for a few compounds, on the rat colon (1-Asp-AT_{II} > 1-Asn^2-AT_{II} > AT_I > 7-Ala-AT_{II} > 6-Ala-AT_{II}) (248).

It can be therefore concluded that identity or dissimilarity between receptors of AT_{II} in the heart and in smooth muscle has not yet been established. The analysis of the data shown above raises another interesting point: in fact, the order of potencies we have found for angiotensin and analogues in the rat stomach strip and rabbit aorta strip would not allow the conclusion that receptors of AT_{II} in the two tissues are of the same type. This conclusion is however supported by the

[3] Since submission of this paper Baudouin-Legros *et al.* (351) have reported that endogenous prostaglandins participate in the contractile-response of oestrogen-treated rat uterus to angiotensin II.

results with antagonists (second criterion): in fact, pA_2 values for competitive antagonists of AT_{II} are similar in rat stomach and rabbit aorta strip (see table 6).

Receptors for AT_{II} in the peripheral sympathetic nervous system, the adrenal medulla and the adrenal cortex have been studied by Peach *et al.* (227). Structure-activity relationships studies on inhibition of noradrenaline uptake by angiotensins in perfused rabbit hearts showed differences that induced these authors (225) to propose another type of receptor for angiotensin. In fact, the activities displayed by several 8-substituted analogues of AT_{II} on this function (inhibition of NA uptake) did not correlate with activities of the same compounds on the rat blood pressure, rat uterus, or cat papillary muscle.

The rabbit heart *in vitro* is a pharmacological preparation inadequate for precise and repeated evaluation of dose-response curves and maximum effects (30) of AT_{II}. This inadequacy should be taken into account particularly when 8-substituted analogues of AT_{II} are used to identify receptors, because these compounds may act as agonists, partial agonists or antagonists (see table 4). As far as the biological effect (block of noradrenaline uptake by angiotensin) is concerned, some contradictory results have been recently reported, which will be discussed in section VII.

Release of catecholamines from the adrenal medulla of cats and dogs by angiotensin has been studied by Feldberg and Lewis (74, 75) and by Staszewska-Barczak and Vane (315). The order of potency of a limited number of angiotensin analogues fall very close to that found *in vivo* (rat blood pressure).

Antagonists of ACh such as atropine and hexamethonium do not affect the response of the adrenal medulla to angiotensin (75, 315) nor is the depolarization of chromaffin cell by angiotensin affected by these drugs (66).

Peach (227) has shown that AT_I is as active as AT_{II} on the adrenals and acts directly (is apparently not converted to AT_{II}). Moreover, an aromatic amino acid (Phe) is not essential in position 8, because 8-Ala-AT_{II} maintains 25% of activity (226). In this respect, 8-Ala-AT_{II} is apparently an exception: several other 8-substituted analogues of AT_{II} (8-Leu-AT_{II}, 8-Val-AT_{II}, 8-Ile-AT_{II}, and 8-Cha-AT_{II}) inhibit the action of the native hormone (227). Before concluding that receptors for angiotensin mediating the release of adrenal catecholamines are different from those of smooth muscle, it would be essential to know whether the effect of AT_I is blocked by the specific and competitive antagonists of AT_{II} and what pA_2 values will be found for the same inhibitor against AT_I and AT_{II}.

The stimulation of aldosterone release and synthesis by angiotensin has been studied by Hageman *et al.* (108) and Blair-West *et al.* (26) in dogs. Order of potencies of angiotensin derivatives in this system were similar to those found in isolated smooth muscles and on the rat blood pressure. The only exception was the heptapeptide 2–8 which is apparently as active as AT_{II}. 8-Substituted analogues of AT_{II} act as competitive antagonists in this system (see section IV).

The above analysis of the quantified relative potencies of several angiotensins in various target tissues indicate that there is reason to suspect multiple receptor types for angiotensin, but definite demonstration has not yet been provided.

We have recently characterized receptors of AT_{II} in vascular and intestinal smooth muscle (219), with the criteria suggested by Schild (277), namely a) order of potency of agonists, b) estimation of pA_2 values for competitive antagonists, and c) measurement of myotropic effects of analogues in tissues desensitized with AT_{II}.

Results are summarized in table 6. Full agonists were chosen to establish the order of potency that was similar in the three tissues. pA_2 values for 8-Gly-AT_{II}, a specific and competitive antagonist of AT_{II} (259), were similar in the three tissues, the difference did not exceed 0.5. Moreover, all agonist analogues of AT_{II} were inactive on tissues desensitized with AT_{II} (table 6).

TABLE 6

Compilation of data to compare receptors for angiotensin in three smooth muscle preparations

No.	Compound[a]	Rabbit Aorta Strip			Rat Stomach Strip			Rat Colon		
		R.P.[b]	pA_2[c]	R.A.D.[d]	R.P.	pA_2	R.A.D.	R.P.	pA_2	R.A.D.
I	AT_{II}	100	7.42		100	6.90		100	7.40	
II	1-Sar-AT_{II}	175	7.15	0	280		0			
III	4-Phe-AT_{II}	1.10	7.35	0	2.8	6.90	0	13	7.42	0
IV	7-Ala-AT_{II}	0.74		0	1.1		0	8		0
V	6-Ala-AT_{II}	0.17		0	0.6		0	0.7		0

[a] All compounds have Ile in position 5 and compounds I, III, IV, V have Asp in position 1 (see tables 2 and 3).

[b] R.P., relative potency compared to AT_{II} = 100%.

[c] pA_2 values were obtained with 8-Gly-AT_{II}, as competitive antagonist of AT_{II} (219).

[d] R.A.D., residual activity on tissues *desensitized* with AT_{II} (10^{-7} M). (Modified from Park *et al.*, Brit. J. Pharmacol. *48:* 288–301, 1973.)

These results indicate that receptor for angiotensin in vascular and intestinal smooth muscle of two different species are of the same type.

B. Progress Towards Isolation of Receptors for Angiotensin

Work in this field is still in a preliminary phase primarily due to lack of a specific irreversible inhibitor. Such a compound is essential for labelling receptors in living tissues and for obtaining a drug-receptor complex that remains stable during the fractionation procedures required for isolation of macromolecules (186, 237).

Before analyzing the few studies on receptors for angiotensin, we want to show that at least three types of macromolecules (proteolytic enzymes, plasma proteins, and specific antibodies) share with the receptors a strong affinity for angiotensin and its analogues. Proteolytic enzymes are present in a large number of mammalian tissues and appear to be very active against short peptides, such as angiotensin. This has been demonstrated by Cain *et al.* (41) in man and by Osborne *et al.* (199) in isolated rat hearts and kidneys perfused with an artificial medium. With iodinated or tritiated (both in position 4) 1-Asn^2, 5-Val-AT_{II} with high specific activities, it was found that, during the time of a single circulation through a peripheral vascular bed, angiotensin is broken down to heptapeptide 2–8 (199), hexapeptide 3–8 (41), tetrapeptide 1–4 (199), and other unidentified fragments.

From these experiments, it may be concluded that: a) even though plasma degrades angiotensin at a relatively slow rate (330), most tissues contain active peptidases which degrade the octapeptide very rapidly (330); and b) the enzymes involved in this metabolic breakdown are aminopeptidases, chymotrypsin, and possibly carboxypeptidases. These results confirm previous findings *in vitro*, on the degradation of AT_{II} by plasma and homogeneates of tissues (238).

Because of these facts, several essential criteria for identifying a given tissue component as a receptor should be applied, as suggested by Ehrenpreis *et al.* (70). In particular, the tissue macromolecule that shows some affinity for angiotensin must exhibit a high specificity of combination with both angiotensin and its specific antagonists. In addition, this macromolecule must be found in target organs and not in tissues insensitive to the octapeptide: it must also originate from those regions of the cell where angiotensin presumably acts (the plasma membrane, as discussed in section II). Affinities of angiotensin, specific inhibitors, and analogues less potent than the native hormone for the presumed receptor, should be similar to those found *in the same tissue* with pharmacological assays. Finally, nonspecific binding must be

excluded, particularly with polypeptidases, by inactivating or preventing the action of these enzymes: this is particularly important when agonists are used, that undergo rapid metabolic degradation and release intermediate products (*e.g.*, heptapeptide 2–8, hexapeptide 3–8) that are still labelled, and have strong affinities for receptors, proteolytic enzymes and antibodies.

Two groups of workers, Goodfriend and Lin (99) and Meyer *et al.* (185) have tried to apply some of these criteria in order to localize specific binding sites for angiotensin at the cellular and subcellular level. With highly purified monoiodo AT_{II} (with the biological activity of approximately 80%), Lin and Goodfriend (173) found specific binding in slices, particles and intact tissues of rat, rabbit, and ox. Specific binding measured after 15 minutes of contact of the tissue with the radioactive angiotensin varied from one tissue to another, averaging 33% in rat uterus and colon, and it was present in tissues insensitive to angiotensin (*e.g.*, rat esophagus). Baudouin *et al.* (16), with [^{3}H] 1-Asn^2, 5-Val-AT_{II} (with a biological activity of 100%), prepared according to Morgat *et al.* (187), found no specific binding in rat esophagus and higher specific binding (85% instead of 25%) (16) in rabbit aorta. In this experiment, the time of contact of [^{3}H] AT_{II} was 2 minutes. From these results, it is impossible to conclude that binding sites for angiotensin are found only in target organs.

Both groups of workers used as criterion of specific binding "the portion (in percent) of the total binding which could be prevented by simultaneous presence of a relative excess of unlabelled angiotensin" (173), presumably 1-Asn^2,5-Val-AT_{II}. If one assumes that the labelled derivative reacts with the same macromolecules as angiotensin and in the same way, then one could expect that the two peptides have the same affinity for receptors and for proteolytic enzymes. The presence of peptidases (in particular, aminopeptidases) cannot be excluded by using the radioimmunoassay for angiotensin II, as described by Lin and Goodfriend (173) and Lin *et al.* (172), because specific antibodies against AT_{II} bind heptapeptide 2–8 (102), hexapeptide 3–8 (41), and possibly other metabolic products of AT_{II} (7). The use of a short period of incubation [2 min in the experiment of Baudouin *et al.* (16)] to exclude any consistent degradation of AT_{II} by homogenates of rabbit aorta is also subjected to criticism, because binding to the enzymes can occur before any enzymatic activity is detectable. It is therefore difficult to conclude that specific binding sites for angiotensin in intact tissues or subcellular particles are distinct from peptidases.

Affinity constants for several analogues of angiotensin, including 8-Ala-AT_{II}, a specific inhibitor (243), were compared with biological activities of the same compounds *in vivo* (101, 185). We have mentioned above that a comparison of this type should possibly be made either simultaneously with receptor labelling or separately, but at least on the same tissue: otherwise, the comparison is inappropriate. In addition, the parameter for comparison of specific competitive antagonists should be the pA_2 and not the angiotensin-like activity. A recent report by Catt (43) shows that inhibitors of angiotensin prevent the binding of labelled hormones to slices of adrenals *in vitro*. This has recently been confirmed by Devynck *et al.* (61) with 8-Ile-AT_{II} and 8-Ala-AT_{II} on the rabbit aorta.

In conclusion, the identification of specific binding sites involved in the physiological response of angiotensin is still incomplete and must await for specific long acting antagonists that possibly have no affinity for polypeptidases. The binding sites at the cellular level described by Baudouin *et al.* (16) and by Goodfriend *et al.* (101) show similarities to the specific antibodies of AT_{II}, explored by Vallotton (326). In fact, the two macromolecules have no affinity for 8-Ala-AT_{II}, contrary to the receptors of AT_{II} in target organs and tissues (see section IV).[4]

[4] Since submission of this paper Devynck *et al.* (352, 353) and Glossmann *et al.* (354) have reported that binding of [^{3}H]-AT_{II} to smooth muscle cell membranes or solubilised receptors (352, 353) and the

Few reports have recently appeared with "indirect methods," according to the definition of Ehrenpreis *et al.* (70). Goodfriend *et al.* (101, 103) measured the binding of monoiodo AT_{II} to macromolecules in serum or plasma of a number of patients. It was found that these macromolecules have strong affinity for heptapeptide 2–8, hexapeptide 3–8, and Des^1-8-Ile-AT_{II}, while 1-Sar,8-Ala-AT_{II} (a compound presumably not degraded by aminopeptidases) was bound to a much lower degree. This suggests that aminopeptidases account for a large portion of the binding sites.

The only information available on the chemical nature of receptors for angiotensin derives from *in situ* modification by chemical or physical means. After the report by Khairallah *et al.* (152) that urea and several amino acids at concentrations higher than 0.2% could inhibit selectively the myotropic action of angiotensin on rat uterus or guinea-pig ileum, Fleisch and Ehrenpreis (81, 82) explored the influence of urea (2 M) and heating (at 47°C for 20 min) on receptors for angiotensin in the rat stomach strip. It was shown that exposure to urea for about 20 minutes greatly diminished the contractile response of the stomach without modifying that to KCl. The treatment produced a depression of the maximal response to angiotensin, while those of the other agents could still be elicited. It was concluded that receptors for angiotensin are at least partly of protein nature, since urea and heating denaturate proteins.

The function of the presumed receptors for angiotensin has also been investigated by Goodfriend *et al.* (100, 101) as well as by Baudouin *et al.* (17, 18). This topic will be discussed in section VI.

VI. Mechanism of Action of Angiotensin

We have reported in section II B, that angiotensin II originates from the action of renin of an α_2-globulin (angiotensinogen) and from the subsequent conversion of AT_I by the lung. The vasoconstrictor octapeptide does not bind or binds very little to plasma proteins (7, 102) and can therefore diffuse in the extracellular fluid to reach the cell membrane, where it probably acts (see section II B). Angiotensin is one of the endogenous agents for which a single type of action (stimulation) has been demonstrated. Besides its widespread effect on smooth muscle, angiotensin acts as a secretagogue, increasing the release of catecholamines and aldosterone from the adrenal glands. One would be tempted to speculate that nature would use a similar or identical mechanism to elicit contraction and secretion. Unfortunately, the data published to date are insufficient to substantiate this hypothesis, which will therefore remain a profitable venture for the workers in this field.

The mechanism of action of angiotensin at the cellular level has been studied in four types of tissues: smooth muscle, adrenal medulla, adrenal cortex and rat neurohypophysis.

A. Smooth Muscle

The mechanism of action of any chemical on excitable tissues, such as smooth muscle can be studied by measuring electrical changes of the transmembrane potential, particularly if the agent is expected to act on the cell membrane. Culture of smooth muscle cells and recording of transmembrane potential with intracellular microelectrodes would be ideal: to our knowledge, this type of experiment with angiotensin has not yet been carried out. It has also been difficult to use intracellular microelectrodes in intact tissues, because the microelectrodes are easily displaced by the spontaneous or the provoked contractions of the tissues. This could be partially avoided by the use of the sucrose gap technique (313) or of floating microelectrodes (310). The two approaches have been used by several workers to study the effect of drugs on smooth muscle cells, enabling the recordings of electrical activity during the course of large contractions. Similar resting

binding of [^{125}I]-AT_{II} (354) to particulate fraction from bovine adrenyl cortex are prevented by antagonists of AT_{II} (8-Ala-AT_{II} and 1-Sar,8-Ala-AT_{II}).

membrane potential values were obtained with intracellular microelectrodes or sucrose gap technique in several vascular smooth muscle preparations (309). Therefore, results of experiments with the two techniques are comparable. However, the limitations of both techniques have been clearly underlined by Burnstock *et al.* (40) and Somlyo and Somlyo (308). By using the sucrose gap technique to establish the effect of vasoactive agents on rabbit mesenteric veins, Cuthbert and Sutter (54) showed that the action potential discharge (AP), induced by angiotensin or K^+, occurs in parallel with the contractile response. The effect of AT_{II} under a condition where AP was blocked (by high K^+ in the medium), was greatly diminished, compared to noradrenaline: it was suggested that the action of angiotensin is more dependent on membrane excitation than that of noradrenaline (54). In other studies on rabbit and canine pulmonary arteries and aortae, AT_{II} and 5-hydroxytryptamine (5-HT), noradrenaline (NA) and histamine (H) were found to produce a graded depolarization, somewhat proportional to the amplitude of the contraction (308, 309).

Conflicting results were obtained by Keatinge (146) on sheep carotid arteries. AT_{II}, acetylcholine (ACh), NA and bradykinin (B) caused depolarization and simultaneous contraction of this tissue. Contractions could be maintained with each of these drugs, despite the fact that repolarization had started. During exposure to high K^+ medium, the tissue could still be contracted by four agents, without any detectable electrical changes. It was therefore concluded that the myotropic effect of AT_{II} and of the other three agents is, at least in part, independent of changes of membrane potential (146).

More recently, Somlyo and Somlyo (308) have reported that angiotensin depolarizes rabbit or dog mesenteric arteries and increases the frequency of the spontaneous spike potentials. However, no correlation was found between spike frequency and tension development. Furthermore, the difference in attainable maximum contractions of dog abdominal aortae after application of AT_{II}, adrenaline (A) and vasopressin (VSP), was maintained in the presence of K^+ rich medium, indicating that the difference in maximum contraction for the three agents is not dependent on changes of transmembrane potential (308).

Finally, Shibata and Briggs (299) reported that angiotensin contracted rabbit aorta strips independently of any electrical phenomenon and produced the same effect on polarized or depolarized tissues. It was therefore suggested (311) that vasoconstrictors can induce contractions of vascular smooth muscle by at least three mechanisms: the first, by increasing the frequency of spike potentials; the second, through a graded depolarization of the cell membrane; and the third, by "pharmaco-mechanical coupling," as defined by Somlyo and Somlyo (311).

The main conclusion to be drawn from the electrophysiological experiments is that the interaction of angiotensin with its specific receptors on vascular smooth muscle generally induces changes of transmembrane potential. However, it cannot be yet established whether these electrical events are causally related to the stimulant action, or are just an "epiphenomenon" (308), accompanying the drug-receptor interaction.

The question that naturally arises is what is the influence of ions on the myotropic action of AT_{II}? A large number of studies (23–25, 116, 153, 167, 300) have been carried out to establish the role of Na^+, because of the preponderant contribution of this ion in the electrophysiological events. Conflicting results were obtained on perfused rat tail arteries (115), guinea-pig ileum (153) and cat papillary muscle (167) on the one hand, and rabbit aorta strips (190) and rat uterus (153), on the other. It was shown that decrease of Na^+ in the medium reduced the action of AT_{II} in the first series of tissues, while it increased or did not influence the effect on rabbit aorta and rat uterus. Possibly because of different preparations and techniques employed, the

results of these and other similar studies are highly variable and do not allow any definite conclusion.

A large number of experiments have also been published on changes of Na^+ flux produced by angiotensin in several smooth muscles (31, 98, 107, 126, 321). These results have been challenged by Keatinge (147), who showed that a considerable portion of a slowly exchanging Na^+ may be extracellular and indistinguishable from true cellular efflux by simple compartmental analysis. Therefore, these studies will not be analyzed in detail.

More consistent indications have emerged from studies designed to establish the role of Ca^{++} as cofactor for the myotropic action of angiotensin. Reduction or elimination of Ca^{++} from the medium abolished the response of the guinea-pig ileum, rat uterus (153), and rat tail artery (116) to AT_{II} and reduced that of dog mesenteric arteries (39).

A similar depression of the response to 5-HT, NA, and A was observed in some of these experiments (39), suggesting that Ca^{++} is probably a common essential cofactor for the myotropic action of several agents.

The essential requirements for Ca^{++} during excitation-contraction coupling in smooth muscle has been extensively studied (14, 73, 120, 166, 309, 311) and appears to be well established. The fundamental mechanism by which drugs and Ca^{++} interact during stimulation or inhibition of smooth muscle is still unresolved. One important question that will not be discussed here is the possible involvement of Ca^{++} in the binding of drug to receptors on smooth muscle, as suggested by Paton and Rothschild (223). No studies have been carried out with angiotensin to explore this point. Maximum contractions of smooth muscle by vasoactive drugs have been shown to depend on the total Ca^{++} content of the tissue [*e.g.*, rabbit aorta (5)]. Moreover, the fact that different drugs acting on the same preparation vary considerably in the intensity of the contractile response and degree of depolarization has been related to a differential ability to utilize Ca^{++}. This interpretation has been proposed by Somlyo and Somlyo (311) to explain the different efficacies of 5-HT and NA on strips of rabbit portal-mesenteric veins, and of NA and AT_{II} on the strips of rabbit aorta.

In vivo or under ordinary experimental conditions *in vitro*, the depolarization of the cell membrane by drugs, with the consequent passive influx of Ca^{++} from the extracellular fluid can account for the increase of intracellular Ca^{++}, which appears to be the prerequisite for any agent to contract smooth muscle or stimulate the secretion of catecholamines (67). When tissues *in vitro* are depolarized by K^+, no measurable change of transmembrane potential can account for Ca^{++} influx (65). However, ACh has been shown to stimulate Ca^{++} uptake in depolarized smooth muscle (67, 128). To explain the effect of vasoconstrictors on depolarized smooth muscle, it has recently been proposed that myotropic agents including AT_{II} may mobilize Ca^{++} firmly bound to the membrane, to increase the intracellular concentration of Ca^{++}, in order to activate the ATP-ase of the contractile protein (311). This hypothesis is supported by the results of Van Breemen *et al.* (327, 328) with lanthanum (La^{+++}) and by Kalsner *et al.* (145) with SKF 525 A. After exposure to La^{+++}, AT_{II}, Na or H can induce only one contraction of the rabbit aorta strip. Repeated applications of these agents after the first contraction are ineffective. These results led Van Breemen *et al.* to conclude that La^{+++} inhibits the repletion of the tightly bound intracellular Ca^{++} pool, which probably takes part to a common pathway for the action of several myotropic drugs. With SKF 525 A, which inhibits the movement of extracellular and/or loosely bound Ca^{++} in rabbit aorta strips, Kalsner *et al.* (145) showed that the response to AT_{II} is only slightly decreased, unlike the responses to K^+ and to NA. They concluded that the contraction induced by AT_{II} appears to involve almost exclusively the firmly bound

Ca^{++}. This hypothesis is supported by several observations reviewed extensively by Somlyo and Somlyo (311) who proposed the model of "pharmaco-mechanical coupling."

The possible applications of this model to explain the action of angiotensin on smooth muscle have been discussed by Meyer and Baudouin (184). Recently, Baudouin-Legros and Meyer (18) have reported several interesting observations, suggesting that the action of angiotensin on the rabbit aorta could be, at least partially, mediated by a translocation of Ca^{++} from binding stores in the cell membrane. With a highly purified subcellular fraction (membrane vesicles of rabbit aorta), these authors found that AT_{II} (10^{-8}–10^{-6} M) increased the release of membrane-incorporated Ca^{++}, while reducing the binding of Ca^{++} to the same fraction. They suggested that the two processes cooperate to increase the cytoplasmic concentration of Ca^{++} to approximately 4×10^{-6} M, a concentration much higher than the threshold required for contraction (18). The same workers (8, 17, 18), have attempted to explore the relationship between Ca^{++} and cyclic AMP, the hypothetical second messenger, involved in the stimulus-secretion coupling. They showed that AT_{II} inhibits the stimulant effect of adrenaline on adenyl cyclase in rat uterus (8), while Volicer and Hynie (338) reported that AT_{II} tends to decrease intracellular cyclic AMP in rat tail arteries. However, the relationships between cyclic AMP and Ca^{++} on both vascular and intestinal smooth muscle is still controversial, as underlined by the recent reviews by Somlyo *et al.* (312) and Andersson *et al.* (6).

Further studies on this area are undoubtedly a prerequisite to the understanding of the mechanism of action of angiotensin and can be extremely valuable to explain the basic mechanisms of vasoconstriction and vasodilatation.

B. Adrenals and Other Organs

The outstanding studies by Douglas *et al.* (66), some years ago, on isolated chromaffin cells from gerbils and by Peytremann *et al.* (229) on isolated bovine adrenal cells have contributed most interesting data on the mechanism subserving the release of catecholamines and steroids by angiotensin. Transmembrane potentials were recorded by Douglas *et al.* (66) from adrenal chromaffin cells maintained *in vitro* with a special technique (66). It was shown that angiotensin, in common with other drugs (H, ACh, *etc.*) depolarized adrenal chromaffin membranes, by acting on receptors different from those of acetylcholine. The effect of high doses of AT_{II} (10^{-4} g/ml) was not influenced by atropine and hexamethonium. Calcium was shown to be required for release of catecholamines by AT_{II} as well as by other agents. The possible involvement of Ca^{++} in the stimulus secretion-coupling has been discussed by Douglas (67).

Peytremann *et al.* (229) have recently compared the effect of angiotensin and ACTH on adrenal cyclic AMP and steroidogenesis *in vitro*, with isolated cells from the zona fasciculata of bovine adrenals. Cyclic AMP level increases within 1 minute after application of AT_{II} and correlates very well with the increase of the fluorogenic corticosteroids. Angiotensin is less potent than ACTH in increasing the cellular content of cyclic AMP, and its effect is of short duration. However, the octapeptide appears to affect cyclic AMP formation by a mechanism different from ACTH, because the two peptides appear to interact synergistically. Angiotensin does not inhibit phosphodiesterase or activate adenyl cyclase. In spite of the relatively small increase of cyclic AMP and of its rapid reversibility, the effect on steroidogenesis is quantitatively similar to that of ACTH.

Recent findings by Hornych *et al.* (121) provide indirect evidence that the second messenger may be involved in the action of angiotensin on sodium and water transport. With everted rat ascending and descending colon, these authors found that very low concentrations of AT_{II} (10^{-10} M) stimulate Na^+ and H_2O fluxes across the mucosa of

the ascending colon, in a similar way to vasopressin and cyclic AMP. The opposite effect (reduction of Na^+ and H_2O fluxes) was observed in the descending colon with the three agents.

Imidazole (10^{-3} M) inhibited the effect of AT_{II}, while theophylline failed to potentiate. It is suggested that this effect of AT_{II} can be at least in part mediated by endogenous cyclic AMP (121).

It has been recently found by Gagnon *et al.* (91) that: a) 1-Asn^2-AT_{II} (10^{-9} M) increases significantly the spontaneous release of vasopressin from rat neurohypophysis *in vitro;* b) 8-Ala-AT_{II} and 8-Leu-AT_{II} do not change or slightly reduce the spontaneous release, but prevent the effect of 1-Asn^2-AT_{II}. With the same preparation in presence of theophylline (10^{-2} M) to block degradation of cyclic AMP by phosphodiesterases, Gagnon and Heisler (92) were able to demonstrate an accumulation of cyclic AMP by small doses (10^{-10}, 10^{-9} M) of 1-Asn^2-AT_{II}. Again, inhibitors of AT_{II} (*e.g.*, 8-Gly-AT_{II}) did not show any stimulating effect, but prevented the action of 1-Asn^2-AT_{II}. Recent findings in the same laboratory (Gagnon, personal communication) indicate that the accumulation of cyclic AMP (maximum after 5 min of incubation) precedes the release of vasopressin into the medium (maximum after 10 min of incubation). These results suggest that the two phenomena may be related. Evidence is therefore accumulating in favour of the hypothesis that angiotensin may act through a system (cyclic AMP) that is common to several other peptide hormones.

VII. Effects of Angiotensin on the Nervous System

A vast literature has accumulated in recent years on the actions of angiotensin on the nervous system and a review has been recently published (298). In reviewing this literature, one is immediately struck by the variety of the interpretations proposed to explain findings which occur under circumstances in which there are striking differences in experimental conditions, biological preparations, and dosage of the drug. We want to summarize the most consistent data relating to the action of angiotensin: a) on the central nervous system; b) on the adrenal medulla; c) on the sympathetic ganglion; and d) on the sympathetic nerve terminals. Particular attention will be given to the concentrations of angiotensin used in the various experiments, considering that in general the physiological concentration of endogeneous angiotensin in blood is of the order of 2.5 ng/100 ml (41).

A. Central Nervous System

The possibility that endogenous angiotensin maintains elevated blood pressure through its action on the central sympathetic system was considered by McCubbin and Page (178) to provide an explanation of the neurogenic phase of renal hypertension. Chronic infusion of subpressor doses of angiotensin for several days in rabbits were shown to produce a progressive increase of sympathetic nervous tone and of blood pressure (62, 63). More recently, Zimmerman (347) concluded that the increase of blood pressure observed after intracarotid or intervertebral injection of angiotensin (1–10 μg) to anesthetized dogs could be attributed to the peripheral vasoconstriction brought about when the peptide gained access to the systemic circulation, rather than to a centrally mediated pressor effect. Morrison and Pickford (188) recorded the firing frequency of cervical sympathetic nerves in anesthetized cats and dogs after intravenous injection of angiotensin: they concluded that the increase of sympathetic activity observed at high blood pressure levels were reflex in nature and not due to a central action of angiotensin.

Many cross-circulation experiments have been performed by Buckley (34) in dogs. Blood from a donor animal was used to perfuse a vascularly isolated head of a recipient dog, under controlled flow. Nervous connections between the head and the rest of the body were left intact and blood pressure

was recorded both from the donor and the recipient. Injection of relatively high doses (0.2–4.0 μg/kg) of angiotensin directly into the carotid artery of the recipient, apparently did not increase the resistance of the cerebral vessels, but induced a significant increase of blood pressure in the recipient animal. The pressor effect was prevented by piperoxan and was attributed to a centrally mediated increase of sympathetic tone. The same group of workers obtained similar results in anesthetized cats, after injection of angiotensin (0.01–4.0 μg/kg) into the cerebral ventricles. It was suggested that angiotensin may have a centrally mediated effect. However, the authors generally agree that this effect may not account for the overall pressor response evoked by the intravenous administration of the peptide, because of the high doses of peptide used in the experiment. Evidence for a direct central action of angiotensin was not obtained in these experiments, probably because the peptide was administered at such high doses that systemic vasoconstriction resulted.

Recent experiments by Lowe and his colleagues (142, 293) in dogs have shown that small doses of angiotensin (0.2–0.8 ng/ml), infused into the vertebral artery produce a significant increase of heart rate, cardiac output, and blood pressure. The effect was abolished by the ablation of the area postrema, a region which is accessible to angiotensin and other drugs (142), bebecause of the gap in the blood-brain barrier (337, 341, 342). It was suggested that the area postrema is a chemoreceptor zone (49) relatively sensitive and specific for angiotensin, but not to noradrenaline (142). The doses of peptide used in this experiment were well within the range of physiological concentrations: any peripheral effect could also be excluded because the ablation of the area postrema prevented the action of angiotensin without modifying resting heart rate or other central autonomic effects (142).

B. Adrenal Medulla and Sympathetic Ganglia

The discovery by Khairallah and Page (148) that angiotensin stimulates intramural ganglia of the guinea-pig ileum and by Feldberg and Lewis (74, 75) that the octapeptide is a potent stimulant of the release of catecholamines from the adrenal medulla in cats, stimulated great interest on the action of angiotensin on ganglionic structures. Extensive pharmacological analyses of the release of medullary catecholamines by angiotensin have shown that the effect is not modified by ganglion blocking agents (75, 315), chronic denervation of the gland (75), spinal block (315) or intravenous administration of morphine (170). These observations led Lewis and Reit (170) to compare the action of angiotensin on the adrenal medulla and on the superior cervical ganglion of the cat. Retrograde injection of angiotensin into the carotid artery of the spinal cat, to reach the superior cervical ganglion, induced contractions of the ipsilateral nictitating membrane (169). Angiotensin has no direct myotropic effect on this tissue and therefore the effect was attributed to the stimulation of the ganglion cell (169). The effect was abolished by morphine (170, 320), cocaine (320), depolarizing doses of nicotine (170, 320), and postganglionic denervation (169): potentiation was observed during the nondepolarizing phase of the block by nicotine (170, 320) and after intensive preganglionic stimulation (170, 213, 320). It was concluded that angiotensin acts as a nonnicotinic ganglion stimulant, similar to histamine and 5-hydroxytryptamine. The same conclusions could be drawn from the analysis of the experiments demonstrating an indirect effect of angiotensin on the guinea-pig ileum, through stimulation of the intramural ganglia (see section I). In addition, angiotensin has been shown to increase by 30 to 50% the output of acetylcholine from the superior cervical ganglion of the cat in the absence and by 200 to 300% in the presence of preganglionic stimulation (213, 214). The preparation was perfused with oxygenated plasma and the effect of angiotensin was partially blocked by hexamethonium, contrary to the finding reported by Lewis and Reit (170) and by Trendelenburg (320). No satisfactory explanations can be given so far

for this discrepancy, but it would be hazardous to postulate a direct action of angiotensin on the preganglionic nerve terminals, based on a single type of observation.

The demonstration that angiotensin may increase sympathetic peripheral tone by stimulating the ganglia and by releasing medullary catecholamines is suggestive of a possible participation of the nervous system to the vasoconstrictor action of angiotensin. The magnitude of the doses required to stimulate the adrenal medulla and the ganglia as well as the rapid desensitization of these structures by angiotensin, has led some authors (75, 315) to conclude that these pharmacological actions do not necessarily reflect a physiological role of the peptide. However, this peptide appears to increase the action of the sympathetic system at several separate sites (see following paragraph), particularly in the presence of physiological nervous impulses. Therefore, the possibility cannot be excluded that at least one part of the overall vasoconstrictor effect of AT_{II} is mediated *via* the sympathetic nervous system.

C. Sympathetic Nerve Terminals

Evidence for a permissive action of angiotensin on the sympathetic nerve terminals has accumulated during the last decade. Interest in this field of study was arisen: a) from the observation of Zimmerman (346) that acute sympathectomy reduces the vasoconstrictor response to angiotensin in the perfused hind-quarter of the dog; and b) from the report of McCubbin and Page (178) that the pressor effect of angiotensin increases in parallel with the release of endogenous catecholamines. Experiments performed in a large variety of pharmacological preparations have been interpreted as indicating that angiotensin may release noradrenaline from nerve endings (tyramine-like effect) (64, 95, 284, 285) or may block the re-uptake of the neurotransmitter (cocaine-like effect) (56, 57, 127, 157, 158, 208–210, 215, 224, 225). These findings have been recently challenged by the continuously increasing number of observations that angiotensin facilitates the release of neurotransmitter *only in the presence of sympathetic nerve stimulation* (21, 59, 60, 123, 124, 143, 286, 314, 348–350). More recent experiments on vascular tissues (123, 124, 348–350) have definitely shown that angiotensin potentiates the release of labelled noradrenaline induced by sympathetic nerve stimulation. Interference with the re-uptake process or consistent increase of the release of neurotransmitter in the absence of nervous stimulation could not be substantiated. In addition, angiotensin apparently acts on the same pool of neurotransmitter that is used by nerve impulses (143, 350). Bell's ingenious experiments (21) have further supported the finding that angiotensin potentiates *only* the response induced *by sympathetic nerve stimulation*. According to Bell (21), angiotensin "increases the degree of facilitation of successive excitatory junction potentials during repetitive low frequency nerve stimulation leading to greater depolarization of the muscle cells than under control conditions." It has been repeatedly shown that the potentiation of sympathetic impulses by angiotensin is more evident at low frequencies (21, 348–350). This low frequency range is apparently close to that concerned for the maintenance of the sympathetic tone (21). If this observation is considered together with the fact that the concentration of angiotensin required to potentiate sympathetic nerve stimulation (21) and to increase the release of noradrenaline during stimulation (124) are close to the physiological concentrations (21, 124, 350), one could be tempted to speculate on the possible role of the peptide as *modulator* of sympathetic transmission.

The precise mechanism by which angiotensin potentiates sympathetic nerve impulses is not yet known. However, in view of the numerous data reviewed by Rubin (266) that the release of noradrenaline from the nerve endings depends on ionic movements (particularly Ca^{++}) into the nerve terminals, it seems reasonable to think that angiotensin could affect Ca^{++} movement through the membrane of nerve terminals

(124) and in this way potentiate the release of neurotransmitter.

Finally, several recent reports suggest that angiotensin may accelerate the synthesis of noradrenaline from precursors (28, 46, 57, 264, 265) in various tissues. It is still unclear whether angiotensin stimulates the synthesis of enzymes involved in the biosynthesis of noradrenaline or liberates these enzymes from feedback inhibitions, as discussed by several authors (28, 46, 57).

Apparently, there is no correlation between acceleration of noradrenaline biosynthesis in the presence of angiotensin and a small inconsistent release of neurotransmitter, associated with the contraction induced by the peptide in some tissues during the first minutes of contact (29, 265). Stimulation of synthesis by angiotensin is maximal after 60 minutes of incubation, at the time when most of the peptide has been metabolized (29). It is possible that single amino acids released from the octapeptide by the action of proteolytic enzymes enter the cell and induce the metabolic changes required to stimulate noradrenaline biosynthesis. This is, of course, highly speculative and further evidence is required to substantiate the hypothesis.

VIII. Conclusions

1. The occupation theory, as formulated by Ariens (11) with the concept of affinity and intrinsic activity, provides an acceptable basis for the interpretation of the interactions between angiotensin and its specific receptors in smooth muscle. Dose-response curves of classical hyperbolic shape and linear relations between stimulus and effect, excluding threshold phenomena and spare receptors, have been obtained with AT_{II} in two effector systems. The absence of spare receptors has been confirmed in rabbit aorta with the prolonged occupation of a fraction of receptors by a long acting inhibitor of AT_{II} (1-Sar,8-Leu-AT_{II}). Linear relations between the observed effect of AT_{II} and the actual stimulus make highly tenable the assumption that extent of contraction is a function of the concentration of drug-receptor complex. It follows that maximum responses are obtained when the totality of receptors are activated: the concept of intrinsic activity, as initially formulated by Ariens in 1964 (11), has therefore been applied in the analysis of the interactions between angiotensin and its receptors in structural terms.

2. The analysis of structure-activity relations among a large number of analogues and fragments of AT_{II} has allowed the identification of side chains primarily involved in binding, stimulation or in the maintenance of the critical conformation required for drug-receptor interaction. The discovery of the active group responsible for stimulation has been rapidly followed by the development of specific antagonists, very similar to angiotensin in regard to chemical structure, physicochemical properties and presumably secondary structure.

Models of secondary structure, proposed by several workers (78, 233) have contributed in raising the study of structure-activity relationships from a purely empirical to a rational and systematic approach. More specifically, these models have contributed to the improvement of the design of analogues with a view to exploring receptor topography and to expressing pharmacological parameters, such as affinity and intrinsic activity, in molecular terms. A first attempt in this direction has been presented in this paper, assuming that changes of secondary structure influences primarily affinity, because 7 of the 8 amino acids constituting AT_{II} are either involved in binding to receptors or in preserving the critical conformation of the octapeptide, while only Phe in position 8 is essential for triggering changes at the receptor sites. Full intrinsic activity is therefore maintained even by compounds that show extremely low affinities, provided the hydrophobic phenyl ring is present in position 8. Only drastic changes of conformation can affect intrinsic activity. Minor changes in position 8, on the contrary, modify substantially the pharmacological

spectrum of analogues from that of a full agonist to that of a partial agonist or of an antagonist.

3. Little is known as yet about tissue proteolytic enzymes, as regard to their possible influence on the potency and rate of dissociation of AT_{II} from receptors. The study of the relations between tissue metabolism and development or disappearance of biological responses constitutes a promising approach for future work in this field.

At present, duration of action of agonists and antagonists can be prolonged by substitution in position 1. The possibility therefore emerges that other modifications, particularly in position 4 and 8, may confer resistance to proteolytic enzymes and long duration of action. Knowledge of receptor topography, on the other hand, and precise assessment of the distances between groups essential for binding and stimulation could lead to the discovery of structures simpler than the peptides, but able to occupy irreversibly the receptors for angiotensin.

4. Much work has still to be done to identify receptors for angiotensin in various organs and systems. This may be a prerequisite for the development of antagonists against specific functions of the natural peptide. Effects such as the stimulation of the central nervous system, the stimulation of catecholamine synthesis in sympathetic nerve terminals and the effects on transport of water and sodium across epithelia (121) should be investigated further, in order to extend our knowledge of the physiological and pathological actions of this peptide.

5. The ultimate aim of any pharmacological study is the understanding of the process that leads to biological action when the drug comes in contact with its specific receptors. Recent observations tend to emphasize the possible role of Ca^{++} for the stimulant actions of AT_{II} on smooth muscle and on the release of catecholamines from the adrenal glands. The involvement of the second messenger (cyclic AMP) in smooth muscle is still unclear, while it is offered as possible intermediate step for the action of angiotensin on the adrenal cortex and on the neurohypophysis.

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