Effect of Lipophilic Character on the Biological Activity of Synthetic Angiotensin Peptides

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[Ile⁵]angiotensin II (angiotensin) derivatives bearing acetyl, propionyl, butyryl, pentanoyl or hexanoyl moieties at the *N*-terminal amino group were synthesized. The myotropic effects *in vitro* (on guinea-pig ileum and rat uterus) of desamino-angiotensin and of the above compounds did not correlate with their partition coefficients in butan-1-ol/acetic acid/water. The pressor effects *in vivo* in rats showed a negative correlation with the partition coefficients, discouraging further attempts to raise the pressor potency of angiotensin analogues by increasing their hydrophobicity. The half-times for onset and reversal of the biological responses also did not correlate with partition coefficients, but reversal was retarded by the presence of a free amino group. It is concluded that partition between aqueous medium and the lipophilic receptor environment is not a limiting factor for angiotensin activity.

Structure-activity relationships in the angiotensins have been explored through the study of numerous synthetic peptide analogues (Khosla *et al.*, 1974), yielding a great deal of information about electronic and steric requisites for biologically active molecules. However, little is known about the third of the physicochemical factors which are widely accepted as determinants of biological activity (Hansch, 1969), namely the hydrophobic component. To learn more about this aspect, we have evaluated the smooth-muscle-contracting (myotropic) and blood-pressure-raising (pressor) activities of synthetic angiotensin ([5-isoleucine]angiotensin II) derivatives in which the *N*-terminal amino groups were acylated by normal fatty acids of different chain lengths. This method of obtaining a series of analogues with different degrees of hydrophobicity was chosen because a free amino group is not essential for the biological activity of angiotensin (Khosla *et al.*, 1974). The present results indicate that partitioning between the aqueous external medium and the non-polar 'biophase' (Furchgott, 1964) is not a limiting factor for angiotensin activity and that a free amino group is important for the duration of its effects.

Experimental

Materials

Angiotensin, desamino-angiotensin and acetyl-angiotensin were synthetic products of this laboratory (Paiva *et al.*, 1974c). The *t*-butoxycarbonyl

derivatives of the amino acids were from Bachem Inc. (Marina Del Rey, CA, U.S.A.) and the Merrifield resin (co-polymer of styrene and 2%, w/w, divinylbenzene) was from Bio-Rad Laboratories (Richmond, CA, U.S.A.). Dicyclohexylcarbodi-imide was from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). The precoated 0.2 mm-thick silica-gel plates for t.l.c. were from E. Merck (Darmstadt, Germany).

Peptide synthesis

The acylated angiotensin derivatives were synthesized by the solid-phase method (Stewart & Young, 1969; Erickson & Merrifield, 1976). *t*-Butoxycarbonylphenylalanine was attached to the resin as described by Merrifield (1964). The sequential coupling of the residues of proline, *N*-tosylhistidine, isoleucine, *O*-methyltyrosine, valine, nitroarginine and aspartic acid β -benzyl ester was performed as previously described (Paiva *et al.*, 1974c). The coupling of the fatty acid to the last residue was done with a 2.5 molar excess of the acid and of dicyclohexylcarbodi-imide in dichloromethane/dimethylformamide (3:1, v/v). The couplings were monitored with the ninhydrin reaction (Kaiser *et al.*, 1970) and were all completed within 3 h. The peptide-resin was cleaved and deprotected with anhydrous HF containing 5% (v/v) anisole. After removal of HF and anisole by vacuum distillation and washing with ethyl acetate, the peptide was extracted with acetic acid and freeze-dried. Purification was by counter-current distribution in an automatic machine (H. O. Post, Middle Village, NY, U.S.A.) with the solvent

system butan-1-ol/acetic acid/water (4:1:5, by vol.), and the peptide material was located by a quantitative Pauly reaction (Stewart & Young, 1969). As many transfers were made as were necessary for obtaining peaks with the expected shape for homogeneous material. The number of transfers ranged from 400 to 600 for the different peptides. The fractions were pooled with the aim of obtaining pure peptides rather than good yields. After vacuum evaporation of the solvent and freeze-drying, the peptides were found to be homogeneous by the following criteria: (a) the amino acid analysis of the acid hydrolysates (6M-HCl for 72h at 110°C), carried out in a Beckman model 120C amino acid analyser, yielded molar ratios within 4% of the theoretical value for all the amino acid residues; (b) only one spot was detected with Pauly, ninhydrin and hypochlorite reagents (Stewart & Young, 1969) after t.l.c. on silica-gel plates with the following solvent systems: A, butan-1-ol/acetic acid/water (5:1:1, by vol.); B, butan-1-ol/ethyl acetate/acetic acid/water (1:1:1:1, by vol.); C, butan-1-ol/pyridine/acetic acid/water (15:10:3:12, by vol.); (c) only one component could be detected after electrophoresis on Whatman 3MM paper between plates at 30V/cm for 60min in three different buffers: 1M-acetic acid (pH2.8), 0.1M-pyridine acetate (pH4.9) and 0.2M-Na₂CO₃/NaHCO₃ (pH9.9). The peptides used in the present study are shown in Table 1.

Partition coefficients

Butan-1-ol/acetic acid/water (4:1:5, by vol.) was equilibrated for 90 min and the two phases were separated by centrifugation for 30min at 700g. The pH of the lower phase was 2.5. The peptide was dissolved in a mixture of 5 ml of each phase and was gently shaken (35 inversions/min) at room temperature (18–22°C) for 90 min. This was five times the maximum time for equilibration, determined by preliminary experiments in which the partition kinetics were followed at 5 min intervals. After 90min rest, the lower aqueous phase was centrifuged for 90min at 700g, which was important for obtaining reproducible

results, even if no emulsion was visible in the samples (Hansch *et al.*, 1962). The peptide concentration was then determined by reading the A_{275} . The molar absorptivity of all the peptides, determined in standard solutions in pure water or in the centrifuged lower phase of the solvent system, did not vary significantly from the value 1.37×10^3 . The concentration in the upper phase was estimated by difference, because absorbance measurements in that phase were subject to much larger errors than in the lower phase. The partition coefficient (K) was calculated as the ratio of the concentrations in the upper and in the lower phase.

Bioassays

The biological activity of the peptides was assayed *in vitro* in two isolated smooth-muscle preparations described in detail elsewhere [the guinea-pig ileum (Aboulafia *et al.*, 1976) and the rat uterus (Paiva *et al.*, 1974a)]. The activity *in vivo* was assayed in the rat blood-pressure preparation, also described elsewhere (Paiva *et al.*, 1973). The activity of each analogue was compared directly with that of angiotensin by using a four-point assay, and the data were analysed as described by Schild (1942). The relative potencies were expressed on a molar basis. The $t_{\frac{1}{2}}$ values for the responses were defined as the time for the response to reach half of its maximum value during treatment with the analogue. The $t_{\frac{1}{2}}$ for the dissipation of the effect (recovery), for smooth muscles, was the time taken for the muscle to relax to half of its response, after the organ was washed with free medium to remove the peptide. In the assays, *in vivo*, the recovery $t_{\frac{1}{2}}$ was the time elapsing between the moment when the blood pressure reached its maximum and the moment when it had fallen to halfway between that maximum and the basal value.

Results and Discussion

The partition coefficients determined for angiotensin and the other peptides (Table 2) showed the

Table 1. *Peptides used in this study*

The amino acid residues are of the L configuration and are abbreviated as recommended by the IUPAC-IUB Commission on Biochemical Nomenclature [*Biochem. J.* (1972) **126**, 773–780; (1973) **135**, 9]. Abbreviation: Suc, succinyl (3-carboxypropionyl).

Peptide	Amino acid sequence
Angiotensin	Asp-Arg-Val-Tyr-Ile-His-Pro-Phe
Desamino-angiotensin	Suc-Arg-Val-Tyr-Ile-His-Pro-Phe
Desaspartyl-angiotensin	Arg-Val-Tyr-Ile-His-Pro-Phe
Acetyl-angiotensin	CH ₃ -CO-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe
Propionyl-angiotensin	CH ₃ -CH ₂ -CO-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe
Butyryl-angiotensin	CH ₃ -CH ₂ -CH ₂ -CO-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe
Pentanoyl-angiotensin	CH ₃ -CH ₂ -CH ₂ -CH ₂ -CO-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe
Hexanoyl-angiotensin	CH ₃ -CH ₂ -CH ₂ -CH ₂ -CH ₂ -CO-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe

Table 2. *Partition coefficients and biological activities*

The partition coefficients were determined in the system butan-1-ol/acetic acid/water (4:1:5, by vol.). Bioassay results are means $\pm$ S.D., with numbers of observations in parentheses. Data for angiotensin, desaspartyl-angiotensin and desamino-angiotensin were taken from Paiva *et al.* (1974c).

Peptide	Partition coefficient	Biological activity (% relative to angiotensin)		
		Guinea-pig ileum	Rat uterus	Rat blood pressure
Angiotensin	0.34	100	100	100
Desaspartyl-angiotensin	0.36	34 $\pm$ 1	66 $\pm$ 2	47 $\pm$ 4
Desamino-angiotensin	1.19	11 $\pm$ 2	47 $\pm$ 3	101 $\pm$ 3
Acetyl-angiotensin	0.99	25 $\pm$ 1 (5)	71 $\pm$ 6 (6)	101 $\pm$ 2 (3)
Propionyl-angiotensin	1.33	27 $\pm$ 2 (6)	68 $\pm$ 4 (4)	89 $\pm$ 13 (7)
Butyryl-angiotensin	1.79	22 $\pm$ 4 (11)	52 $\pm$ 3 (10)	93 $\pm$ 4 (5)
Pentanoyl-angiotensin	2.7	20 $\pm$ 5 (7)	53 $\pm$ 8 (8)	49 $\pm$ 7 (7)
Hexanoyl-angiotensin	3.9	17 $\pm$ 4 (7)	46 $\pm$ 4 (8)	47 $\pm$ 5 (4)

expected dependence on the size of the acyl group coupled at the *N*-terminus. The increment caused by the introduction of the acetyl group was smaller than that resulting from removal of the amino group. This is understandable in view of the polar nature of the amide group. In the series acetyl-, propionyl-, butyryl-, pentanoyl-, hexanoyl-angiotensin there was linear dependence between the logarithm of the partition coefficient and the number of methylenic groups. A least-squares treatment yielded a correlation coefficient (r) of 0.997 ± 0.003 , and the equation:

$$\log K_n = -0.02 + 0.150n$$

where K_n is the partition coefficient of the analogue with n methylenic groups in the acyl moiety.

The biological activities of the peptides are also shown in Table 2. Before discussion of the correlation between these values and partition coefficients (K) it should be pointed out that $\log K$ is related to free energy of partition, and is an additive constant as long as strong group interactions are absent (Iwasa *et al.*, 1965), which was shown to be the case in angiotensin peptides (Juliano & Paiva, 1974). Except for angiotensin and desaspartyl-angiotensin, the peptides studied here have identical ionizable groups, whose ionization should make equal contributions to $\log K$. Therefore, we shall assume that the difference between the physiological pH and that of the solvent system used to obtain K does not affect the relative values of that parameter in the series desamino-, acetyl-, propionyl-, butyryl-, pentanoyl-, hexanoyl-angiotensin.

When all the data of Table 2 were considered, no significant correlation between biological activities (or their logarithms) and $\log K$ was found by using the t test for the significance of correlation coefficients ($P > 0.05$). In the above series of six peptides, there was also no correlation between $\log K$ and the activi-

Table 3. *Half-times for onset of, and recovery from, pressor effects*

Results are expressed as means $\pm$ S.D., with numbers of observations in parentheses.

Peptide	Dose (ng/100 g body wt.)	$t_{\frac{1}{2}}$ (s)	
		Onset	Recovery
Angiotensin	30	12 $\pm$ 2 (4)	61 $\pm$ 12 (4)
	60	12 $\pm$ 2 (8)	48 $\pm$ 9 (8)
	150	18 $\pm$ 2 (4)	74 $\pm$ 10 (3)
Desamino-angiotensin	30	13 $\pm$ 2 (8)	25 $\pm$ 4 (7)
	60	11 $\pm$ 2 (9)	32 $\pm$ 6 (7)
Hexanoyl-angiotensin	60	13 $\pm$ 1 (3)	27 $\pm$ 5 (3)

ties on the ileum and on the uterus, but a significant negative correlation was found between the rat blood pressure and K ($r = -0.93 \pm 0.06$, $P < 0.01$) or $\log K$ ($r = -0.938 \pm 0.003$, $P < 0.01$).

Jorgensen & Weinkam (1973) have found a direct relationship between the pressor activities of angiotensin analogues modified at residues 5 or 7 and the logarithms of the partition coefficients in octanol/water. These results, however, cannot be considered a clear indication of the importance of lipophilic character for activity. Alterations of angiotensin side chains at those positions cause steric and electronic effects (Juliano & Paiva, 1974; Deslauriers *et al.*, 1975, 1976) that may be responsible for the observed changes in biological potency. Even if these changes could be attributable to lipophilicity, it is not possible to determine whether they are due to partition or to receptor-binding effects. In our acyl-angiotensin series, on the other hand, the increase in chain length should not affect receptor-binding, because the important groups for biological activity are not

Table 4. *Half-times for onset of, and recovery from, guinea-pig ileum-contracting effect*
Results are expressed as means $\pm$ S.D., with numbers of observations in parentheses.

Peptide	Concn. (nM)	$t_{\frac{1}{2}}$ (s)	
		Onset	Recovery
Angiotensin	10	11 $\pm$ 3 (15)	35 $\pm$ 13 (15)
Desamino-angiotensin	100	4.3 $\pm$ 0.4 (8)	6 $\pm$ 1 (8)
Hexanoyl-angiotensin	40	4.8 $\pm$ 0.8 (8)	9.5 $\pm$ 0.5 (8)

altered along the series. Further, it is known that bulky or polymeric substituents at the *N*-terminal amino group do not significantly affect the pressor activity, as compared with that of desamino-angiotensin (Arakawa *et al.*, 1962; Khosla *et al.*, 1974). Thus we believe that the negative correlation that we have found between pressor response and log *K* indicates that, in that series of peptides, the partition coefficients are above an optimum value. Such optima have been proposed by Hansch (1969) to occur in complex systems very much of the kind involved in the pressor assay *in vivo* used here. This discourages further attempts to raise the pressor potency of angiotensin analogues by increasing their lipophilicity.

Concerning the isolated organ assays, the lack of correlation between activity and partition coefficient indicates that diffusion from the aqueous external medium into the hydrophobic environment of the membrane receptors is not a limiting factor for angiotensin potency in these organs.

We have also investigated the role of hydrophobic character in the onset and recovery kinetics of angiotensin pressor and myotropic effects. The half-times ($t_{\frac{1}{2}}$) estimated for the pressor effects, shown in Table 3, did not appear to depend on the peptide dose that was administered. The onset $t_{\frac{1}{2}}$ was not significantly different for the three peptides listed in Table 3, indicating that neither a free amino group nor the hydrophobicity of the molecule is a limiting factor for transport to, or binding at, the angiotensin receptors. As for the recovery from pressor effects, the absence of the amino group, or its blocking by the hexanoyl moiety, decreased the $t_{\frac{1}{2}}$ to about the same extent (Table 3). This suggests that the free amino group retards the reversal of angiotensin effects, and that this reversal is not influenced by the hydrophobicity of the molecule. Similar conclusions, with the guinea-pig ileum, may be drawn from the data of Table 4. These findings agree with previous observations concerning the acute and specific desensitization (tachyphylaxis) of smooth muscles towards angiotensin peptides. It has been shown that the amino group in its protonated form is an important factor for the manifestation of tachyphylaxis (Paiva *et al.*, 1974b). The kinetics of recovery from the pressor and smooth-muscle-containing effects, sum-

marized in Tables 3 and 4, give more support to the hypothesis that the amino group of angiotensin is responsible for a slowly reversible alteration of the receptors (Paiva *et al.*, 1977). Partition effects between external medium and lipophilic biophase do not appear to play a determinant role in the biological activity of angiotensin.

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