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Circular Dichroism of Bradykinin and Related Peptides

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1. The circular dichroism of bradykinin and a number of its analogues and homologues was measured over the spectral range 200–300nm. All of the biologically active peptides showed maxima at 220nm and minima at 235nm. The spectra were independent of solvent and temperature. The vibronic transitions of phenylalanyl residues in the 250–280nm range showed no evidence of intra- or inter-molecular interactions. We take this as evidence that bradykinin and its biologically active analogues and homologues exist in solution as disordered chains. 2. None of the analogues with spectra unlike bradykinin possessed biological activity. However, peptides such as retro-bradykinin, des-6-serine-bradykinin, des-1-arginine-bradykinin and des-9-arginine-bradykinin produced spectra like that of bradykinin but were devoid of biological activity. Although we could not identify spectral features that were clearly correlated with biological activity, it appears unlikely that highly ordered peptides of the same amino acid composition as bradykinin would possess bradykinin-like effects.

Bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg, all residues in the L-form) is released from its plasma protein precursor(s) by enzymes with the specificity of trypsin. Its pharmacological actions include induction of acute arterial hypotension, vasodilation, increased capillary permeability, leucocyte migration and accumulation and pain, suggesting that bradykinin may be a mediator of conditions ranging from functional vasodilation to acute inflammation (Erdos, 1966).

Since the determination of its primary structure, a large number of analogues and homologues of bradykinin have been synthesized (Stewart, 1968). These synthetic compounds have allowed substantial progress in understanding the sequence and specific amino acid residues required for full pharmacological activity. A free carboxyl group is essential, but little activity is lost by modification of the α-amino group. The molecule must contain N- and C-terminal arginine residues. Aromatic rings are important at positions 5 and 8. Tyrosine or tyrosine *O*-methyl ether residues can be substituted for phenylalanine with preservation of biological activity, but all activity is lost if either phenylalanine residue is replaced by isoleucine. Serine and glycine can be replaced by other small amino acids but not deleted. Proline residues 2 and 7 cannot be replaced. Proline residue 3 can be replaced with alanine but not with valine (Schröder, 1964).

There is little information on the conformation of bradykinin in solution or, for that matter, on the possible relevance of a regular conformation to biological activity. Bradykinin is a nonapeptide, and previous statistical work initiated by Zimm & Bragg (1959) and later experimental studies (Goodman & Schmitt, 1959; Goodman, Schmitt & Yphantis, 1960) raise the possibility of helix conformation in peptides of seven or eight residues. However, bradykinin contains three proline residues, distributed at either end of the molecule such that only three amino acid residues intervene between proline residues. An α-helical structure can be considered highly unlikely on this basis. Even accommodating the first proline residue in the first turn of the amino end of a helix permits only six residues to be α-helical. In the present study, we investigated the question of whether bradykinin exists in solution as a random coil, or in some rigid conformational state other than α-helix. We compared the circular-dichroism spectra of all-L-, all-D- and mixed L- and D-analogues and homo-

logues of bradykinin. Special attention was given to long-wavelength ($\lambda > 250$nm) bands of the phenylalanyl residues, and attempts were made to correlate these spectral features with biological activity.

MATERIALS AND METHODS

Bradykinin and its analogues and homologues were synthesized by the solid-phase method (Stewart, 1968; Merrifield, 1969; Stewart & Young, 1969). The peptides obtained after deprotection were purified by countercurrent distribution on a 100-transfer Craig–Post device (butan-1-ol–water–trifluoroacetic acid, 100:99:1, by vol.) or by continuous-flow electrophoresis (Elfor FF, Brinkman Instruments, Westbury, N.Y. 11590, U.S.A.) with 0.1M-acetic acid adjusted to pH 5 with pyridine. After purification each peptide migrated as a single ninhydrin- or Sakaguchi-positive spot on paper electrophoresis (Whatman no. 1 paper, 0.1M-acetic acid adjusted to pH 5 with pyridine) and could be hydrolysed (6M-HCl) to appropriate component amino acids in whole molar ratios. Paper electrophoresis was carried out at 22V/cm, 10–20mA, for 1h. Amino acid analyses were performed on a Spinco model 120B amino acid analyser.

Circular-dichroism measurements were performed on a Cary 60 spectropolarimeter with a circular-dichroism attachment. U.v.-absorption spectra were obtained with a Cary 15 spectrophotometer. Except where noted, the peptides were dissolved in 0.03M-K_2HPO_4 at pH 7.2. All solvents were spectral grade. The spectropolarimeter was standardized with an aqueous solution of d-10-camphorsulphonic acid (K & K Laboratories Inc., Plainview, N.Y., U.S.A.), giving a coefficient of dichroic absorption, $\Delta\epsilon = \epsilon_L - \epsilon_R = 2.20 \pm 0.05$ at 290nm. Spectra were always checked for reproducibility and strict conformity to Beer's law, thus ruling out spurious effects such as aggregation. Peptide spectra were measured on solutions of 1–2mg/ml.

Assays for biological activity were performed as described previously (Stewart, 1968).

RESULTS AND DISCUSSION

Circular dichroism at wavelengths below 250nm. The circular-dichroic spectra of bradykinin and its related peptides at wavelengths below 250nm are remarkably uniform, with maxima at 220nm and minima at 235nm. Figs. 1 and 2 show representative spectra. With the exceptions noted below, the spectra of peptides listed in Table 1 are identical with that of bradykinin. The spectrum of des-6-serine-bradykinin is identical with that of retro-bradykinin, i.e. bradykinin in which amino acid residues are coupled in reverse sequence, and [3-alanine]-bradykinin cannot be distinguished from Polistes kinin. There are negligible changes in dioxan, dioxan–methanol (1:1, v/v) and 2-chloroethanol. Lysyl-lysyl-bradykinin shows the most marked pH dependence. Lysyl-lysyl-bradykinin and bradykinin have identical spectra at pH 7. No differences arise over the temperature range of 10–50°C. However, at high pH (Fig. 2), where the charged amino groups are neutralized and Coulombic repulsion between chain ends should diminish, the circular-dichroism maximum (220nm) of lysyl-lysyl-bradykinin lessens in intensity.

Although the origins of the circular-dichroism maxima at 220nm and minima at 235nm are not clear, we consider it significant that the spectra of bradykinin and its analogues and homologues are similar to the spectra of polylysine and polyglutamic acid in their random-coil states. The absence of temperature and solvent effects on the

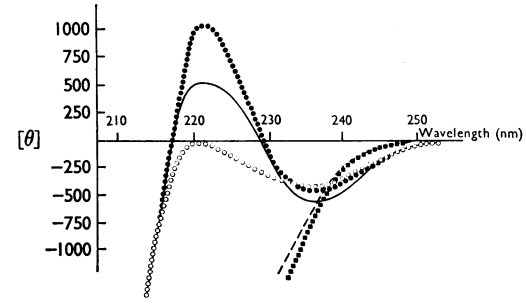

Fig. 1. Circular dichroism of bradykinin and several analogues in the region 210–250nm. The [D-Phe^8] substitution has a small effect on optical properties of the peptide by comparison with the [D-Phe^5] substitution, which yields a spectrum very close in ellipticity to [D-Phe^{5,8}]-bradykinin. Ellipticity is calculated on the basis of a mean residue weight of 115. ——, Bradykinin; ●, [D-Pro^7]-bradykinin; ○, [D-Phe^8]-bradykinin; ----, [D-Phe^5]-bradykinin; ■, [D-Phe^{5,8}]-bradykinin.

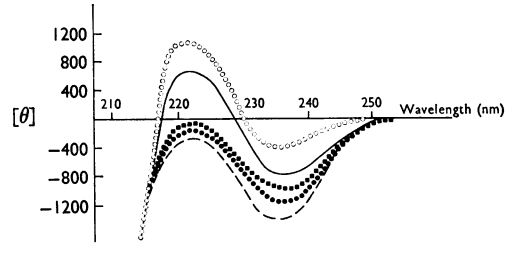

Fig. 2. Circular dichroism of analogues and homologues in the region 210–250nm. Lysyl-lysyl-bradykinin at neutral pH is identical with bradykinin at the same pH. The change in lysyl-lysyl-bradykinin with pH is the largest noted among the analogues. Ellipticity is calculated on the basis of a mean residue weight of 115. ○, Retro-bradykinin; ——, Lys-Lys-bradykinin and bradykinin, pH 7; ■, Lys-Lys-bradykinin, pH 13; ●, Polistes kinin, pH < 6; ----, Polistes kinin, pH > 10.

Table 1. *List of bradykinin analogues*

	1	2	3	4	5	6	7	8	9
Bradykinin	Arg-	Pro-	Pro-	Gly-	Phe-	Ser-	Pro-	Phe-	Arg
Retro-bradykinin	Arg-	Phe-	Pro-	Ser-	Phe-	Gly-	Pro-	Pro-	Arg
Lys-Lys-bradykinin	Lys-Lys-Arg-	Phe-	Pro-	Ser-	Phe-	Gly-	Pro-	Pro-	Arg
Des-Ser-bradykinin	Arg-	Pro-	Pro-	Gly-	Phe-	....	Pro-	Phe-	Arg
[Di-Phe^{4,6}]-bradykinin	Arg-	Pro-	Pro-	Phe-	Phe-	Phe-	Pro-	Phe-	Arg
[Phe^{6}]-bradykinin	Arg-	Pro-	Pro-	Gly-	Phe-	Phe-	Pro-	Phe-	Arg
[D-Pro^{2}]-bradykinin	Arg-	D-Pro-	Pro-	Gly-	Phe-	Ser-	Pro-	Phe-	Arg
[D-Pro^{2,3}]-bradykinin	Arg-	D-Pro-	D-Pro-	Gly-	Phe-	Ser-	Pro-	Phe-	Arg
[D-Pro^{7}]-bradykinin	Arg-	Pro-	Pro-	Gly-	Phe-	Ser-	D-Pro-	Phe-	Arg
[D-Pro^{2,3,7}]-bradykinin	Arg-	D-Pro-	D-Pro-	Gly-	Phe-	Ser-	D-Pro-	Phe-	Arg
[Ala^{3}]-bradykinin	Arg-	Pro-	Ala-	Gly-	Phe-	Ser-	Pro-	Pro-	Arg
[D-Phe^{5}]-bradykinin	Arg-	Pro-	Pro-	Gly-	D-Phe-	Ser-	Pro-	Phe-	Arg
[D-Phe^{8}]-bradykinin	Arg-	Pro-	Pro-	Gly-	Phe-	Ser-	Pro-	D-Phe-	Arg
[D-Phe^{5,8}]-bradykinin	Arg-	Pro-	Pro-	Gly-	D-Phe-	Ser-	Pro-	D-Phe-	Arg
Des-Arg^{9}-bradykinin	Arg-	Pro-	Pro-	Gly-	Phe-	Ser-	Pro-	Phe-	
Des-Arg^{1}-bradykinin		Pro-	Pro-	Gly-	Phe-	Ser-	Pro-	Phe-	Arg
		Pro-	Pro-	Gly-	Phe-	Ser			
		Pro-	Pro-	Gly-	Phe				
	Arg-	Pro-	Pro-	Gly-	Phe				
Polistes kinin	PyroGlu-Thr-Asn-Lys-Lys-Lys-Leu-Arg-Gly-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg								

circular dichroism support this view. An objection can be raised in drawing this comparison because of the relatively low intensities of the minima and maxima of bradykinin. However, Adler, Hoving, Potter, Wells & Fasman (1968) have described the circular dichroism of a derivative of polyglutamic acid, poly(hydroxyethyl-L-glutamine), exhibiting band intensities in its random-coil state that correspond closely to that of bradykinin. Thus we interpret these data as indicating that bradykinin exists in solution as a nearly random coil. The qualification nearly is inserted because it appears from Corey–Pauling models of bradykinin that rotation of the bond between the α-carbon atom and the carbonyl carbon atom of Pro^{2} is more severely restricted than the corresponding bond of other residues.

Circular dichroism at wavelengths above 250nm. We carried out a detailed study of the circular dichroism of bradykinin and related peptides in the spectral region 250–280nm, paying particular attention to phenylalanine bands. It was expected that stable interactions of the π-electron systems would alter the characteristic circular dichroism of free L-phenylalanine. Any interaction of this type could be taken as evidence of a regular secondary structure.

Several analogues and lower homologues were studied to explore possible variations of the phenylalanyl vibronic bands. The u.v. absorption and circular dichroism of the phenylalanyl portion of bradykinin is essentially that described by Simmons, Barel & Glazer (1969). The u.v.-absorption spectrum of L-phenylalanine is essentially the same as that of bradykinin except the extinction of the short-wavelength limb of phenylalanine is not as large as that of bradykinin. The correction for phenylalanine is shown by the broken line in Fig. 4.

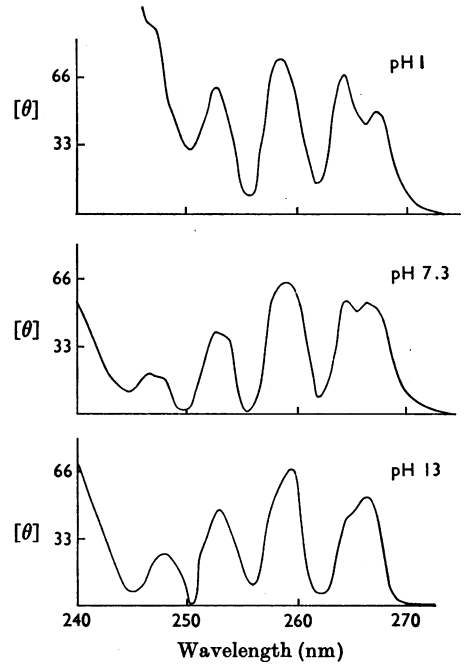

Fig. 3. Circular dichroism of free phenylalanine at pH 1, 7.3 and 13. Each maximum represents a doublet which can be separated to some extent by change in pH and solvent, but bands always remain positive. Spectra were obtained on solutions containing 1–2mg/ml in a 1.0cm cell.

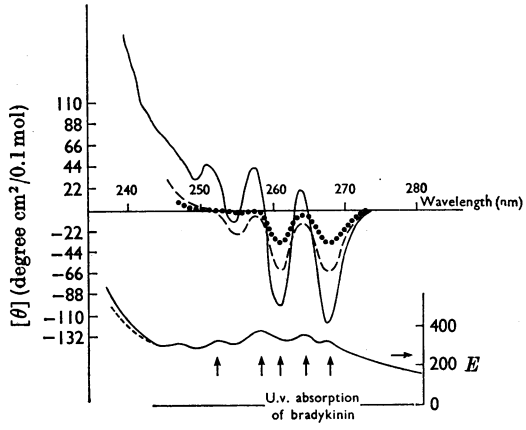

Fig. 4. Circular dichroism of several dipeptides. Small vertical arrows indicate positions of circular-dichroism maxima of free phenylalanine at pH 7. The doublet character of each of the circular-dichroism maxima noted in free phenylalanine (Fig. 3) is particularly apparent in glycyl-L-phenylalanine (——) where the doublet separates into positive and negative bands. Ellipticity is calculated on the basis of phenylalanine molecular weight and phenylalanine molarity. The circular dichroism of L-prolyl-D-phenylalanine (----) is uniformly reflected about the abscissa in comparison with L-prolyl-L-phenylalanine (●) above. The u.v.-absorption spectrum of L-phenylalanine is essentially the same as that of bradykinin except the extinction of the short-wavelength limb of phenylalanine is not as large as that of bradykinin. The correction for phenylalanine is shown by the dotted line on the 'tail' of the absorption curve.

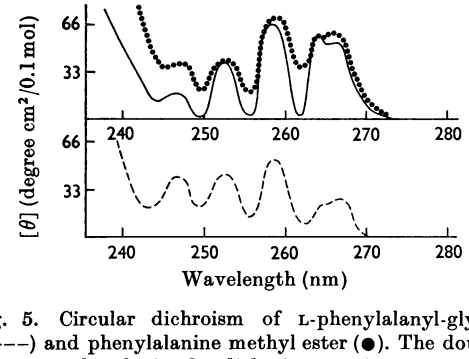

Fig. 5. Circular dichroism of L-phenylalanyl-glycine (----) and phenylalanine methyl ester (●). The doublet character of each circular-dichroism maximum noted for free phenylalanine (——) in Fig. 3 is also discernible with these compounds. Despite changes in pH, temperature and solvent the bands remain positive.

Fig. 3 shows circular-dichroism spectra of L-phenylalanine at acid, neutral and alkaline pH. At each pH value the phenylalanyl bands of bradykinin are inverted. In the large group of peptides studied, phenylalanyl spectra fell into two distinct categories: (1) exhibiting all positive bands and (2) exhibiting mainly negative bands. These categories are illustrated by Figs. 3, 4 and 5. The negative or positive direction of bands correlated well with the presence or absence of a substitution of the amino group of phenylalanine, the unsubstituted group giving rise to positive bands (Figs. 3, 4 and 5).

The circular-dichroism spectra of L-phenylalanine methyl ester and L-phenylalanyl-glycine (Fig. 5) show that substitution of the phenylalanyl carboxyl group results in rather limited alterations, not unlike the small changes of the spectra of L-phenylalanine subjected to changes of pH (Fig. 3). These spectra also show that each major band in the region 250–270 nm is actually a doublet, the character of which can be modified by carboxyl substitution or change of pH.

Of the peptides in which phenylalanine is not the first residue, bands associated with π–π^* vibronic transitions (Goodman, Davis & Benedetti, 1968) are mainly negative and are essentially invariable over broad pH and temperature changes (Fig. 4). Bands of L-prolyl-L-phenylalanine are entirely negative. The spectrum of glycyl-L-phenylalanine retains a partially positive sequence of bands (Fig. 4), but the bands become entirely negative with increasing *N*-substitution (e.g. as in the tetrapeptide L-prolyl-L-prolyl-glycyl-L-phenylalanine). In contrast with results with glycyl-L-phenylalanine, the phenylalanine bands of L-phenylalanyl-L-phenylalanine are negative, emphasizing the lack of equivalence of a carboxyl-substituted phenylalanine residue and an amino-substituted phenylalanine residue. L-Phenylalanyl-L-phenylalanine in particular verifies the negative dominance of the amino-substituted phenylalanine residue over the positive ellipticity of a carboxyl-substituted phenylalanine. It is unlikely that these changes result from phenyl ring interactions because there are neither shifts of absorption or dichroic band nor drastic changes in band intensities.

Results with the analogues of bradykinin listed in Table 1 indicate that none of the residues of bradykinin constitute an environment affecting the optical activity of the phenylalanine transitions. The spectrum of retro-bradykinin is the same as that of bradykinin. Removal of serine has no effect, and the addition of one or two phenylalanine residues ([6-phenylalanine]-bradykinin and [4,6-diphenylalanine]-bradykinin) does not give rise to a new species of circular-dichroism band. Replacement of proline residues by D-proline yields the same spectrum. Further removal of either arginine residue (des-1-arginine-bradykinin or des-9-arginine-bradykinin) leaves the phenylalanine spectrum unchanged. Except for change of band direction,

substitution of L-phenylalanine by its D-isomer does not generate new transitions. The spectrum of L-prolyl-D-phenylalanine is uniformly reflected about the abscissa when compared with the spectrum of L-prolyl-L-phenylalanine. Near-equivalence of the two phenylalanyl residues of bradykinin is shown by results with [5-D-phenylalanine]-bradykinin and [8-D-phenylalanine]-bradykinin. The phenylalanine bands of these two analogues are almost cancelled except for a small ellipticity (approx. 5°cm²/0.1mol) near 250nm, a result explained by the non-equivalence of the glycyl-L-phenylalanine and L-prolyl-L-phenylalanine units described above. In terms of positions of bands we found no evidence of interactions of the π-electron system of phenylalanine, and we regard it as highly unlikely that the phenylalanine rings of bradykinin participate in fixed intramolecular interactions.

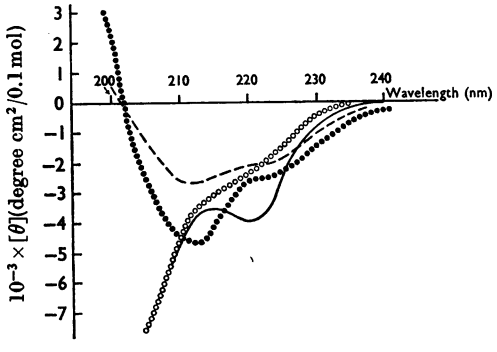

Fig. 6. Circular dichroism of two analogues in the region 200–240nm. The change in [D-Phe^{5,8}]-bradykinin with pH may be attributed to the movement of a strong band associated with phenylalanine. Generally this band appears as a positive maximum near 207nm. With D-phenylalanine we may expect the band to become negative, although the reasons for its shift to longer wavelengths are not explained. Ellipticity is calculated on the basis of a mean residue weight of 115. [D-Pro^{2}]-bradykinin: ----, pH13; ●, pH<7; [D-Phe^{5,8}]-bradykinin: ——, pH12; ○, pH<4.

Peptides with anomalous spectra. From Corey–Pauling models it was clear that some amino acid substitutions would remove much of the conformational freedom of bradykinin. [5-D-Phenylalanine]-bradykinin and [5,8-D-phenylalanine]-bradykinin are strained in extended-chain conformations. In bradykinin the N–C$^{\alpha}$ bond of serine is only slightly restricted in respect to rotation. Substitution of phenylalanine in position 5 with its D-isomer removes much of this freedom, and the resulting analogue has a circular-dichroism spectrum below 250nm (Figs. 1 and 6) clearly different from that of bradykinin. L-Phenylalanine has a positive band near 207nm, and it is possible that the spectra of [5-D-phenylalanine]-, [8-D-phenylalanine]- and [5,8-D-phenylalanine]-bradykinin are accounted for by band inversion. Neither [5-D-phenylalanine]-bradykinin nor [5,8-D-phenylalanine]-bradykinin has significant biological activity (Table 2).

[2-D-Proline]-bradykinin also exhibits a spectrum unlike that of bradykinin and most of its analogues and homologues. This analogue is not biologically

Table 2. *Activities and partition coefficients of bradykinin analogues*

	Biological* activity	Butanol–trifluoroacetic acid partition coefficients
Bradykinin	1	1.72
Retro-bradykinin	0	1.65
Des-Ser-bradykinin	1/3000	4.15
[Di-Phe^{4,6}]-bradykinin	1/10000	14.1
[Phe^{6}]-bradykinin	1/1000	1.72†
[D-Pro^{2}]-bradykinin	1/500	2.1
[D-Pro^{2,3}]-bradykinin	1/10000	1.5
[D-Pro^{7}]-bradykinin	1/75	1.80
[D-Pro^{2,3,7}]-bradykinin	1/∞	1.51
[Ala^{3}]-bradykinin	1	0.61
[D-Phe^{5}]-bradykinin	1/100	1.01
[D-Phe^{5,8}]-bradykinin	1/300	1.11
Des-Arg^{9}-bradykinin	1/100	2.38
Des-Arg^{1}-bradykinin	1/100000	1.58
Lys-bradykinin	1/2	0.23
Lys-Lys-bradykinin	1/3	0.08
Polistes kinin	1	<0.02

* Expressed as fraction of bradykinin activity on rat uterus.
† In butan-2-ol–trifluoroacetic acid. Cannot be compared with others.

active and its circular-dichroism spectrum is unique in relation to all other peptides studied. The shape of its circular-dichroism curve is remarkably similar to that of a peptide with a small degree of helix, but the ellipticity at 220nm is much too small. Since phenylalanine possesses bands in this region, it is probable that the negative ellipticity at 220nm represents mostly phenylalanine contributions. Corey–Pauling models of this peptide are always more extended than those of bradykinin, a finding inconsistent with helical conformation.

Solution conformation and biological activity. Table 2 lists the relative biological activities of the analogues and higher homologues of bradykinin surveyed in this study. All of the peptides of high biological activity, e.g. Polistes kinin, lysyl-lysyl-bradykinin and [3-alanine]-bradykinin, exhibited circular-dichroism spectra like that of bradykinin. None of the peptides with spectra unlike that of bradykinin possessed biological activity. However, peptides such as retro-bradykinin, des-6-serine-bradykinin, des-1-arginine-bradykinin and des-9-arginine-bradykinin produced spectra like that of bradykinin but were devoid of biological effects. In sum, although we could not identify spectral features that were clearly correlated with biological activity, it appears unlikely that highly ordered peptides of the same amino acid composition as bradykinin would possess bradykinin-like effects.

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