Conformation of Angiotensin II. Evidence for a Specific Hydrogen Bonded Conformation†

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ABSTRACT: The peptide amide hydrogen exchange rates of human angiotensin II in H_2O have been measured at room temperature by the transfer of solvent saturation method. The data are consistent with the assumption of a highly motile dynamic equilibrium between folded and highly solvated conformations. The NH of His^6 is observed to exchange more slowly than predicted, suggesting that it is a participant in an internal hydrogen bond. Several models previously suggested in the literature for the conformation of the peptide in aqueous solution are examined, and most are found to be inconsistent with the exchange data. Evidence in support of a structure for the Ile^5–His^6 fragment of the hormone involving a C_7^{eq}–C_5 bend is presented.

Human angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) is an octapeptide hormone with a broad range of biological activities [for reviews, see Schroder & Lubke (1966), Khosla et al. (1973), Marshall et al. (1974), and Regoli et al. (1974)]. An equally potent analogue is the synthetic derivative angiotensinamide ([Asn^1,Val^5]angiotensin II). Since this peptide has until recently been more readily available, many investigators have focused their efforts on conformational studies of the synthetic analogue. There is evidence to support the belief that the two molecules exist in similar conformations in aqueous solutions (cf. Table II). Their solution conformations have been studied by a variety of methods such as potential energy calculations (DeCoen & Ralson, 1977; Premilat & Maigret, 1979), circular dichroism (Greff et al., 1976; Fermandjian et al., 1971; Lintner et al., 1977), infrared and Raman spectroscopy (Fermandjian et al., 1972a), ^{1}H NMR (Pitner et al., 1974; Glickson et al., 1972, 1973, 1974; Marshall et al., 1973), ^{13}C NMR (Zimmer et al., 1972; Deslauriers et al., 1975, 1977), and the measurement of isotopic exchange kinetics (Thiery et al., 1977; Bleich et al., 1973; Printz et al., 1972b; Nabedryk-Viala et al., 1975). On the basis of the results of these and other studies, various models for the conformation of the angiotensin II hormones in aqueous solution have been proposed. These include an α helix (Smeby et al., 1962), a random coil (Paiva et al., 1963), β-turn, γ-turn, and cross-β conformers (Fermandjian et al., 1972a,b; Printz et al., 1972a), and a number of other more complex models (DeCoen & Ralston, 1977; Premilat & Maigret, 1979; Weinkam & Jorgensen, 1971; Glauser et al., 1970; Fermandjian et al., 1976). As yet, no consensus on the solution conformation of angiotensin has been reached. A key element in many of these models is the existence of one or more intramolecular hydrogen bonds which serve to stabilize the three-dimensional structure of the peptide.

It is generally recognized that the accessibility of an NH hydrogen to water is one of the factors determining its rate of exchange (Schellman & Schellman, 1964; Hvidt & Nielsen, 1966; Harrington et al., 1966; Willumsen, 1971; Otteson, 1971; Englander et al., 1972). Consequntly, the amide proton exchange rate of angiotensin II might be sensitive to the secondary and tertiary structural details of this hormone in aqueous solution. For this reason, there have been several studies directed at examining the NH exchange rates of angiotensin II (Thiery et al., 1977; Bleich et al., 1973; Printz et al., 1972b; Nabedryk-Viala et al., 1975) by monitoring the isotopic exchange with either tritium or deuterium. Such techniques are limited to the determination of exchange half-lives of the order of minutes and longer. As a result, these studies were performed under acidic conditions or in trifluoroethanol and at temperatures between 0 and 20 °C in order to sufficiently slow the exchange rates. The results obtained under these decidedly nonphysiological conditions have been ambiguous. Also, if the conformation of the peptide is dependent on pH, as is believed to be the case with angiotensin II (Lintner et al., 1977; Glickson et al., 1973; Craig et al., 1964), the results of such studies can be misleading.

A few reports have appeared which describe the perturbations observed in the NH resonances of [Asn^1,Val^5]angiotensin II upon solvent saturation without a quantitative analysis (Glickson et al., 1974; Pitner et al., 1974, 1975). Recently, a double-resonance ^{1}H NMR method has been developed by which exchange rates (with lifetimes in the range $\sim$10 ms to $\sim$1 s) of peptide/solvent hydrogens can be measured at pHs more closely approximating those found in vivo (Waelder et al., 1975, 1977; Bleich & Glasel, 1975). This technique has been applied recently to the study of several biologically active peptides (Krishna et al., 1979, 1980b,c). We present here the direct measurement by this technique of the exchange rates of five of the NH protons of the peptide backbone at pHs between 5.0 and 6.5 and present evidence that one of these may be participating in an internal hydrogen bond.

Materials and Methods

Human angiotensin II was purchased from Vega Chemicals and was used without further purification. Transfer of solvent saturation experiments and measurement of apparent spin–lattice relaxation rates were performed at ambient probe temperature (25 °C) on an 8 mM angiotensin II sample in H_2O. The pH of each sample was adjusted with NaOH and HCl. Spectra were recorded on a Bruker WH-400 spectrometer by using the 2-1-4-1-2 pulse sequence of Redfield

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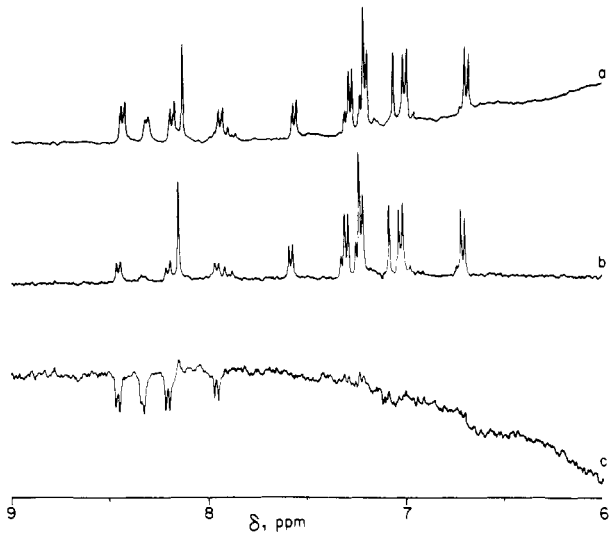

FIGURE 1: Transfer of solvent saturation experiment performed on 8 mM angiotensin II in H_2O (pH 6.1). (a) Control spectrum with decoupler frequency 4000 Hz upfield of the water resonance. (b) Same as (a) with saturating radio frequency applied to the solvent peak. (c) Difference spectrum (b − a) × 2.

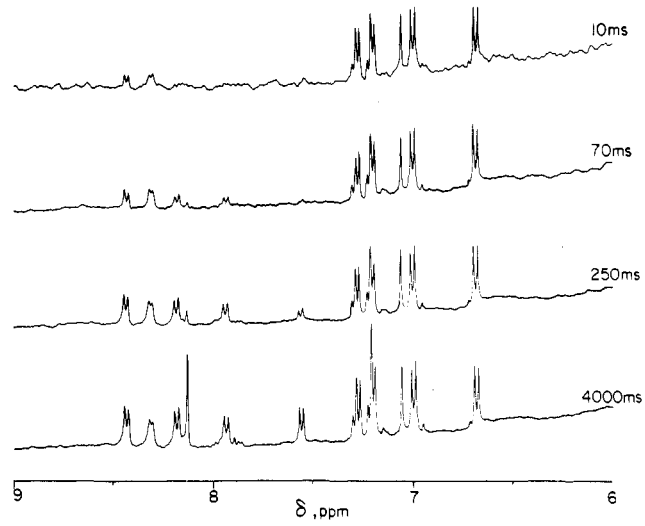

FIGURE 2: Typical saturation recovery experiment (8 mM, pH 6.1); only the resonances in the ppm range 7.5–8.5 have been saturated. The interval between completion of the saturation and initiation of the 2–1–4–1–2 observation pulse is indicated at the right of each spectrum.

et al. (1975) and Redfield (1976, 1978).[1] Typical spectra represent 32 accumulations obtained with a total pulse length of 495 μs with the carrier frequency offset 2000 Hz downfield of the solvent peak. The apparent longitudinal relaxation rates were measured by the saturation recovery method.

Results and Discussion

A typical transfer of solvent saturation experiment on angiotensin II is shown in Figure 1. Significant saturation transfer indicative of proton exchange with the solvent is evident in the negative peaks of the difference spectrum in Figure 1c, corresponding to NH's of Tyr^4, and His^6, Val^3, and Ile^5 (8.47, 8.34, 8.22, and 7.97 ppm, respectively) (Glickson et al., 1973, 1974). Positive peaks indicating intermolecular NOE's resulting from dipolar interaction with the solvent are observed for most of the aromatic CH resonance (Pitner et al., 1974, 1975; Krishna & Gordon, 1973).

A typical saturation recovery experiment from which the apparent T_1 values ($1/\langle R_{app}\rangle$) of the peptide NH's are measured is shown in Figure 2. The recovery curves were single exponential within experimental error. The apparent relaxation rates were obtained from a least-squares fitting of the data to a straight line on a semilogarithmic plot. The single-exponential nature of the relaxation curves and the observed pH dependence of the apparent relaxation rates (not shown) suggest that the dynamical behavior of angiotensin II is consistent with the assumption of high motility for the conformational transitions. Under this limit, exchange with the solvent is rate limiting (Hvidt & Nielsen, 1966), and the observed exchange rate is simply a weighted average over the individual conformations (Krishna et al., 1980a,b). The experimental exchange rates listed in Table I are given by the product of the apparent relaxation rate ($1/T_1$) and the fractional intensity change in a saturation transfer experiment (Krishna et al., 1980a). Theoretical treatments for the analysis of these experiments under two-state and multistate conformational transitions are available elsewhere (Krishna et al., 1979, 1980a,b).

Table I: Observed NH Exchange Rates (s^{-1}) of Angiotensin II

pH	Arg^2	Val^3	Tyr^4	Ile^5	His^6
5.06	58.64[a]	0.239	0.532	0.0	0.529
5.70	87.96[a]	0.468	1.106	0.788	1.056
6.10	*b*	2.92	2.37	2.64	6.20
6.41	*b*	3.665	2.942	2.550	4.685
6.57	*b*	7.046	6.574	4.304	7.766

[a] Calculated as $\pi\nu_{1/2}$. [b] Not observed.

Figure 3 shows the pH dependence of the exchange rates of the NH hydrogens of Val^3, Tyr^4, Ile^5, and His^6. The positive unit slope of the linear semilogarithmic plot of the Val^3, Tyr^4, and Ile^5 exchange constants is characteristic of simple hydroxide catalysis. The magnitudes of amide hydrogen exchange rates are largely governed by the inductive effects of neighboring groups (Molday et al., 1972). Thus, peptide NH protons next to positively charged groups (α-amino, His^+, etc.) are more acidic and hence exhibit larger base-catalyzed exchange rates than those adjacent to uncharged groups (e.g., Val, Ile). Similarly, negatively charged groups have the reverse effect.

The rates observed for the Val^3, Tyr^4, and Ile^5 NH hydrogens are in good agreement with those predicted from the data of Molday et al. (1972), indicating that they are in a fully solvated state. The observed rate for the Tyr^4 amide proton at a pH of 5.06 is somewhat larger than that predicted. This may result from a slight error in intensity measurements caused by overlap with the NH resonance of His^6. The observed rate for His^6 is consistently smaller than that predicted by a factor of 8–10. Since Deslauriers et al. (1975) observed no evidence for the aggregation of angiotensin II in their ^{13}C studies, which were performed at a significantly higher concentration than ours, there should be no significant effect from sample aggregation on the exchange rates (e.g., through intermolecular hydrogen bonding). These observations suggest that the His^6 NH hydrogen is partially shielded from the solvent or is a participant in an intramolecular hydrogen bond.

We can estimate the mole fraction of the hydogen bonded conformers from Figure 3c if we assume the Linderstrom-Lang two-state model (Linderstrom-Lang, 1958) (corresponding to

[1] The 2–1–4–1–2 sequence of Redfield et al. (1975) is a long and weak composite observation pulse that enables one to observe weak solute resonances without the dynamic range problems associated with the strong solvent resonance. If the total pulse duration is 10τ, the pulse sequence may be represented as $(2\tau)_x$–$(\tau)_{-x}$–$(4\tau)_x$–$(\tau)_{-x}$–$(2\tau)_x$. It provides a fairly uniform excitation for the peaks near the carrier frequency, and the excitation is negligible for resonances (e.g., solvent peak) at frequencies $\pm 1/10\tau$ from the carrier.

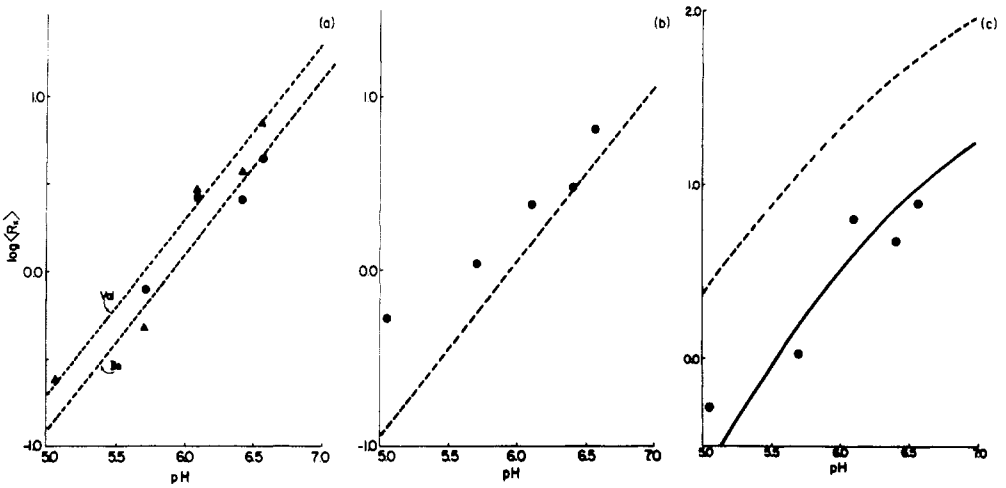

FIGURE 3: Variation of log $\langle R_x \rangle$ with pH for the amide protons of (a) Val[3] (▲) and Ile[5] (●), (b) Tyr[4], and (c) His[6]. The dotted lines represent the exchange rates predicted from the parameters of Molday et al. (1972). The solid line represents the best fit to the experimental data.

Table II: Predicted and Observed Coupling Constants (Hz) for Angiotensin II

	Tyr[4]		Val[5]		His[6]		Tyr[4]		Ile[5]		His[6]	
	ϕ	ψ	ϕ	ψ	ϕ	ψ	J_B[f]	J_R[f]	J_B[f]	J_R[f]	J_B[f]	J_R[f]
type I[a]	−60	−30	−90	0			3.1	3.8	7.8	7.6		
type II[a]	−60	120	80	0			3.1	3.8	7.0	5.7		
type III[a]	−60	−30	−60	−30			3.1	3.8	3.1	3.8		
Printz β turn[b]	55	−99	−142	33	−156	165	8.6	6.3	9.5	8.4	7.4	6.9
C_7^{eq}–C_5[c]			−80	80	−160	160			6.3	6.4	6.3	6.4
C_7^{eq}–C_5[d]			−90	80	−160	160			7.8	7.6	6.3	6.4
J_{obsd}[e]							8.1 ± 0.4		8.5 ± 0.4		6.6 ± 0.4	

[a] Lewis et al. (1973). [b] Printz et al. (1972a). [c] DeCoen & Ralston (1977) and Lewis et al. (1973). [d] C_7^{eq}–C_5 slightly distorted from the values of Lewis et al. (1973) and DeCoen & Ralston (1977). [e] R. E. Lenkinski and R. L. Stephens, unpublished experiments. [f] J_B and J_R refer to coupling constants derived from the Karplus equation by using the constants proposed in Brystrov et al. (1973) and Ramachandran et al. (1971), respectively.

a folded state with no exchange, and a fully solvated state) for angiotensin II. Under this model, the observed diminution in exchange by a factor of 9 for His[6] NH implies a mole fraction of ~0.9 for the hydrogen bonded conformer.

On this basis, we can evaluate a number of previously proposed models of angiotensin II in aqueous solution. Fermandjian et al. (1972a,b) have proposed a cross-β-type conformation for the hormone defined by a turn involving the Val[3], Tyr[4], and Val[5] sequence and a second turn at the His[6]–Pro[7] linkage. This model places the histidine NH away from the interior of the molecule in a position allowing ready exchange with solvent protons and is therefore inconsistent with our results. Fermandjian et al. (1976) have also proposed a second, more complex conformation featuring strong hydrogen bonds involving the NH's of Tyr[4] and His[6], and in which three of the other amide protons in the peptide are "weak" interaction participants. Since the observed exchange rate of the Tyr[4] NH is in agreement with that predicted for the fully solvated proton, we see no evidence for any hydrogen bond involving the NH of the tyrosine residue, nor do we observe any evidence for the three "weak" interactions postulated in this model.

Printz et al. (1972a) have suggested two models for angiotensin II in aqueous solution. The first of these is characterized by a γ turn in residues 3–5. This model postulates a hydrogen bond between the Val[3] NH and the carbonyl of Ile[5], and a hydrogen bond involving the amide proton of Ile[5] and the Val[3] carbonyl. This model, too, is unsupported by our findings, which indicate that both of these protons are fully solvated.

The second model proposed by Printz et al. (1972a) is the β-turn model, which also has two hydrogen bonds: one between the amide hydrogen of Val[3] and the carbonyl of His[6], and the second between the NH of His[6] and the Val[3] carbonyl. The above evidence argues against the participation of the valine amide proton in a hydrogen bond, and, therefore, this model as it stands is inconsistent with our results. However, it should be noted that the slow rate of exchange of the His[6] NH is consistent with part of this β-turn model. Marshall et al. (1973) have calculated the ^{1}H NMR coupling constants predicted by the torsional angles of this conformer and determined that they were inconsistent with the observed coupling constants. In view of this and the above argument, parts of the β-turn model of Printz et al. (1972a) can be discounted. Nevertheless, the essential feature of this model, the β turn, might be retained if the hydrogen bond between the Val[3] NH and the His[6] carbonyl is discarded. Such a model, featuring a β turn stabilized by a hydrogen bond between the His[6] NH and the carbonyl of the valine residue, would be consistent with the hydrogen exchange data presented here.

However, as shown in Table II, neither a simple type I nor a type II turn is consistent with the NH–C$^\alpha$H coupling constant observed for Tyr[4], the i + 1 residue of the turn. The observed coupling constants for residues 4–6 of angiotensin II are shown in Table II, together with the torsional angles defining several different types of turns for the peptide backbone (Lewis et al., 1973). The coupling constants predicted from the torsional angles defined by the type I, II, and III turns are inconsistent with the coupling constant observed for the tyrosine residue although there is reasonable agreement between the predicted and observed values for Ile[5]. In addition, the NH–C$^\alpha$H coupling constants predicted from the β-turn model of Printz et al. (1972a) and calculated by Marhsall et al. (1973) for Arg[2] and Val[3] (~10 and 9.5 Hz, respectively) do not agree well with the observed values (Glickson et al.,

1974; Marshall et al., 1973) (6.5 ± 0.3 and 7.9 ± 0.3 Hz, respectively).

The observed coupling constants could be accounted for by significant distortion of the β turn from the torsional angles described by Lewis et al. (1973). Indeed, the angles defining the β-turn model of Printz et al. (1972a) bear little resemblance to the turn as described by others (see table II). In view of this, it is possible that angiotensin II contains a highly distorted β turn in residues 3–6 stabilized by a hydrogen bond between the amide proton of His^6 and the carbonyl of Val^3.

DeCoen & Ralston (1977) have carried out theoretical calculations of the possible conformation of Ala_6-Pro-Ala and [Asn^1,Val^5]angiotensin II. At a low dielectric constant (ϵ = 1.0), the most probable conformers of Ala_6-Pro-Ala are stabilized by C_5 and C_7 structures in which the Ala^6 NH interacts with the carbonyls of both residues 4 and 6 simultaneously. The authors note that these conformers become much less important at higher values of the dielectric constant and when the side chains of [Asn^1,Val^5]angiotensin II are included in the calculations. These calculations also predict that the amide proton of the tyrosine residue would experience interactions similar to those of the histidine. There is no evidence in our results of such interactions. However, the calculations of DeCoen and Ralston take into account only nonspecific interactions with the solvent. Specific solvation effects have not been incorporated into the calculations.

In examining all of the above models, we find that a C_7^{eq}–C_5 structure for the Ile^5–His^6 fragment of angiotensin II is the most consistent with the experimental results. The observed ^1H NMR coupling constants are in good agreement with those predicted for this structure (see Table II). This model places the His^6 NH in a hydrogen bond with the carbonyls of Tyr^4 and His^6, providing an explanation for the experimental observation that the exchange rate of the histidine amide proton is slower than expected. In addition, in a recent NMR study, a C_7 structure has also been proposed to be a dominant form in the conformational equilibrium of an active pentapeptide fragment of thymopoietin (Krishna et al., 1980c). The possibility of this structure for this two-residue fragment in angiotensin II merits further examination.

Conclusions

We have determined the exchange rates of the amide protons of human angiotensin II at several pHs and compared them with the rates predicted for the solvated peptides from the data of Molday et al. (1972). The rates observed for Val^3, Tyr^4, and Ile^5 are in good agreement with those predicted, but the NH of His^6 exchanges more slowly than would be expected from a diffusion-controlled model. This is interpreted as indicative of the participation of the histidine amide proton in a hydrogen bond.

None of the previously proposed models for the solution conformation of angiotensin that we have examined were consistent with these results. We have proposed a structure for the Ile^5–His^6 fragment of the peptide that is consistent with both the reported coupling constants and our present findings, but further work is necessary to evaluate the validity of this model.

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Calcium/Magnesium Specificity in Membrane Fusion: Kinetics of Aggregation and Fusion of Phosphatidylserine Vesicles and the Role of Bilayer Curvature†

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ABSTRACT: We have investigated the relative abilities of Ca^{2+} and Mg^{2+} to induce the fusion of phospholipid vesicles composed of pure bovine brain phosphatidylserine (PS). Vesicle aggregation was monitored by light-scattering measurements, fusion by the terbium/dipicolinic acid assay for mixing of internal vesicle volumes, release of vesicle contents by carboxyfluorescein fluorescence, and changes in vesicle size by freeze–fracture electron microscopy. Either small unilamellar vesicles (SUV, diameter 250 Å), prepared by sonication, or large unilamellar vesicles (LUV, diameter 1000 Å), prepared by reverse-phase evaporation and extrusion through a polycarbonate membrane, were used. Measurements were done in a 0.1 M NaCl medium at 25 °C and pH 7.4. In the presence of either Mg^{2+} or Ca^{2+} at above-threshold concentrations, both types of vesicles massively aggregate. Mg^{2+} does not induce the fusion of LUV or any release of vesicle contents. It does induce the fusion of SUV, which is accompanied by a transient release of vesicle contents, but the extent of fusion is limited to a small increase in vesicle size, beyond which there is no further mixing or release of vesicle contents. By contrast, Ca^{2+} induces rapid and extensive fusion of LUV or SUV, while eventually all internal contents are lost due to collapse of the vesicles. The Mg^{2+}-induced fusion of SUV proceeds less rapidly and is more leaky than the initial Ca^{2+}-induced fusion of SUV. Mixtures of Ca^{2+} and Mg^{2+} behave either competitively or cooperatively, depending on the relative cation concentrations and on the type of vesicle used. The initial rate of Ca^{2+}-induced fusion of LUV is inhibited by Mg^{2+} at any Ca^{2+} concentration. At low, near-threshold Ca^{2+} concentrations, Mg^{2+} cooperates in inducing the fusion of SUV, whereas at higher Ca^{2+} concentrations, Mg^{2+} is inhibitory. The results are discussed in terms of the degree of dehydration and the change in lipid fluidity induced by interaction of the divalent cations with the vesicles. Crucial to the specificity of Ca^{2+} in inducing the fusion of PS vesicles seems to be its ability to form an anhydrous complex between apposed bilayers.

The well-documented involvement of Ca^{2+} in various biological membrane fusion phenomena (Poste & Allison, 1973; Rubin, 1974; Douglas, 1975) has prompted extensive studies on the interactions of divalent cations with phospholipid vesicles composed of phosphatidylserine (PS)[1] or other acidic phospholipids. Several of our recent studies have emphasized the contrasting effects of Ca^{2+} and Mg^{2+} on the physical properties of acidic phospholipid bilayers (Jacobson & Papahadjopoulos, 1975; Newton et al., 1978), and we have proposed correlations of these discrepancies with the diverse abilities of the two cations to induce vesicle fusion (Papahadjopoulos et al., 1977,

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[1] Abbreviations used: PS, phosphatidylserine; DPA, dipicolinic acid; CF, carboxyfluorescein; Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; SUV, small unilamellar vesicles; LUV, large unilamellar vesicles.

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