hybrids may also reflect the intermolecular interactions. For example, consider a strongly ligand-linked association in which each M subunit can interact once to form an intermolecular bond and the intrinsic affinity of the M subunit for bis(ANS) is independent of the subunit composition of the tetramer. In this model H_3M dimerizes, H_2M_2 undergoes indefinite linear association, while M_3H and M_4 branch indefinitely in three and four directions, respectively. On a relative basis, the overall binding of bis(ANS) to M-type subunits would be thermodynamically favorable for M_4, with many modes of association, and unfavorable for H_3M, which could only dimerize. Further hydrodynamic studies may clarify the actual mechanism of association and the roles of intermolecular and intramolecular interactions.

Acknowledgments

I thank Mr. Robert Schuyler and Professor I. Isenberg for the measurements of the fluorescence time decay, Professor K. E. Van Holde for the use of the circular dichroism spectrophotometer, and Professor Gregorio Weber for the gift of bis(ANS).

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Complexes of Phosvitin with Poly-L-lysine and Protamine. Conformational Analysis*

Gertrude E. Perlmann† and Kärt Grizzuti

ABSTRACT: The interactions between the phosphoprotein phosvitin and the polybases poly-L-lysine or protamine have been studied with optical rotatory dispersion (ORD) and circular dichroism (CD). The data suggest that phosvitin–poly-L-lysine or phosvitin–protamine complexes form a β-pleated-sheet structure at pH 3.0–5.0, characterized by Cotton effects with a 230- to 232-nm trough, a 202- to 204-nm peak, and dichroic bands at 192–195 nm and 216 nm. Under the same conditions phosvitin, poly-L-lysine, and protamine by themselves are in an unordered conformation. Maximum values of $[m']_{202}$ and $[\theta']_{192}$ of the complexes are found when the ratio, r, of the negatively charged phosphoserine residues of phosvitin and the positive ϵ-NH_2 groups of poly-L-lysine or arginine residues of protamine is unity, *i.e.*, $r = 1$. On addition of methanol to a final concentration of 46% or 74% (v/v), $[m']$ and $[\theta']$ of the complexes increase further, whereas the presence of 0.2–1.0 N NaCl in the solutions prevents complex formation. Thus electrostatic interactions, in part, are apparently of importance in the stabilization of the complex. No interactions were detected in phosvitin–poly-L-lysine mixtures at pH 7.0–9.5 where the unordered conformation of the two constituents prevails.

When poly-L-lysine or protamine is added to phosvitin at pH 3.0–5.0, optical rotatory dispersion (ORD)[1] and circular dichroism (CD) measurements indicate that the basic polymers interact with the acidic phosphoprotein to form a β-pleated sheet, whereas each constituent is present initially in an unordered conformation (Beychok, 1967; Grizzuti and Perlmann, 1970).

The purpose of the present study is to examine this interaction in detail and to answer questions such as the effect of solvent composition, pH, and polymer chain length on the complex formation of polyelectrolytes carrying predominantly opposite charges.[2]

We will show that the interaction and formation of these complexes display a characteristic stoichiometry of 1:1 phosphoseryl to lysyl or arginyl residues and that under these conditions the largest changes of the optical rotatory dispersion patterns and circular dichroism spectra are observed.

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[1] Abbreviations used are: $[m']$, reduced mean residue rotation; $[\theta']$, reduced mean residue ellipticity; ORD, optical rotatory dispersion; CD, circular dichroism; Psr, phosphoserine; PL, poly-L-lysine; PR, protamine.

[2] It has been discussed by Perlmann and Grizzuti (1970) that some of the properties of phosvitin can best be explained by considering this protein as a polyelectrolyte.

Materials and Methods

Materials. Phosvitin, with a nitrogen and phosphorus content of 13.50 and 12.28%, respectively, was prepared from fresh hens' eggs according to the procedure of Joubert and Cook (1958).

Protamine from salmon sperm (free base), lot 30C-4750, Grade IV, was obtained from Sigma Chemical Co. On polyacrylamide gel electrophoresis by the method of Reisfeld *et al.* (1962) this preparation migrated as several bands. Its average molecular weight as determined by ultracentrifugation was 4000; it had an arginine content of 79.8%, serine 6.8%, proline 9.6%, glycine 3.0%, and valine 3.9%. Traces of lysine, alanine, and leucine were detected on amino acid analysis. The amino acid composition of our preparation agreed well with those found in the literature (Dixon and Smith, 1968; Ando and Watanabe, 1969; Ling *et al.*, 1971). From the results of the amino acid analysis the average residue weight of 135 was calculated.

Poly-L-lysine hydrobromide (code no. 71-120 A, control no. LY 146) with the average degree of polymerization, $\overline{DP} \sim 24$, and average molecular weight 5000 came from Miles Laboratories; poly-L-lysine hydrochloride (Lot 4494) with $\overline{DP} \sim 156$ and an average molecular weight of 24,000 (ultracentrifugation) was obtained from Schwarz/Mann Biochemicals; poly-L-lysine hydrochloride, $\overline{DP} \sim 20$, average molecular weight 3200, and a preparation with an average molecular weight of 125,000 were gifts from Dr. G. Fasman; the chromatographically purified poly-L-lysine hydrochlorides, $\overline{DP} = 5$, $\overline{DP} = 10$, and $\overline{DP} = 17$, were gifts of Dr. H. A. Sober.

All chemicals used were commercial analytical reagents and were not further purified. Methanol, Spectral Grade, was purchased from Eastman Organic Chemicals.

Solutions. Stock phosvitin and poly-L-lysine solutions (0.5%) were prepared in distilled water while protamine was dissolved in 0.02 N HCl to lower the pH of the solution to 2.0. The concentrations of the solutes are based on nitrogen determinations.

For the optical rotatory dispersion (ORD) and circular dichroism (CD) measurements the concentrations used were 0.01–0.02%.

Complexes were obtained by adding solutions of the complexing agent (poly-L-lysine or protamine) to the phosvitin solution. The ratios of phosvitin to the complexing agent are reported either as molar ratios or as that of phosphoserine residues of phosvitin (Allerton and Perlmann, 1965) to that of lysine or arginine residues of the poly-L-lysine and protamine,[3] respectively. Since it was found that the order of addition of reagents affects the results in solvents other than water, as a general rule, and, unless specified, phosvitin was diluted with the appropriate solvent close to the volume desired. The complexing agent was then added and the solutions adjusted with the solvent to the final volume.

Methods. In precipitation experiments poly-L-lysine (3.6–4.0 mg/ml) or protamine (4.7 mg/ml) was added in varying amounts to a series of tubes containing 2 ml of phosvitin solution (3.7 mg/ml). The mixtures were allowed to stand at 25° for 15–20 min. The suspensions were then centrifuged at 8000 rpm for 30 min, using the Sorvall SS-1 rotor. After centrifugation the supernatant was carefully pipetted off, and the sediment was dissolved in 2 ml of 50% H_2SO_4 with slight heating. Nitrogen and phosphorus were determined on aliquots of the supernatants and the dissolved precipitates.

Nitrogen analysis was done by the Pregl micro Kjeldahl method with a mercuric catalyst (Hiller *et al.*, 1948). For the phosvitin and protamine solutions the nitrogen factors, based on separate nitrogen, ash, and moisture determinations, were used for conversion to dry weight.[4] For the various poly-L-lysines the factor based on the theoretical nitrogen content of 17% was used to obtain the dry weight equivalent.

Phosphorus was determined as described by Allerton and Perlmann (1965).

Amino acid analysis was performed on a Spinco Model 120 amino acid analyzer as developed by Spackman *et al.* (1958), equipped with the AA-15 and AA-27 resins (Spackman, 1967) and a range card to increase the sensitivity of the analysis to 0.01 μm (Fruchter and Crestfield, 1965).

Optical rotatory dispersion and circular dichroism measurements were carried out at 25° in a Cary Model 60 recording spectropolarimeter, equipped with the 6001 circular dichroism attachment in 10-mm, 1-mm, and 0.5-mm path-length cells. The results of the optical rotatory dispersion are given as mean residue rotation, $[m]$, where the mean residue weight was calculated for each mixture from the concentrations of each constituent. The mean residue weight of phosvitin was taken as 161 (Allerton and Perlmann, 1965), whereas the values of 128 and 135 were used for poly-L-lysine and protamine, respectively. The mean residue weight for each mixture varied from 150 to 161. The circular dichroism is recorded in terms of ellipticity in degrees and in analogy to $[m]_\lambda$ expressed as $[\theta]_\lambda$. All our results were corrected for the variations of the index of refraction as described elsewhere (Grizzuti and Perlmann, 1970; Perlmann and Grizzuti, 1971) and are reported as reduced mean residue rotation, $[m']_\lambda$, and reduced mean residue ellipticity, $[\theta']_\lambda$.

Since the protamine and the poly-L-lysine have significant optical activity in the wavelength range of 300–190 nm, the reference solution in each experiment was always that of the corresponding protamine or poly-L-lysine solution without added phosvitin.

Some of the solutions showed slight opalescence. However, this did not interfere with the optical rotatory dispersion and circular dichroism measurements (*cf.*, Lenard and Singer, 1966; Wallach and Zahler, 1966; Steim and Fleischer, 1967; Hammes and Schullery, 1968, 1970; Perlmann and Grizzuti, 1971).

The pH of all solutions was measured with the Radiometer pH meter Model 4, calibrated with the standard buffers recommended by Bates (1954). The differing amounts of complexing agents that were added to the phosvitin solutions produced variations in the pH values of the reaction mixtures by about ±0.5 pH unit. However, since the changes of the optical rotatory dispersion and circular dichroism of phosvitin, poly-L-lysine, and protamine in the pH range of 3.5–4.5 are insignificant when compared to those observed during the complex formation, these variations were ignored. The pH values reported throughout this paper are average values.

[3] Residue ratio, r, is defined as the ratio of the number of phosphoserine residues of phosvitin to the ϵ-NH_2 groups of the poly-L-lysines or to the arginine residues of the protamine.

[4] We are indebted to Mr. T. Bella for these analyses.

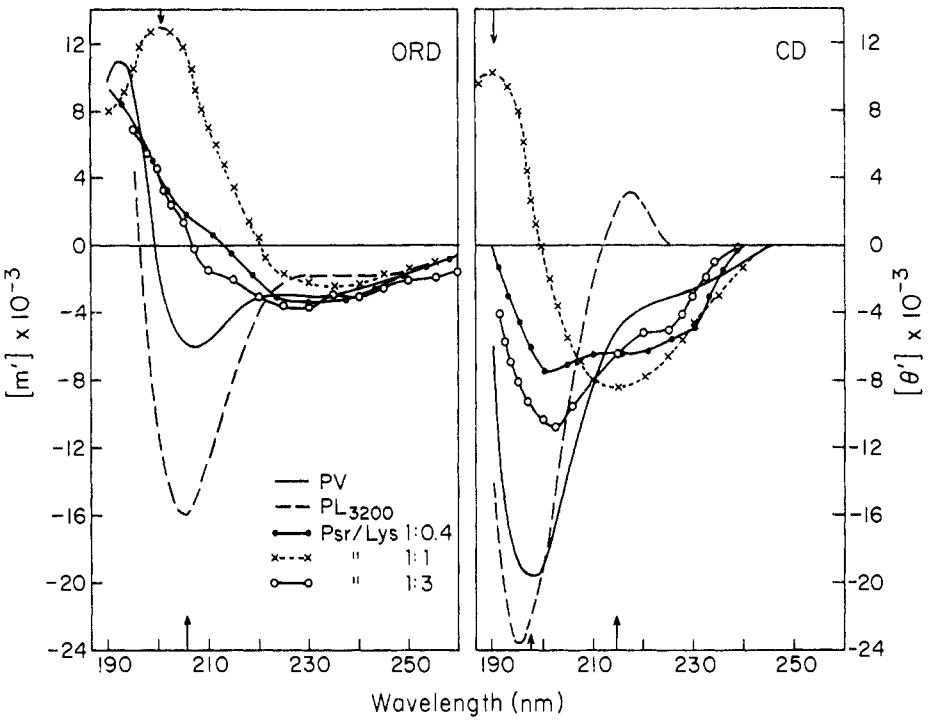

FIGURE 1: Optical rotatory dispersion and circular dichroism of phosvitin, PV, poly-L-lysine, PL_{3200}, and phosvitin–poly-L-lysine complexes at pH 3.8 and at various ratios of Psr/Lys.

Results

Addition of poly-L-lysine of various degrees of polymerization or of protamine to an aqueous phosvitin solution of pH 3.8 produces marked changes in the optical rotatory dispersion curves and circular dichroic spectra of the protein. As has been reported elsewhere, the optical rotatory dispersion patterns of phosvitin in the pH range of 3.5–5.0 display two minima at 207 nm and at 232 nm; $[m']_{207} = -5200$ and $[m']_{232} = -3400$. The maximum at 192 nm has a $[m']_{192}$ of 9500 (Perlmann and Allerton, 1966; Timasheff *et al.*, 1967b; Grizzuti and Perlmann, 1970). On complexing cationic polymers with the acidic phosphoprotein spectral characteristics of β structure are obtained, *i.e.*, a trough at 232–240 nm, a crossover point between 220 and 230 nm, and a maximum at 200–207 nm. Similarly, the circular dichroism spectra of the complexes resemble those of β structure with the typical negative band at 215 nm and a positive band at 190–195 nm.

The extent of the changes observed is influenced by: (a) the ratio of phosvitin to the complexing agent added, (b) the nature and chain length of the complexing agent, (c) pH, (d) ionic strength, (e) presence of organic solvents and order of mixing.

Changes of the Optical Rotatory Dispersion and Circular Dichroism of Phosvitin as a Function of Phosvitin–Poly-L-lysine Ratio for the Complexes Formed. Formation of complexes between phosvitin and increasing amounts of poly-L-lysine, PL_{3200},[5] at pH 3.8 causes progressive changes in the ORD patterns and CD spectra. Figure 1 illustrates the optical rotatory dispersion patterns and circular dichroism spectra of phosvitin and poly-L-lysine, PL_{3200}, as well as the effect of poly-L-lysine added to a phosvitin solution of pH 3.8. As the molar ratio of the polyamino acid to the protein is increased, the typical trough of phosvitin at 207 nm is gradually transformed into a maximum centered at 200 nm with $[m']_{200}$ of 12,000, while the small trough at 232 nm remains relatively unchanged.[6] In the circular dichroism spectra the negative band at 197 nm with $[\theta']_{197}$ of $-20{,}000$ has disappeared and has been replaced by a positive band at 190 nm with $[\theta']_{190} = 11{,}000$. The negative band in these spectra is at 216 nm and $[\theta']_{216} = -8000$. The maximum change observed with the poly-L-lysine preparation, PL_{3200}, is at a molar ratio of 7.4. If the molar ratio of the two constituents of the complex is expressed as the ratio, r, of phosphoserine residues of phosvitin (Allerton and Perlmann, 1965) to the lysine residues of PL_{3200}, the greatest effect on the spectra is observed when $r = 1$. This can be taken as an indication that at $r = 1$ all of the phosvitin and poly-L-lysine is transformed into the complex brought about through electrostatic interaction of the residues carrying opposite charges. It should be pointed out that in the pH range of 3.0–5.0 in which complex formation occurs only one hydroxyl of each phosphomonoester group of the phosphoserine residues is ionized; hence the net electronic charge corresponds roughly to *one* negative charge per phosphoserine. On increasing the concentration of PL_{3200}, $[m']_{200}$ and $[\theta']_{197}$ decrease.

In these experiments it was also observed that the opalescence of the mixtures increases as the amount of PL_{3200} added is enhanced. The maximum spectral change observed in the mixtures with the ratio $r = 1$ corresponded to the

[5] The average molecular weight of the poly-L-lysine used is indicated by a subscript. Thus, PL_{3200} is the poly-L-lysine preparation with $\overline{DP} \sim 20$ and the average molecular weight of 3200.

[6] In the experiments the molar ratio of the polyamino acid to phosvitin was increased from 0 to 20. For the sake of clarity only 3 spectra are given in Figure 1.

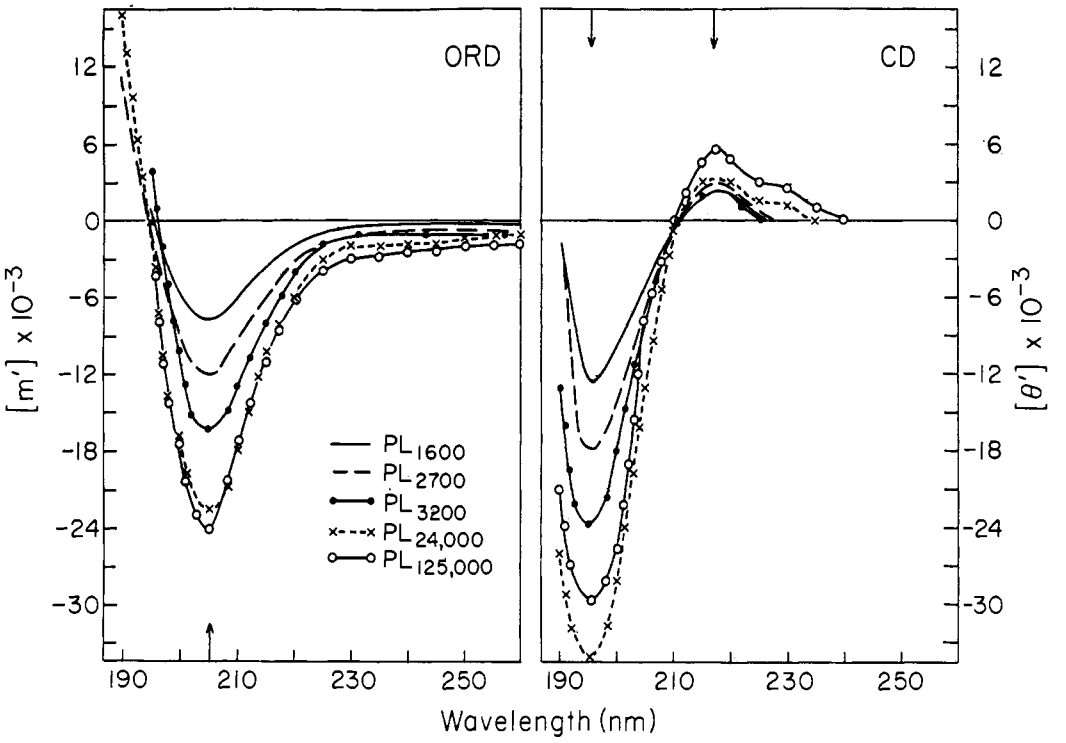

FIGURE 2: Optical rotatory dispersion and circular dichroism of poly-L-lysines at pH 3.8. Molecular weight range of the poly-L-lysines varied from 1600 to 125,000.

point of highest opalescence which, however, gradually disappeared on further addition of PL_{3200}.[7]

The depression of the spectra, however, cannot be explained in terms of aggregation[8] as has been suggested by Davidson and Fasman (1969). In the cases described here dissociation of the complexes occurs due to the excess of positive charges contributed by the poly-L-lysine molecules once the negatively charged phosphate groups of the phosvitin have been neutralized.

Effect of Chain Length of Poly-L-lysine on the Optical Rotatory Dispersion and Circular Dichroism of Phosvitin–Poly-L-lysine Complexes. In the pH range of 1.0–10.0 poly-L-lysine has an unordered conformation characterized by a trough at 205 nm and a dichroic band at 195 nm (Beychok, 1967; Timasheff *et al.*, 1967a). However, as shown Figure 2, the chain length of the poly-L-lysine influences the optical rotatory dispersion and circular dichroism. Although the positions of the minima remain at the same wavelengths, *i.e.*, at 205 nm (ORD) and at 195 nm (CD), the magnitude of [m'] and [θ'] changes considerably. Similarly, the chain length (molecular weight) of the polyamino acid has also an effect on the spectral properties of the complexes.

Figure 3 illustrates the optical rotatory dispersion patterns and circular dichroism spectra of phosvitin–poly-L-lysine complexes at pH 3.8 and $r = 1$, using poly-L-lysine preparations of an average molecular weight of 1600 to 125,000 as complexing agent. From Figure 3 it is apparent that bands characteristic of β structure are present in each case. In addition, the degree of polymerization of the different poly-L-lysines used determines the magnitude of $[m']_{200}$ and $[\theta']_{195}$. Thus, at $r = 1$ the reduced mean residue rotation, [m'], is 13,500 and 30,000 if PL_{1600} and $PL_{24,000}$, respectively, are added to the phosvitin solution. Included in Figure 3 are also the optical rotatory dispersion patterns and circular dichroism spectra of a mixture of phosvitin and a lysine hydrochloride solution of pH 3.8 at $r = 1$, *i.e.*, at the corresponding molar excess of this amino acid. The patterns of the phosvitin–lysine monomer mixture are almost superimposable on those of phosvitin, which indicates that no complex is formed between the phosphoprotein and the lysine monomer. This observation, therefore, raises the question as to which degree of polymerization or molecular weight of the complexing agent is a prerequisite for complex formation to occur. With the aid of chromatographically purified poly-L-lysines of $\overline{DP} = 5$, $\overline{DP} = 10$, and $\overline{DP} = 17$, it could be ascertained that no complexes with typical β-structure patterns could be obtained with polymers that have a $\overline{DP}$ smaller than 17 (Table I).

Phosvitin–Protamine Complexes. If phosvitin–protamine complexes are prepared, similar changes in the optical rotatory dispersion and circular dichroism parameters are observed as in the phosvitin–poly-L-lysine complexes. The results of some representative experiments are shown in Figure 4. Optimal conditions are again found at a ratio, $r = 1$, of phosphoserine residues of the phosphoprotein to the arginines of the protamine. Thus, it appears that the spectra are not dependent on the nature of the complexing agent as long as the two components of the complex contain prevalent amounts of oppositely charged amino acid residues.

Stoichiometry of Complex Formation. In the study of the optical rotatory dispersion of phosvitin–poly-L-lysine and phosvitin–protamine mixtures reported in the preceding sections we have established that a transition of the unordered

[7] Additional evidence that light scattering has no effect on the ORD and CD measurements is provided by experiments on phosvitin–protamine mixtures at $r = 1$ in which the respective initial concentrations of the solutes were varied. [m'] and [θ'] remain unchanged.

[8] Aggregation of the complex occurs only if the solutions containing 1:1 residue ratio are allowed to stand for one hour at room temperature.

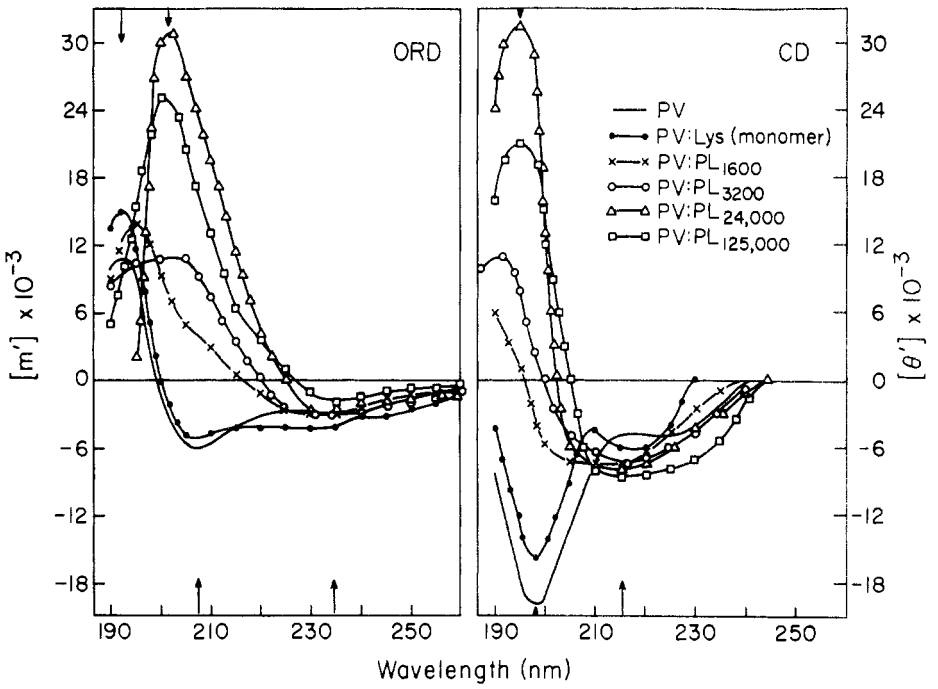

FIGURE 3: Optical rotatory dispersion and circular dichroism of phosvitin–poly-L-lysine complexes at pH 3.8 and at $r = 1$, using poly-L-lysines of different molecular weights.

TABLE I: Position of Cotton Effects and Dichroic Bands of L-Polypeptides and of Phosvitin–Poly-L-lysine Complexes using Poly-L-lysines of Various Degrees of Polymerization, at $r = 1$.

Conformation	ORD (nm)			CD (nm)		
	Trough	Cross-over	Peak	Negative	Cross-over	Positive
Phosvitin, pH 3.0–5.0, unordered	207 (235)[a]	199	192	197	189	NR[b]
L-Polypeptides, unordered	204–205	198	190	199–200		
Complex with poly-L-lysine,[c] $\overline{DP} = 5$	(235)[a]	217	192	200	NR	NR
Complex with poly-L-lysine,[c] $\overline{DP} = 10$	(232)[a]	216	195	214	199	NR
Complex with poly-L-lysine, $\overline{DP} = 17$	232	220	200	215	200	190
L-Polypeptides, β form	229–230	221	204	218	208	197

[a] Minor trough always present in phosvitin at pH 3.0–5.0. [b] NR, not recorded. [c] These spectra show a progressive transition from "unordered" conformation to β structure; *cf.* Figure 1 patterns Psr/Lys = 1:0.4.

conformation of the constituents to β structure occurs on forming a complex through electrostatic interaction of the oppositely charged constituents. As illustrated in Figure 5, on addition of the different poly-L-lysines or protamine the negative reduced mean residue rotation $[m']_{207}$ becomes positive and passes a maximum at $r = 1$. It then decreases if the number of the positively charged lysine or arginine residues present exceeds that of the negatively charged phosphoserines of phosvitin.[9] As judged by the magnitude of $[m']_{207}$, Figure 5 further illustrates that an increased chain length (molecular weight) of the poly-L-lysines used as complexing agent enhances the amount of β structure present in the complex.

Effect of pH. In the pH range of 7.0–9.5 the optical rotatory dispersion patterns and circular dichroism spectra of phosvitin–poly-L-lysine mixtures of a residue ratio varying from 0.5 to 1.0 are those typical of an unordered conformation. This is readily understood since in this pH range both hydroxyls of each phosphate group of the phosphoserine residues of phosvitin have lost their protons and are dissociated; therefore, the electrostatic repulsion of the side chains of these amino acid residues maintains the protein in an extended conformation and thus prevents the formation of complexes with poly-L-lysine, hence also a transition from the unordered conformation to β structure. At pH values greater than 9.5, *e.g.*, 9.5–10.5 at the residue ratio of 0.5 where the number of lysine residues is twice that of phos-

[9] Observed raw ORD measurements (not referred to poly-L-lysine or protamine) also indicate that the observed mean residue rotations also pass a maximum at $r = 1$. It decreases if the complexes are dissociated by further addition of the cationic polymers (*cf.* Table III).

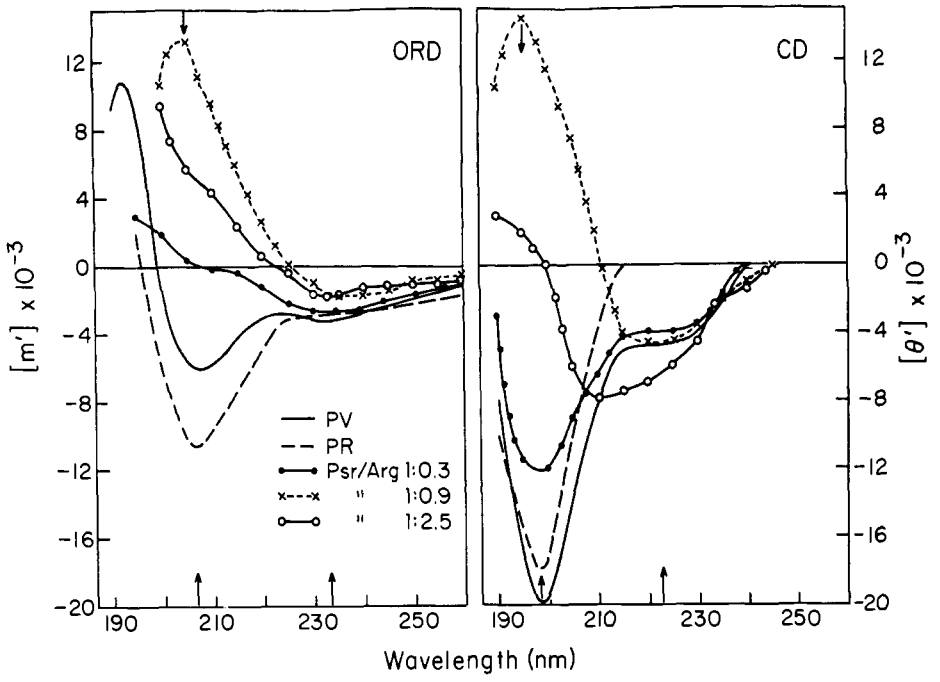

FIGURE 4: Optical rotatory dispersion and circular dichroism of phosvitin, PV, protamine, PR, and phosvitin–protamine complexes at pH 3.8 at various ratios of Psr/Arg.

phate groups, a negative Cotton effect with a trough at 232 nm and two negative dichroic bands at 210 and 222 nm are observed. In this alkaline pH range not all of the ϵ-NH_2 groups of the lysines are dissociated and it may be quite possible that poly-L-lysine at this pH begins to assume an α-helical conformation. The presence of helical regions in the polymeric chain superimposed on the unordered conformation of the phosphoprotein may influence the conformational characteristics of the phosvitin–poly-L-lysine mixtures.

Effect of Ionic Strength. Having demonstrated that addition of poly-L-lysine or protamine to phosvitin at pH 3.8 will result in complex formation accompanied by a transition from the unordered conformation of the constituents to a β structure of the complex, we were interested to investigate the effect of added NaCl on the optical rotatory dispersion patterns. Therefore, the dependence of the complex formation at a 1:1 residue ratio at pH 3.8 on the ionic strength was tested. As shown in Figure 6, the reduced mean residue rotation $[m']_{207}$ of the phosvitin–poly-L-lysine complex is not affected on raising the NaCl concentration from 0 to 0.2 N. However, on raising the salt concentration from

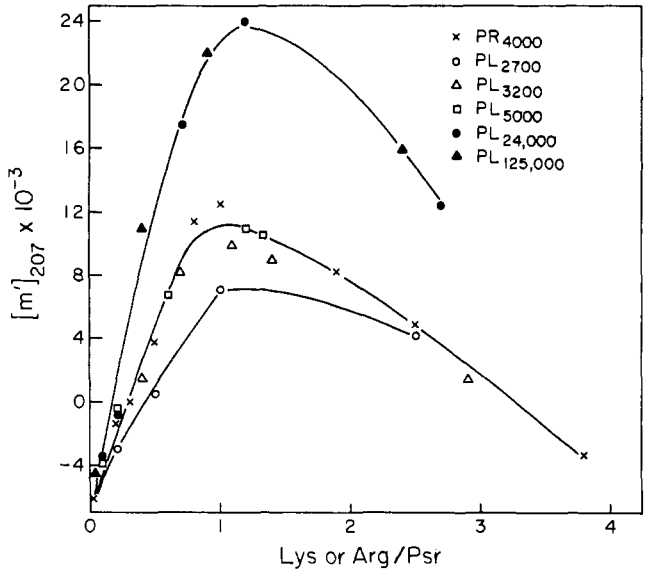

FIGURE 5: Dependence of complex formation of phosvitin with poly-L-lysine, PL, and protamine, PR, on residue ratio, r, and molecular weight of complexing agent.

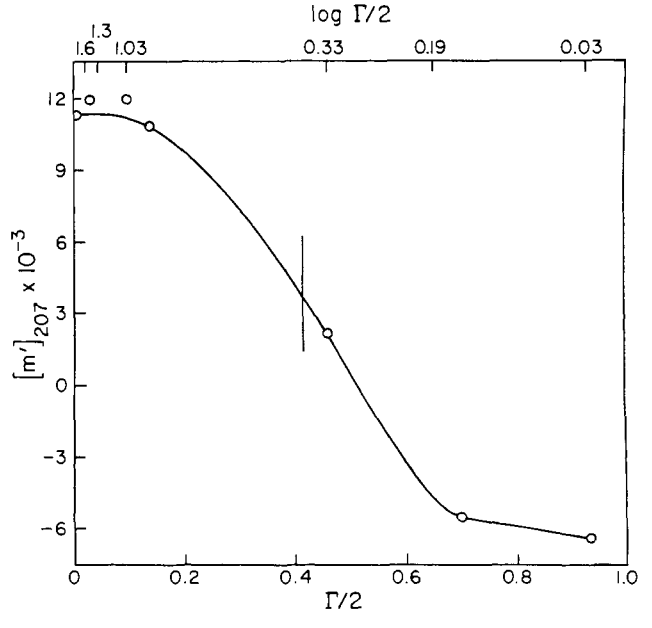

FIGURE 6: Dependence of the reduced mean residue rotation, $[m']_{207}$, of phosvitin–poly-L-lysine complexes on ionic strength; PL_{3200} was used.

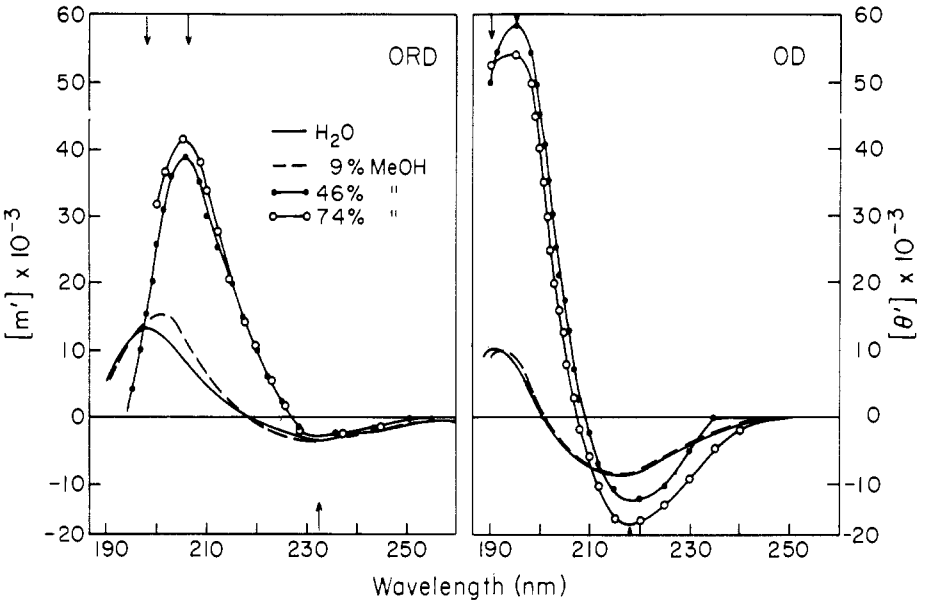

FIGURE 7: Optical rotatory dispersion and circular dichroism of phosvitin–poly-L-lysine complexes in aqueous solution and in methanol of different concentrations at $r = 1$ and at apparent pH 4.6; PL_{3200} was used. The organic solvent was added to the phosvitin solution prior to addition of the complexing agent, *cf.* also Table II.

0.2 to 1.0 N, $[m']_{207}$ decreases from 12,000 to −6400 with a transition midpoint at 0.4 N NaCl. In the same ionic strength interval the reduced mean residue rotation $[m']_{207}$ of phosvitin changes from −6400 to −3500 (Grizzuti and Perlmann, 1970), whereas that of a poly-L-lysine solution varies from −16,200 to −11,500. Similarly, the presence of NaCl also affects complex formation of phosvitin and protamine, although to a lesser degree. Here $[m']_{207}$ decreases from 9000 to −7000 in the same NaCl concentration range with the midpoint of transition at 0.7 N NaCl. It is of interest to note that ionic strength up to $\Gamma/2 = 1.0$ does not affect the reduced mean residue rotation $[m']_{207}$ of a protamine solution at pH 4.0, thus $[m']_{207} = -10{,}400$. We feel, therefore, that the results described in this section fully support the conclusion that an increase of the ionic strength of the solvent diminishes complex formation by repressing the electrostatic interactions between the acidic phosphoprotein and the basic complexing agents.[10]

Effect of Organic Solvents on Phosvitin–Poly-L-lysine Complexes. In a previous investigation (Perlmann and Grizzuti, 1971) it had been found that phosvitin will undergo a transition from an unordered conformation to β structure on transferring the protein from an aqueous solution to an organic solvent. A comparison of the optical rotatory dispersion patterns and circular dichroism spectra of phosvitin–poly-L-lysine complexes at the apparent pH of 4.5 of Figure 7 with those given in Figures 1 and 3 reveals that both the presence and the concentration of an organic solvent enhance the formation of the β form. As the concentration of methanol is increased from 9% to 74% (v/v) methanol, a shift of the optical rotatory dispersion maximum from 202 nm to 205 nm occurs. Simultaneously, $[m']$ increases from 11,500 to 45,000. Similarly, the position of the circular dichroism bands is displaced from 192 nm to 195 nm and $[\theta']$ increases from 10,000 to 55,000. The trough in the optical rotatory dispersion patterns at 232 nm and the negative bands are less affected.

Since addition of methanol or dioxane to a phosvitin–poly-L-lysine complex enhances the formation of β structure, the order of mixing of the constituents has been investigated. As shown in Table II, the amount of β form as judged from the optical rotatory dispersion and circular dichroism parameters is greater if the organic solvent is added prior to the complexing agent. Thus, phosvitin in 46% and 74% (v/v) methanol is already partially in β form (Perlmann and Grizzuti, 1971) which on addition of poly-L-lysine increases further and reaches a maximum.

Precipitation. In an earlier section of this article it was mentioned that addition of poly-L-lysine or protamine to a phosvitin solution in the concentrations used for the optical rotatory dispersion and circular dichroism measurements resulted in opalescence which was most marked at $r = 1$. In an effort to understand what role electrostatic effects may play in the interaction of poly-L-lysine or protamine with phosvitin, a set of precipitation experiments at higher concentrations of the constituents of the mixture was carried out. In the experiments given in Table III the nitrogen and phosphorus distribution found for the precipitated complex and the supernatant, respectively, are listed. It is apparent that at a ratio closest to $r = 1$ precipitation is greatest. Included in this table are also the results obtained in an experiment in which poly-L-lysine was added to a phosvitin solution in methanol to give a final concentration of the organic solvent of 46% (v/v). As shown in Table III, the increased precipitation parallels the enhancement in $[m']_{207}$ and hence also the formation of β structure (*cf.*, Figures 1 and 3).

That complex formation is at its maximum at an equal ratio of oppositely charged amino acid residues is further corroborated by the results of amino acid analyses of the precipitates formed on mixing phosvitin and protamine solutions at pH 3.8 and $r = 1$. In two sets of experiments the micromoles of phosphoserine residues in the precipitates equal those of the arginines of the protamine.

[10] Dissociation of the complexes of phosvitin–poly-L-lysine and phosvitin–protamine at $r = 1$ not only depends on the ionic strength of the solutions but also on the nature of the salt added. For instance, $MgSO_4$ is more effective than NaCl at the same ionic strength.

TABLE II: Dependence of the Optical Rotatory Dispersion and Circular Dichroism of Phosvitin–Poly-L-lysine Complexes in Methanol on the Order of Mixing.[a]

Order of mixing	$[m']_{207}$	$\Delta[m']_{207}$
Phosvitin + poly-L-lysine + 46% methanol	27,000	16,000
Phosvitin + 46% methanol + poly-L-lysine	43,000	
Phosvitin + poly-L-lysine + 74% methanol	24,000	17,000
Phosvitin + 74% methanol + poly-L-lysine	41,000	

[a] Poly-L-lysine, PL_{3200} was used at $r = 1.0$.

TABLE III: Precipitation of Phosvitin by Poly-L-lysine or by Protamine.

Residue ratio, r	Apparent relative concentration (%)			
	Precipitate		Supernatant	
	N_2	P	N_2	P
Psr:Arg	Protamine			
5	30	22	70	78
1	88	87	12	13
0.3	19	36	81	64
Psr:Lys	Poly-L-lysine PL_{3200}			
5	5	6	95	94
0.8	73	66	27	34
0.2	46	66	54	34
0.8	83[a]	97[a]	17[a]	3[a]

[a] Precipitation of phosvitin performed in 46% methanol.

Discussion

The results presented in this article demonstrate that major alterations in molecular structures can occur when the phosphoprotein phosvitin and poly-L-lysine or protamine interact in the pH range of 3.0–5.0. Optical rotatory dispersion and circular dichroism changes indicate a transition from the "unordered" conformation of the unreacted constituents to β structure upon complexing the positively charged poly-L-amino acid or protamine to the acidic protein.

Our data show that under the experimental conditions chosen phosvitin is most strongly complexed at a 1:1 ratio of the phosphoserine residues of the protein to the ϵ-NH_2 groups of poly-L-lysine or to the arginines of protamine. Over the pH range of 3.0–5.0 each phosphoserine residue has lost only *one* proton, thus having only *one* net negative charge. Therefore, it appears that the existence of electrostatic interaction between the amino acid side chains of opposite charge is a fundamental requirement for interaction of phosvitin and poly-L-lysine or protamine. That the complexing process is essentially an electrostatic interaction is further supported by the observation that the stability of the phosvitin–poly-L-lysine or phosvitin–protamine complex is highly sensitive to the ionic strength of the solvent. On raising the NaCl concentration of the medium from 0.2 to 1.0 N, the optical rotatory dispersion patterns and circular dichroism spectra of the complexes revert from those characteristic for β structure to the spectra typical for an unordered conformation. Thus it appears that in salt solution the ionized side-chain charges are shielded by the presence of the "counterions" and the electrostatic interaction is weakened considerably, leading to a dissociation of the complexes.

The fact, however, that no dissociation of the phosvitin–poly-L-lysine or phosvitin–protamine complex occurs at a NaCl concentration below 0.2 N may possibly indicate that other factors, *e.g.*, hydrophobic interactions, may contribute to the stabilization of these complexes.

In a previous communication we have shown that, on transferring phosvitin from a pure aqueous solution to an organic solvent, the dielectric constant of the solution is altered, thus providing a less polar medium which favors formation of β structure (Perlmann and Grizzuti, 1971). Hence, if a transition from the "unordered" conformation to β structure has been initiated, addition of poly-L-lysine increases $[m']$ and $[\theta']$ to values considerably higher than those found for the complexes in an aqueous solvent at $r = 1$. This may be taken as an indication that in aqueous solution only an incomplete conversion of the polypeptide chains present in the complex into the specific structure occurs.

No β structure and complex formation are observed in the pH range of 7.0–10.0 in which each phosphoserine residue is diionic. It appears that the accessibility of the phosphate groups of the phosphoamino acids of phosvitin to the basic groups of the poly-L-lysine is diminished and prevents interaction. A similar pH dependence of complex formation has also been observed by Hammes and Schullery (1968, 1970).

From the foregoing we can conclude that in the pH range of 3.0–5.0 a highly stable complex is formed between the phosphoprotein phosvitin and poly-L-lysine or protamine. These complexes are structurally well defined in that they have β structure and, therefore, are different from the conformation of the constituents alone. These findings may possibly have biological significance since it is physically feasible that in the egg yolk phosvitin may occur as aggregates by association with a basic protein or peptide, resulting in a highly stable but conformationally altered phosvitin. Thus, such phosvitin complexes may be of importance in an attempt to understand the structure and biological role of naturally occurring phosvitin aggregates.

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Pulmonary Alveolar Macrophage. Oxidative Metabolism of Isolated Cells and Mitochondria and Effect of Cadmium Ion on Electron- and Energy-Transfer Reactions*

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ABSTRACT: Pulmonary alveolar macrophages (PAMs), obtained by endobronchial lavage of sheep lungs, manifest an endogenous respiration of 9 nmoles of O_2/mg of protein per min at 30°. A phenomenon similar to the Crabtree effect is produced if glucose, oligomycin, or Cd^{2+} is added to a cell suspension and the phenomenon is removed in the presence of an uncoupler or Wurster's blue. Isolated PAM mitochondria oxidize a number of substrates. Typical rates are 67 and 91 nmoles of O_2 per mg of protein per min at 30°, respectively, for α-oxoglutarate and succinate as substrates. These mitochondria characteristically utilize α-glycerophosphate (74 nmoles of O_2/mg of protein per min) but not β-hydroxybutyrate (6 nmoles of O_2/mg of protein per min). Substrate oxidation is completely inhibited by 50 μM Cd^{2+} and also by classical inhibitors of respiration. Cd^{2+} inhibits substrate oxidation presumably by interfering with dehydrogenases of mitochondrial respiratory chain. Isolated PAM mitochondria carry out coupled phosphorylation. The ADP:O ratios are >1 (approaching 2) for flavin-linked substrates and >2 (approaching 3) for pyridine nucleotide linked substrates. This oxidative phosphorylation is sensitive to 2,4-dinitrophenol, oligomycin, and Cd^{2+}. A concentration of 5–10 μM Cd^{2+} completely abolishes coupled phosphorylation and respiratory control. Cd^{2+} also inhibits adenosine triphosphatase activity of PAM mitochondria and plasma membrane.

The pulmonary alveolar macrophages (PAMs)[1] have specialized properties in common with other cells of the mononuclear phagocyte system, such as amoeboid motility, endocytic processes, and bactericidal activities. Like other mononuclear phagocytes, they are probably derived from marrow stem cells and circulating monocytes (Virolainen, 1968; Brunstetter *et al.*, 1971). However, PAMs have features which are unique compared to cells of most other organs. For example, they (along with other pulmonary cells) reside in high oxygen tensions of the alveolar gas (90–130 mm; *cf.* West, 1970).

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[1] Abbreviations used are: PAM, pulmonary alveolar macrophage; Wurster's blue, semiquindiamine radical of tetramethyl-*p*-phenylenediamine (*cf.* Mustafa *et al.*, 1968); PCP, pentachlorophenol; DNP, 2,4-dinitrophenol; ATPase, adenosine triphosphatase; ADP:O, a ratio of the number of moles of adenosine diphosphate esterified with inorganic phosphate to the number of atoms of oxygen consumed by the system.