

Polarographic behavior of Cd^{2+} and Zn^{2+} –PGA complexes. Polarographic and circular dichroism spectroscopic studies on the Cd^{2+} and/or Zn^{2+} –poly(α -L-glutamic acid) complex in aqueous solution

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Abstract

The helix-coil transition of poly(α -L-glutamic acid) (PGA) induced by Cd^{2+} and/or Zn^{2+} was investigated by direct current (d.c.) polarography and circular dichroism (CD) spectroscopy as a function of pH and the mixing ratio of glutamic acid residue to Cd^{2+} and/or Zn^{2+} . The diffusion current for Cd^{2+} or Zn^{2+} declined sharply in the pH range of *c.* 4–7 due to the formation of the metal ion–PGA helical complex. The helix contents of PGA, estimated by the decrease in polarographic current, are in good agreement with results obtained by circular dichroism in the neutral pH region. The degree of helicity induced by either of these two ions can also be determined independently by polarography. The size of the complex was calculated by the Ilkovič equation and depends strongly on the mixing ratio of glutamic acid to cation. In the neutral pH region, the relationship between the half-wave potentials and the concentration of PGA indicates that two peptide residues coordinate approximately to one Cd^{2+} .

Introduction

It is well known that the helix-coil transition of polypeptide can be induced by a change in the composition of the solvent, temperature or by the addition of metal ion [1–3].

The metal ion induced transition has been investigated extensively by many techniques such as NMR, ESR, IR and CD spectroscopies, and equilibrium dialysis [4–7]. Circular dichroism (CD) spectroscopy in the peptide backbone region, 180–240 nm, has been the primary technique employed to follow change in the secondary structure of PGA [1]. However, CD only measures the helix content averaged over the entire molecule. The information based on CD for this transition, therefore, is not enough for a quantitative discussion. In d.c. polarography, a metal ion can be regarded as a probe which surveys this transition through the behavior of the metal ions attached to the polymer. The shift in the half-wave potential, the diffusion coefficient or the decrease in the reduction wave provides information on the number of polypeptide residues coordinated to a metal ion, the size of the macro-

molecular complex and the helix content, respectively.

Furthermore, the polarographic technique is efficient in providing information on each metal ion's contribution to this transition even when several types of ions co-exist. CD does not provide information on the effect of each type of ligand ion on this transition; only the total effect, from all of the ions, obtainable. Here we investigate the helix-coil transition of PGA solutions containing Cd^{2+} and/or Zn^{2+} which are known to induce the helical structure of PGA [8, 9] and provide well defined polarographic reduction waves.

Experimental

Materials

Sodium poly(α -L-glutamic acid) was provided by the Peptide Institute, Inc., Osaka (degree of polymerization, $DP=175$). Stock solutions of Cd^{2+} and Zn^{2+} were prepared from reagent grade $3\text{CdSO}_4 \cdot 8\text{H}_2\text{O}$ and $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (Wako Pure Chemical Industries, Japan), respectively. The pH of all solutions was adjusted with 0.1 mol dm^{-3} HCl or 0.1 mol dm^{-3} NaOH. The number of ions to PGA was defined as $f = [\text{PGA}]/[\text{Cd}^{2+}]$ or $[\text{Zn}^{2+}]$. The value of f was controlled by adding various

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amounts of PGA to a constant concentration of Cd^{2+} or Zn^{2+} in all measurements*. Reagent grade sodium perchlorate was used as a supporting electrolyte at a concentration of 0.01 mol dm^{-3} .

Apparatus

Polarographic limiting currents were measured using a Yanagimoto model P-8 polarograph with a saturated calomel electrode (SCE) as reference electrode. The dropping mercury electrode had the following characteristics: $m = 0.863 \text{ mg/s}$, $t = 1.28 \text{ s}$ (a forced drop time) in water with a mercury column height of 70 cm. These parameters were obtained with an open circuit at $25 \pm 0.05 \text{ }^\circ\text{C}$. The solutions were de-aerated with pure nitrogen for 5 min and left 1 min before measurements were taken. CD measurements were carried out on a Jasco J-500A spectropolarimeter (Japan Spectroscopic Co., Ltd.) equipped with a DP-500 data processor.

Effect of Hg column height on the limiting current

The effect of the mercury column height on the polarograms, for both helical and random coiled PGA solutions containing Cd^{2+} and/or Zn^{2+} , was studied as a function of Hg column height (50–80 cm). There were linear relationships between the limiting current for these solutions and the square root of the mercury column height. The limiting currents for Cd^{2+} and Zn^{2+} are, therefore, entirely diffusion controlled at a concentration of $0.01 \text{ mol dm}^{-3} \text{ NaClO}_4$.

Results and discussion

Polarograms and CD spectra for solutions of PGA- Cd^{2+} and/or Zn^{2+}

Figure 1 shows the effects of pH variation of mixed polarograms for $5 \times 10^{-4} \text{ mol dm}^{-3} \text{ Cd}^{2+}$ plus $5 \times 10^{-4} \text{ mol dm}^{-3} \text{ Zn}^{2+}$ in the presence and absence of PGA. The wave with a half-wave potential of -0.76 V versus SCE is due to the reduction of Cd^{2+} , and that of -1.40 V versus SCE is due to the reduction of Zn^{2+} . As the pH increases from 3.32 to 6.70, the diffusion current (i_d) for each ion decreases steadily and reaches an almost constant value. In the absence of PGA, however, the diffusion current for each ion remains unchanged in the same pH region. Figure 2 shows the CD spectra of these solutions. In the neutral or alkaline pH region, PGA is known to be in a random coil state [10], having a spectrum with a very weak negative band at 238 nm, a weak positive band at 215 nm and a very strong

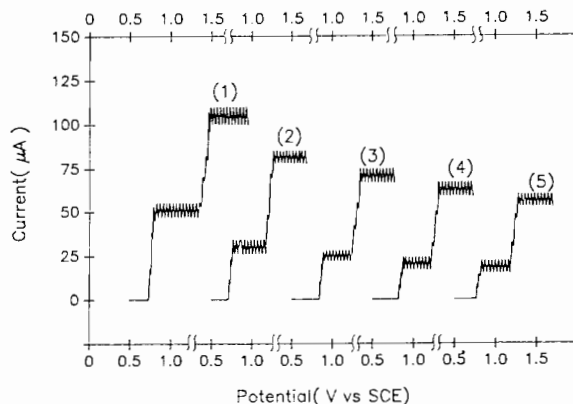


Fig. 1. The pH dependence of polarograms for Cd^{2+} and Zn^{2+} -PGA solution at $f=3.25$ (final f for each ion: $f=0.5$ [PGA]/[Cd^{2+}] or [Zn^{2+}]), [Cd^{2+}]= $5 \times 10^{-4} \text{ mol dm}^{-3}$, [Zn^{2+}]= $5 \times 10^{-4} \text{ mol dm}^{-3}$. (1) pH=3.32 without PGA, (2) pH=4.85, (3) pH=5.71, (4) pH=6.21, (5) pH=6.70.

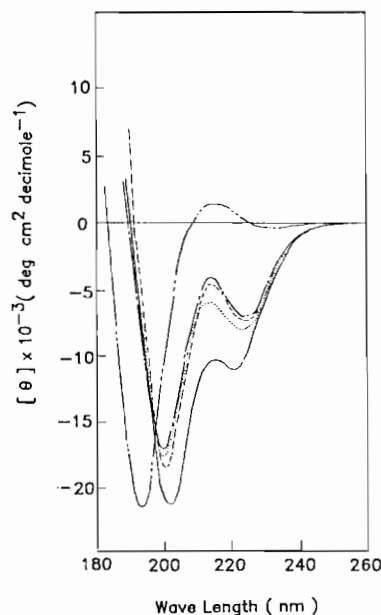


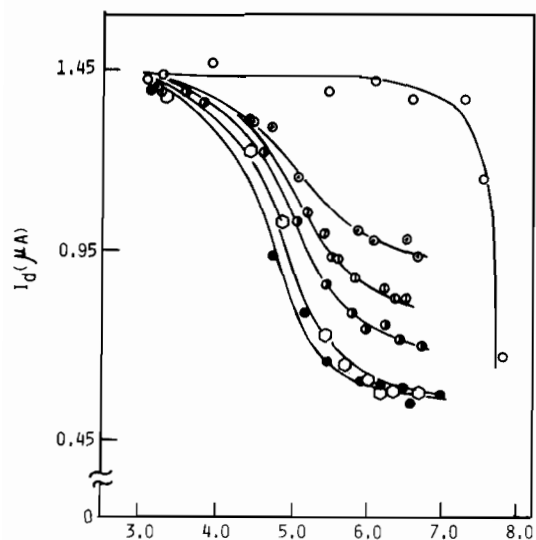
Fig. 2. The pH dependence of CD spectra for Cd^{2+} and Zn^{2+} -PGA solutions at $f=3.25$ (final f for each ion). —, pH=4.85; ·····, pH=5.71; - · - ·, pH=6.21; ---, pH=6.70; - - - -, neither Cd^{2+} nor Zn^{2+} presents at pH=7.3.

negative band at 198 nm. In the acidic pH region, PGA is in a helical state [10], having a spectrum with negative bands at 222 and 208 nm, and a positive band at 195 nm. The CD spectra of all Cd^{2+} , Zn^{2+} -PGA solutions in the pH range of 3.32 to 6.70 clearly show a helix pattern and are essentially the same as those of PGA obtained without any metal ion in the acidic pH region. This result indicates that Cd^{2+} and Zn^{2+} play an important role in the formation of the α -helix structure of PGA in the neutral pH region.

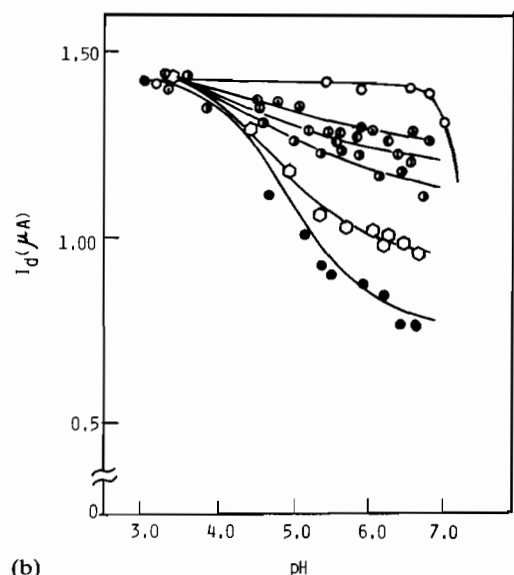
*The value of f for Cd^{2+} or Zn^{2+} in PGA- Cd^{2+} plus Zn^{2+} solutions was calculated on the assumption that PGA is equally allotted between Cd^{2+} and Zn^{2+} . Hence, final f for each ion is given as the ratio of [half the amount of PGA added]/[Cd^{2+}] or [Zn^{2+}] in the case of co-existence of both ions.

Polarographic diffusion current and molecular ellipticity at 222 nm for the solutions of PGA-Cd²⁺ and PGA-Zn²⁺

Figure 3(a) and (b) shows the relationship between the decomposed diffusion currents for Cd²⁺, Zn²⁺ and pH, at various values of *f*. In the absence of PGA, the diffusion currents for both ions remain unchanged in the pH range from c. 3.0 to 7.0. Above pH 7.0, the diffusion current decreases drastically due to the precipitation of Zn(OH)₂ or Cd(OH)₂. In contrast, in the presence of PGA the diffusion currents, at various



(a)



(b)

Fig. 3. The relationships between the decomposed diffusion currents (i_d) and pH for Cd²⁺ and that for Zn²⁺ at various values of *f* (final *f* for each ion: $f = 0.5 [\text{PGA}]/[\text{Cd}^{2+}]$ or $[\text{Zn}^{2+}]$). (a) PGA-Cd²⁺, (b) PGA-Zn²⁺, $[\text{Cd}^{2+}] = 5 \times 10^{-4} \text{ mol dm}^{-3}$, $[\text{Zn}^{2+}] = 5 \times 10^{-4} \text{ mol dm}^{-3}$. ○, $f = 0$; ⊙, $f = 0.65$; ⊕, $f = 0.98$; ⊗, $f = 1.30$; ⊚, $f = 1.95$; ◊, $f = 3.25$; ●, $f = 6.50$.

values of *f*, decrease gradually from pH c. 4.0. The current decreases with *f* especially in the neutral pH region. The effects of pH on the magnitude of ellipticity $[\theta]$ at 222 nm (Figs. 4 and 5) and on the diffusion currents were also investigated on the additionally

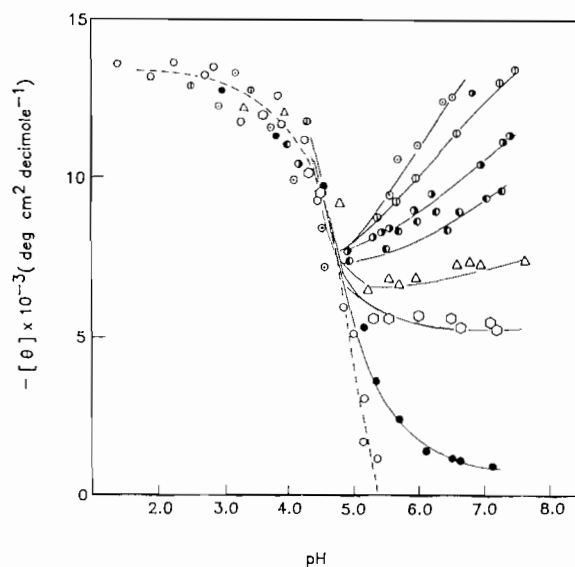


Fig. 4. The relationship between molecular ellipticity at 222 nm and pH for Cd²⁺-PGA solutions at various values of *f*. ○, $f = 0$ (dashed curve); ⊙, $f = 0.65$; ⊕, $f = 0.98$; ⊗, $f = 1.30$; ⊚, $f = 1.95$; △, $f = 2.60$; ◊, $f = 3.25$; ●, $f = 6.50$.

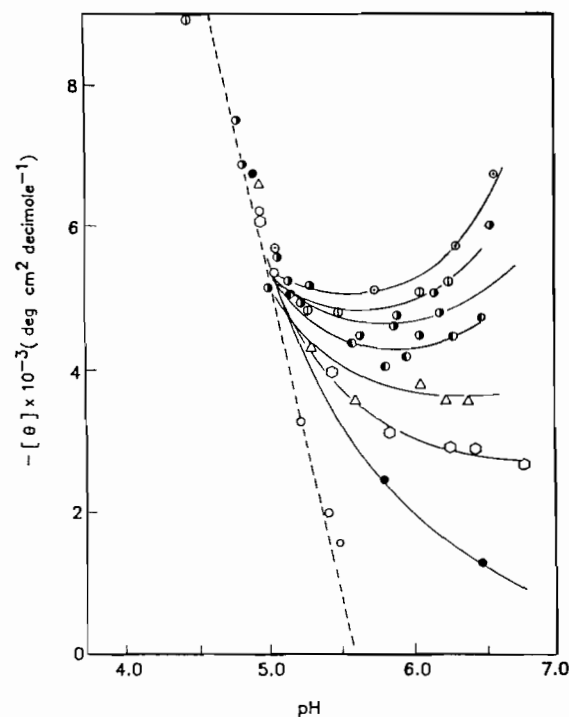


Fig. 5. The relationship between molecular ellipticity at 222 nm and pH for Zn²⁺-PGA solutions at various values of *f*. ○, $f = 0$ (dashed curve); ⊙, $f = 0.65$; ⊕, $f = 0.98$; ⊗, $f = 1.30$; ⊚, $f = 1.95$; △, $f = 2.60$; ◊, $f = 3.25$; ●, $f = 6.50$.

prepared solutions containing $5 \times 10^{-4} \text{ mol dm}^{-3} \text{ Cd}^{2+}$ or Zn^{2+} with various amounts of PGA. The decrease in the current with pH for these solutions is the same as that obtained through the decomposition of the mixed polarograms of Cd^{2+} and Zn^{2+} . This equivalence suggests that PGA is complexed independently with Cd^{2+} and Zn^{2+} to form the α -helix structure. The magnitude of the molecular ellipticity $[\theta]$ at 222 nm is regarded as an indicator of helix content [11]. In Figs. 4 and 5, the helix content of PGA, in the absence of both ions, decreases linearly with an increase in pH from 4.0 to 5.5. In the acidic pH region, the ionization of the carboxylate group is suppressed to maintain the helix structure by hydrogen bonding between NH and CO groups on the polypeptide chain. As the pH increases, the proton on the carboxylate groups will be replaced gradually by Cd^{2+} and/or Zn^{2+} . This corresponds to the dashed curve without Cd^{2+} or Zn^{2+} which begins to deviate from that with Cd^{2+} or Zn^{2+} at pH c. 4.5 and reaches approximately 0 at pH 5.5. In this pH region (c. 4.5–5.5), helix formation is sustained by the co-existence of H^+ and Cd^{2+} which are randomly distributed on the carboxylate groups. Below pH 4.5, the dashed curve without ion is superimposed on that with Cd^{2+} or Zn^{2+} at various f values.

Therefore, below pH 4.5 neither of the two ions contributes to the helix formation of PGA, even though the diffusion currents of both ions in the solutions with PGA are always smaller than that without PGA in the corresponding pH region. This implies that Zn^{2+} and/or Cd^{2+} interact with the carboxylate group in the side chain even in the acidic pH region [4]. Above pH 5.5, PGA forms a helix structure, not by H^+ but by Cd^{2+} or Zn^{2+} which directly correlates with the decrease in i_d . As the pH increases from 5.5 to 7.0, the interaction between PGA and Cd^{2+} or Zn^{2+} was found to become more effective especially at small f . The magnitude of $[\theta]$ at 222 nm for the solutions with large f is almost constant or decreases as the pH increases from 5.5 to 7.0, however that for small f becomes larger. At a constant concentration of Cd^{2+} or Zn^{2+} the relative helix content of PGA becomes smaller with the addition of PGA, since the number of Cd^{2+} or Zn^{2+} attached to PGA is not sufficient. The diffusion currents for small f still maintain a tendency to decrease slightly as the pH increases above 6.0. This corresponds to the increase in the magnitude of $[\theta]$ at 222 nm at small f . Therefore, at pH values higher than 5.5 the relationship between the diffusion current and pH corresponds to that between the helix content by CD and pH at any f value.

Distortion of CD spectra and further transition from helix state

When large amounts of Cd^{2+} compared to PGA residue are present the CD spectra begin to be distorted

such that $[\theta]$ at 208 nm decreases in magnitude and the band at 222 nm is red-shifted to c. 226–228 nm in the neutral pH region. These spectral changes, shown in Fig. 6, might be associated with the presence of a helix aggregate [12, 13]. This CD change from a helix pattern to a further conformational transition is accompanied by the appearance of turbidity of the solution and is also observed for the PGA solution with co-existence of Zn^{2+} and Cd^{2+} but not for the Zn^{2+} -PGA solution. The negative charge on the side chain carboxylate group will disappear as a result of the coordination between the carboxylate and Cd^{2+} . However, other bivalent cations, such as Ba^{2+} , Ca^{2+} or Mg^{2+} [14, 15] do not induce any kind of secondary structure including this further transition. This means that interaction between Cd^{2+} and PGA is rather specific and that the transition depends on the nature of ion. At a pH lower than 3.0, the diffusion current for Cd^{2+} , Zn^{2+} with PGA is the same as that without PGA at any f , which indicates that Cd^{2+} and Zn^{2+} do not bind to any portion of PGA and these ions are free to diffuse to the electrode. The helix content based on the CD spectrum also remains constant. PGA, Cd^{2+} and Zn^{2+} are, therefore, completely independent in this pH region. This low pH, regardless of the presence and absence of Cd^{2+} and Zn^{2+} , caused the turbidity of the solution due to the aggregation of the helix [14]. This aggregation of PGA also caused the slight decrease in magnitude

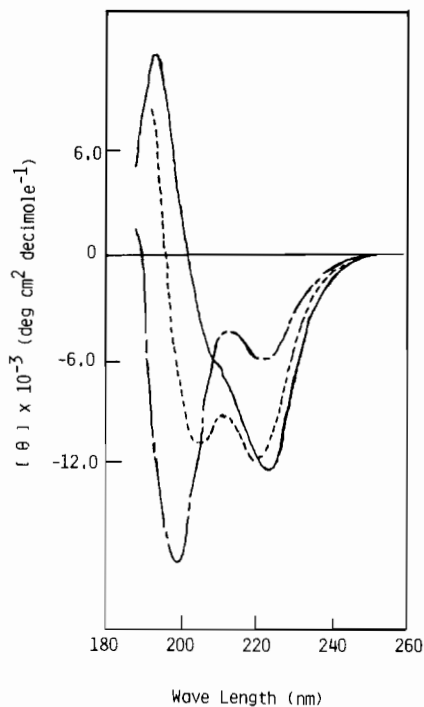


Fig. 6. CD spectra for PGA- Cd^{2+} solutions at pH 6.5. $[\text{Cd}^{2+}] = 5 \times 10^{-4} \text{ mol dm}^{-3}$. —, $f = 1.02$; ---, $f = 1.30$; - · -, $f = 3.20$.

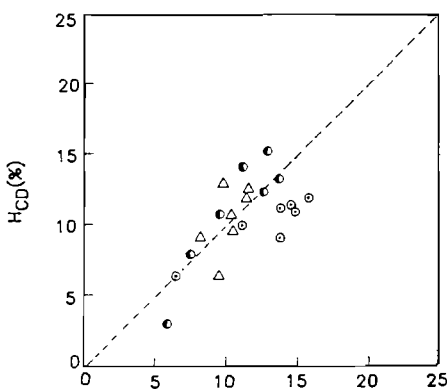
at 208 nm in the CD spectrum like the one in Fig. 6. However, it is not clear from the CD spectrum change only whether further transitions from the helix structure are essentially different from each other at low pH and at neutral pH with the presence of Cd^{2+} . Further study is needed to obtain this information.

Determination of helix content

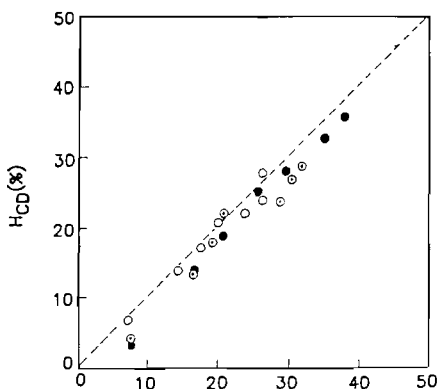
Figure 7(a) and (b) compares the helix content for solutions of PGA- Cd^{2+} and PGA- Zn^{2+} as determined by CD, H_{CD} and polarography, H_{pola} at representative pH values. These techniques all give similar helix content values. This calculation was carried out as follows [3, 16]

$$H_{\text{CD}} = \frac{\text{magnitude of } [\theta] \text{ at } 222 \text{ nm}}{38000} \times 100 \quad (1)$$

$$H_{\text{pola}} = \frac{i_t - i_{\text{red}}}{i_t} \times 1/f \times 100 \quad (2)$$



(a) $H_{\text{pola.}}(\%)$



(b) $H_{\text{pola.}}(\%)$

Fig. 7. The relationship between helix content determined by CD, H_{CD} , and that by polarography, H_{pola} , for PGA- Cd^{2+} and PGA- Zn^{2+} solutions. (a) PGA- Cd^{2+} , (b) PGA- Zn^{2+} . \circ , pH=5.50; \triangle , pH=5.75; \odot , pH=6.00; \bullet , pH=6.50; \bullet , pH=7.00.

where i_t denotes the diffusion current without PGA, and i_{red} the reduced current in the presence of PGA. $1/f$ is a normalizing factor for each solution eliminating the dependence on the concentration of PGA. The helix content due to Cd^{2+} or Zn^{2+} could be calculated separately even for this solution. The total helix content is easily obtained by either CD or a total decrease in diffusion current. Each ion's contribution to the total helix content could be estimated by comparing the ratio of the decrease in current for Cd^{2+} or Zn^{2+} to that for solutions without PGA. For example, at pH=6.0 and $f=1.30$ the helix content in PGA is 24% in the presence of both ions, and the ratio of the helix content due to Cd^{2+} and Zn^{2+} was 0.54 and 0.44, respectively, when their relative concentration was 1:1. This result indicates that Cd^{2+} is more effective than Zn^{2+} in inducing the helix structure of PGA. Although in a limited f and pH region the diffusion current is useful for the determination of helix content, the method proposed here could provide information on the helix content not only for PGA-single metal ion systems, but for systems which contain both Zn^{2+} and Cd^{2+} (possibly other ions as well), and also at any ratio of $[\text{Zn}^{2+}]/[\text{Cd}^{2+}]$.

Estimation of the size of the macromolecular complex

The size of the macromolecular complexes of PGA- Cd^{2+} and/or Zn^{2+} can be estimated with the Stokes-Einstein equation under the assumption that the complex can be regarded as a rigid sphere molecule [17]:

$$r = \frac{1}{6\pi\eta} \frac{RT}{N} \frac{1}{D_{\text{complex}}} \quad (3)$$

where r is the radius of the complex; R is the gas constant; N is Avogadro's number; D_{complex} , the diffusion coefficient for the complex ion; T , the absolute temperature (298 K); η , the viscosity of water (0.8904cP at 25 °C) [18]. In the Ilkovič equation, D_{complex} is expressed as follows:

$$D_{\text{complex}}^{1/2} = \frac{i_{d, \text{complex}}}{607 n C m^{2/3} t^{1/6}} \quad (4)$$

where n is the number of electrons transferred, C is the concentration of the metal ion (mmol dm^{-3}) and $m^{2/3} t^{1/6}$ ($=0.88445$) is the capillary constant. When both free metal ions and those attached to PGA co-exist in a solution, the observed diffusion current for Cd^{2+} or Zn^{2+} becomes an average value in proportion to the ratio of existence as follows:

$$\frac{1}{i_{d, \text{av}}} = \frac{1}{i_{d, \text{free}}} \times (1-H) + \frac{1}{i_{d, \text{complex}}} \times H \quad (5)$$

TABLE 1. Helix contents, diffusion coefficients and radii for PGA-Cd²⁺ and/or Zn²⁺ complexes at neutral pH

Sample	pH	<i>f</i>	Helix (%)	<i>i</i> _{d, complex} (μA)	<i>D</i> _{complex} × 10 ⁷ (cm ² /s)	<i>D</i> _{av, complex, Cd, Zn} × 10 ⁷ (cm ² /s)	<i>r</i> (Å)
PGA-Cd ²⁺	6.0	0.98	34.4	0.59	12.13 ^a		194.8
PGA-Cd ²⁺	6.0	1.30	32.0	0.45	7.03 ^a		336.3
PGA-Cd ²⁺	6.0	3.25	16.4	0.18	1.16 ^a		2037.8
PGA-Cd ²⁺	6.0	6.50	8.0	0.10	0.34 ^a		6952.0
PGA-Zn ²⁺	6.0	0.98	17.1	0.28	2.64 ^b		895.4
PGA-Zn ²⁺	6.0	1.30	15.3	0.23	1.79 ^b		1320.6
PGA-Zn ²⁺	6.0	3.25	10.2	0.10	0.38 ^b		6220.8
PGA-Zn ²⁺	6.0	6.50	6.7	0.05	0.08 ^b		29548.8
PGA-Cd ²⁺ + Zn ²⁺	6.0	0.98	30.0			7.23	326.9
PGA-Cd ²⁺ + Zn ²⁺	6.0	1.30	23.6			4.42	534.8
PGA-Cd ²⁺ + Zn ²⁺	6.0	3.25	17.1			0.77	3077.8
PGA-Cd ²⁺ + Zn ²⁺	6.0	6.50	8.5			0.21	11082.5
Cd ²⁺	3.0			1.42	69.9(70.0 ^c)		
Zn ²⁺	3.0			1.48	70.1(70.0 ^c)		

In eqn. (5), *i*_{d, free} for Cd²⁺ and Zn²⁺ is 1.42 (μA) and 1.40 (μA), respectively.

^aCalculated from Fig. 4 for PGA-Cd²⁺ solution. ^bCalculated from Fig. 5 for PGA-Zn²⁺ solution. ^cRef. 19.

where *i*_{d, av} is the observed diffusion current for the solution of PGA-metal ion at any pH and *f*; *i*_{d, free} and *i*_{d, complex} are the diffusion currents for the free metal ion and the PGA-Cd²⁺ and/or Zn²⁺ macromolecular complex, respectively. *H* is the ratio of helix content between 0 and 1. For solutions containing complexes of both PGA-Cd²⁺ and Zn²⁺, the apparent diffusion coefficient (*D*_{av, complex, Cd, Zn}) averaged over both ions would be expressed at a certain *f* and pH as follows:

$$D_{av, complex, Cd, Zn} = D_{complex, Cd} \times A + D_{complex, Zn} \times B \quad (6)$$

$$A + B = 1 \quad (7)$$

where *A* and *B* are the fractional concentration of each ion.

The size of the complexes of PGA-Cd²⁺ and Zn²⁺ can be obtained using eqns. (3)–(7). Table 1 shows the diffusion coefficients and radii of the complexes and other pertinent data. Maeda *et al.* [13] determined the radius of the aggregated PGA (*DP* = 475) at pH 6.7 in the presence of Cd²⁺, Zn²⁺ and Co²⁺ by light scattering and filtration. The radius of the aggregated PGA was determined to lie between 300 and 2000 Å. Taking into account the difference in *DP* and the aggregation of the samples this value compares favorably with those presented in Table 1.

Coordination number of Cd²⁺-PGA complex in the random coil pH region

If the reaction on the mercury electrode is reversible, the coordination number, *j*, of a complex can be obtained by eqn. (8) [19]:

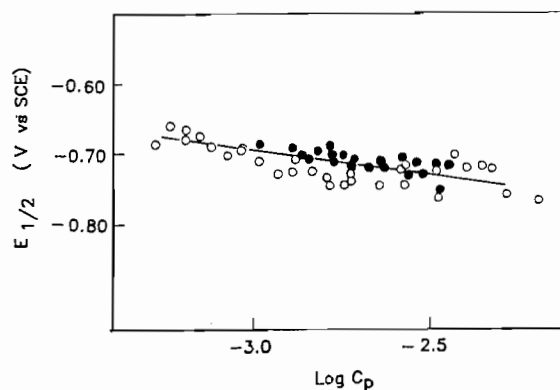


Fig. 8. The relationship between the half-wave potential for Cd²⁺ and the concentration of PGA at pH 6.5. [Cd²⁺] = 5 × 10⁻⁴ mol dm⁻³. O, PGA-Cd²⁺ and Zn²⁺; ●, PGA-Cd²⁺.

$$\frac{d(E_{1/2})}{d(\log C_p)} = \frac{-0.0591 \times j}{n} \quad (8)$$

where *C*_p is the concentration of the free ligand (PGA), which can be approximated by the total concentration of PGA. The value of *E*_{3/4} – *E*_{1/4} for Cd²⁺ is 0.027 V, while that for Zn²⁺ is 0.018 V, instead of 0.028 V as required for a two electron reversible reaction. The plot of *E*_{1/2} for Cd²⁺, in the Cd²⁺ plus Zn²⁺ solution, against log *C*_p at pH 6.5 is shown in Fig. 8. At this concentration of PGA, *j* was 2.03, implying that approximately two peptide residues attach to one Cd²⁺: electrostatically Cd²⁺ is supposed to coordinate to two adjacent carboxylate groups in this pH region. Masujima and Yamaoka [20] also pointed out the existence of this type of complex in the PGA-Cu²⁺ system using UV spectroscopy and the dialysis method.

Conclusions

The polarographic diffusion current is a parameter sensitive to the change in conformation of PGA in the neutral pH region. It gives information not only on the total helix content of PGA summed over that due to a different kind of metal ion, but on the helix content due to each ion independently. It is impossible to distinguish the contribution of each individual type of ion from the CD spectrum. The macromolecular size of the complex can be estimated from the decrease in the diffusion current using the reducible metal ion as a marker or probe. Therefore, the application of polarography provides information not available through other spectroscopic methods, such as CD, NMR and ESR.

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