

# Preparation and properties of poly( $N^\epsilon, N^\epsilon$ -dicarboxymethyl-L-lysine)

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A new ampholytic homopolypeptide, poly( $N^\epsilon, N^\epsilon$ -dicarboxy-methyl-L-lysine), which has one tertiary amino and two carboxyl groups in the side chain has been derived from a hydrochloride salt of poly(L-lysine). The polymer in aqueous solution seems to be in the coil form with locally extended structure (LES) at neutral pH. In both the acidic and alkaline regions, the molar ellipticity of the polymer changes as a result of change in net charge on the side chain. The conformational changes may be from the coil with LES to other coiled forms. 5–7 M NaClO<sub>4</sub> and 80% aqueous methanol induce the  $\alpha$ -helix in the polymer at neutral pH. Divalent cations, Cu<sup>2+</sup> and Ca<sup>2+</sup>, do not induce any remarkably ordered structures such as  $\alpha$ -helix or  $\beta$ -structure in the polymer in aqueous solution at any pH. Ultraviolet absorption studies show an absorption peak of the polymer–Cu<sup>2+</sup> complex near 240 nm. Dependence of the peak intensity on pH at various  $q$  values ( $q = [\text{Cu}^{2+}] / [\text{residue}]$ ) indicates the two steps of the complex formation. At  $q$  less than 0.64, the formation is described only with the first step. An average coordination number for Cu<sup>2+</sup> at the first step was calculated to be about 2 by the method of Mandel and Leyte. The association constant of Cu<sup>2+</sup> with the residue at the step was determined from the absorption data to be far smaller than that for the Cu<sup>2+</sup>–EDTA complex. The second step of the formation occurs in the case of large  $q$  but the absorption data for the second step cannot be obtained exactly due to precipitation.

## INTRODUCTION

Many electrolytic poly( $\alpha$ -amino acids) have been used to elucidate the mechanism of protein conformational changes with pH or ionic strength<sup>1,2</sup>. Studies on pH-induced and counterion-induced conformational changes of acidic and basic poly( $\alpha$ -amino acids) in aqueous media are well known<sup>3–6</sup>. Research in this field has recently been focussed on the conformational changes in poly( $\alpha$ -amino acids) with hydrophobic long side chains: e.g. various derivatives of poly(L-lysine) (PPL) and poly(L-ornithine) (PLO)<sup>7,8</sup>.

Many polyelectrolyte researchers have studied the influences of local charge density on polyelectrolyte properties. The copolymers of maleic acid and non-electrolyte vinyl monomers have been used in such studies<sup>9–11</sup>. The secondary carboxyl groups of maleic acid in the maleic acid copolymers have a considerably larger apparent pK than that of primary groups. This shows the importance of interaction between two ionizable groups in maleic acid. However, few studies on electrolytic homopolypeptides with two or three ionizable groups in the monomer have been reported.

In the present study, we prepared a new electrolytic poly( $\alpha$ -amino acid), poly( $N^\epsilon, N^\epsilon$ -dicarboxymethyl-L-lysine) (PDCML), which has one tertiary amino and two carboxyl groups in the side chain – an ampholytic homopolypeptide. The chemical structure of the side chain is very similar to one half of EDTA (ethylenediamine tetraacetic acid).

The present study was carried out from the following points of view:

(1) how does conformation vary with pH? Does the conformation change in an ampholytic fashion?

(2) how does net charge change as pH changes?

(3) does it form complexes with divalent cations such as Cu<sup>2+</sup>? Is the structure of the complex with Cu<sup>2+</sup> similar to that of EDTA–Cu<sup>2+</sup> complex? Do the divalent cations induce the conformational change in the polymer?

The conformation was studied by circular dichroism (c.d.) measurements in the far ultra-violet region. The net charge was estimated by pH titration. Interaction with the divalent cations was studied by the methods of ultraviolet absorption and pH titration.

## EXPERIMENTAL

### Materials

The polymer PDCML was prepared from PLL, which was made by the usual  $N$ -carboxyanhydride method with an average degree of polymerization of 1500. A hydrochloride salt of PLL ( $3.0 \times 10^{-3}$  monomol) was dissolved in 150 ml of aqueous NaOH (0.02 N), into which 11.175 g ( $60 \times 10^{-3}$  mol) of iodoacetic acid, 30 ml of aqueous NaOH (2 N) and 60 ml of aqueous NaCl (4M) were slowly added with stirring. After homogenizing the solution, aqueous NaOH (2 N) was added dropwise at room temperature so as to retain the solution at pH 10. After complete evolution of hydrogen iodide, the mixture was stirred in a closed vessel for 1 day. It was then dialysed against distilled water. The polymer was freeze-dried and stored in a desiccator. Elemental analysis of the product showed almost complete dicarboxymethylation of  $\epsilon$ -amino groups. Further analysis by an amino-acid analyser demonstrated two minor species in the side chains, which were mono- and tricarboxymethylated

## RESULTS

## Conformation of PDCML in aqueous solution

An example of c.d. spectra of PDCML in aqueous solution at neutral pH is shown over a wavelength range of 210–250 nm in Figure 1. It has a negative peak at about 235 nm and a positive one at 217 nm and it is very similar to the charged poly(glutamic acid) (PGA) spectrum. PDCML at neutral pH may be in the coil form with the locally extended structure (*LES*), according to Tiffany and Krimm<sup>16,17</sup>. Although PGA is in the  $\alpha$ -helical form in the pH region<sup>2,5-4</sup>, the PDCML spectra do not indicate the  $\alpha$ -helical or  $\beta$ -form structure in the same pH region – it is rather in the coil form or in the coil with partly *LES* even in this region. Values of  $[\theta]_{217}$  and  $[\theta]_{235}$  are plotted against pH at 25.0°C in Figure 2, where the conformation is found to change in an ampholytic fashion. That PDCML conformation at the neutral pH is the coil with *LES* is supported by the temperature dependence of the molar ellipticity in a pH region of 4–9 shown in Figure 3<sup>17</sup>. The conformational changes found in both the acidic and alkaline regions may be from the coil with *LES* to the other random coils.

## Changes in the net charge of residue

The isoionic solution of the polymer has a pH value of about 3. The average number  $\bar{r}$  of dissociated protons from a residue in PDCML is shown in Figure 2. PDCML is in the stable conformation (*LES*) at the neutral pH, at which the residue is in the state  $\bar{r} = 1$ , and the conformational change is accompanied by a change of  $\bar{r}$ . The value of  $pK_a$  of  $NH^+$  is larger than 10, and thus the isoionic state at  $\bar{r} = 0$  may have a Zwitterion form. The *LES* ( $\bar{r} = 0$ ) has one ionized carboxyl and one  $NH^+$  groups in the side chain.

## Divalent cation-induced conformation change

Changes in  $[\theta]_{217}$  and  $[\theta]_{235}$  of the aqueous PDCML solution by  $Ca^{2+}$  are shown in Figure 4, where NaCl is added to retain an ionic strength  $I$  of 0.1 M. As the ratio  $q = [M^{2+}]/[P]$  increases, the *LES* structure is converted to the coil especially at the alkaline pH, where  $M^{2+}$  represents the divalent cation, and  $[P]$  expresses residual polymer concentration in mol. However,  $Ca^{2+}$  does not induce the  $\alpha$ -helix in the polymer at  $0 < q < 0.5$  and at any pH.

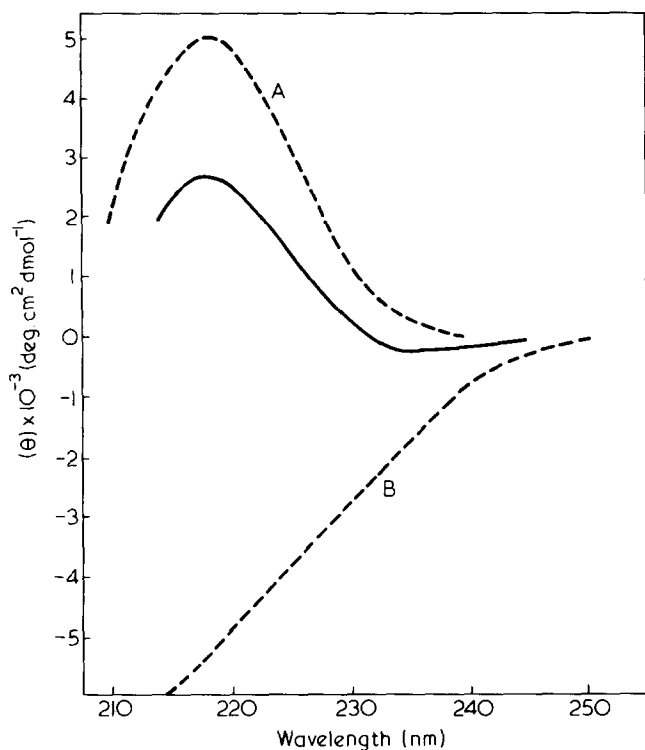


Figure 1 A c.d. spectrum of PDCML ( $[P] = 0.012M$ ) in aqueous solution at pH 7.18 and 16.7°C. C.d. spectra of charged PGA (A) and random coils (B) are quoted from refs 15–17

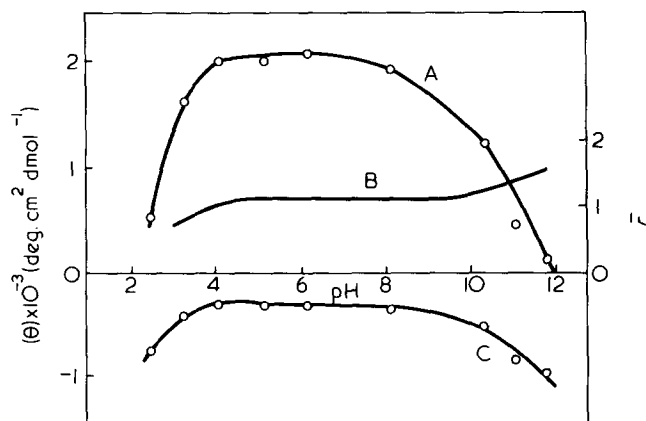


Figure 2 pH-dependence of  $[\theta]_{217}$  (A),  $[\theta]_{235}$  (B) and an average number  $\bar{r}$  (C) of dissociated protons from a residue in PDCML ( $[P] = 0.012M$ ) in aqueous 0.1 M NaCl at 25°C

amino groups. The minor species were found to be less than 8 wt %.

Other chemicals used were of reagent grade (Wako Pure Chem. Ind., Ltd and Nakarai Chem., Ltd).

## Methods

Optical rotatory dispersion (o. r. d.) measurements were carried out with a Yanagimoto Model 3 spectropolarimeter and c.d. with a Jasco J-20 instrument in the manner described previously<sup>12,13</sup>. Both instruments were temperature-controlled. A Beckmann pH meter Model 1019 was used for pH measurements at 25°C. A Beckmann glass electrode E-3 and a Horiba reference electrode 2630-50T with a modified liquid junction were used. pH titration procedures under nitrogen have been described previously<sup>14</sup>. Optical absorption was measured with a Hitachi double-beam ultra-violet spectrophotometer Model 124 with temperature control.

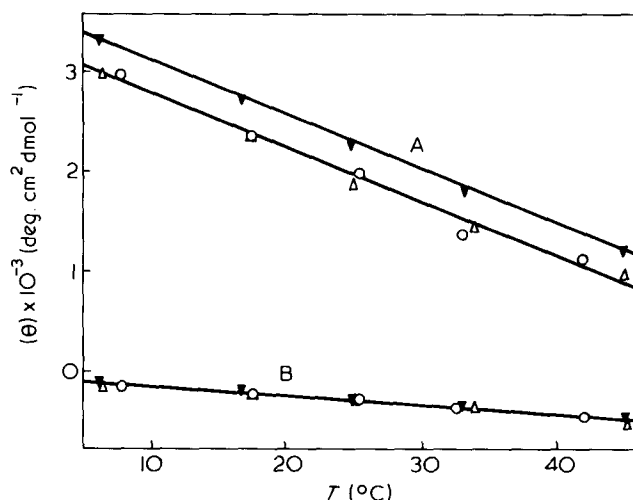


Figure 3 Temperature-dependence of  $[\theta]_{217}$  (A) and  $[\theta]_{235}$  (B) of PDCML ( $[P] = 0.012M$ ) in aqueous solution.  $\circ$ , pH = 4;  $\blacktriangle$ , pH = 7;  $\triangle$ , pH = 9

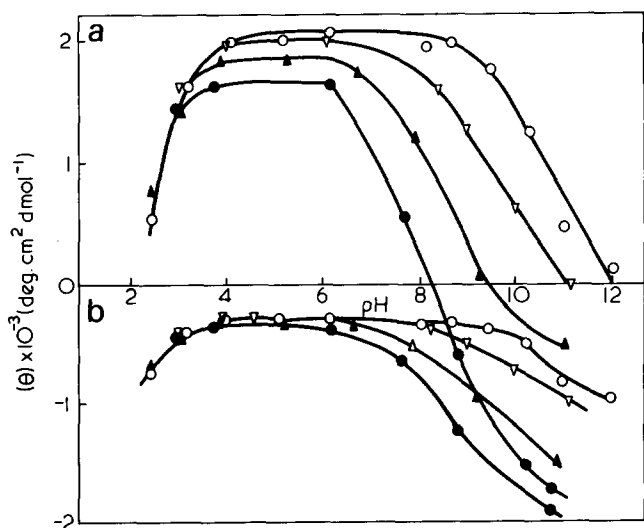


Figure 4 pH dependence of (a)  $[\theta]_{217}$  and (b)  $[\theta]_{235}$  of PDCML ( $[P] = 0.012M$ ) in aqueous  $CaCl_2$ -NaCl solution ( $I = 0.1M$ ) at  $25^\circ C$ .  $\circ, q = 0; \triangle, q = 0.08; \blacktriangle, q = 0.25; \bullet, q = 0.50$

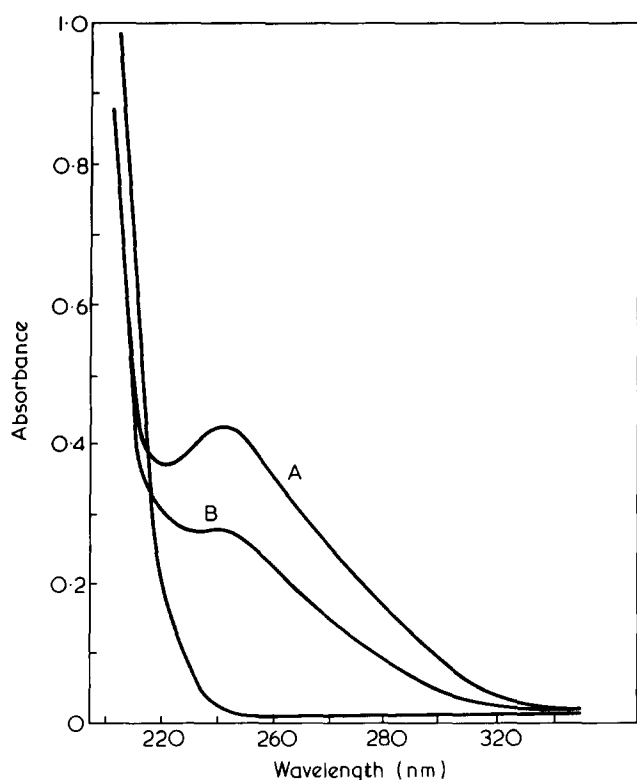


Figure 5 An absorption spectrum of  $Cu^{2+}$ -PDCML complex at  $q = 0.64$ ,  $[P] = 0.002M$ ,  $I = 0.2 M$  [ $Cu(ClO_4)_2$ - $NaClO_4$ ] and  $25^\circ C$ . pH: A, 8.34; B, 2.54. The reference curve taken from absorption data of PDCML and of  $NaClO_4$ - $Cu(ClO_4)_2$  is shown

The backbone structure of the polymer in aqueous solution including  $Cu^{2+}$  was studied via the o.r.d. data in the 340–550 nm wavelength region. The  $b_0$  value is nearly zero irrespective of pH ( $4 < pH < 9$ ) at  $q = 0.5$ . No ordered structure can be induced in PDCML by  $Cu^{2+}$ .

*Absorption spectra of Cu-PDCML complex*

Intense absorption of aqueous  $Cu(NO_3)_2$  at 300 nm may interfere with ultra-violet absorption studies on the Cu-PDCML complex, and thus we used  $Cu(ClO_4)_2$  which exhibits little absorption at wavelengths greater than 240 nm. Figure 5 indicates that an absorption peak of the complex is

near 240 nm. The dependence of the peak intensity on pH at various  $q$  values is shown in Figure 6, where the two steps of complex formation are shown. At  $q$  less than 0.64, the complex formation is described only by the first step. The molar absorption coefficient  $\epsilon$  of the complex formed at this step was determined to be  $3340 M^{-1} cm^{-1}$  at 240 nm from the  $\epsilon_{240}$  values for large  $q$  values at pH = 5. The next step of the formation occurs in case of  $q$  larger than 0.96. The molar absorption coefficient for the second step could not be determined due to precipitation at alkaline pH in the case of large  $q$ .

*pH titration of the Cu-PDCML system*

The pH titration results of the Cu-PDCML system at  $25^\circ C$  are shown in Figure 7.  $Cu^{2+}$  enhances the proton

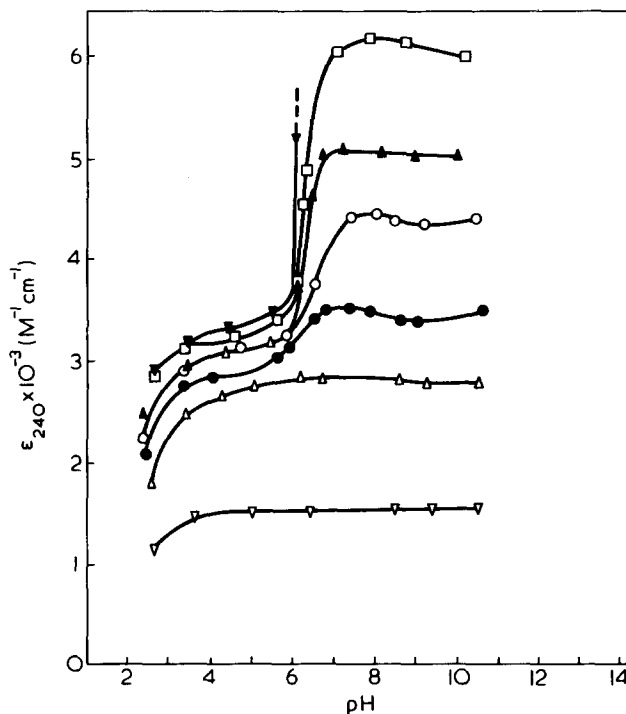


Figure 6 pH-dependence of the peak intensity at 240 nm of  $Cu^{2+}$  PDCML complex at  $[P] = 0.002 M$ ,  $I = 0.2 M$  and  $25^\circ C$ .  $\nabla, q = 0.32; \triangle, q = 0.64; \bullet, q = 0.96; \circ, q = 1.28; \blacktriangle, q = 1.60; \square, q = 1.92; \blacktriangledown, q = 2.56$

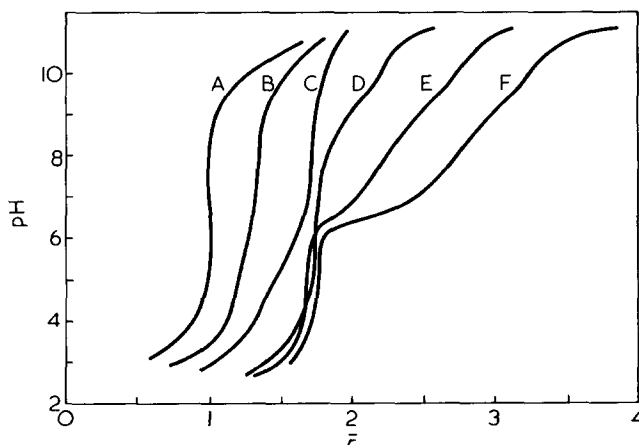


Figure 7 pH dependence of an average number  $\bar{r}$  of dissociated protons from a residue in PDCML ( $[P] = 0.0017 M$ ) in aqueous  $CuCl_2$ -NaCl ( $I = 0.2 M$ ) at  $25^\circ C$ . A,  $q = 0.0$ ; B,  $q = 0.15$ ; C,  $q = 0.29$ ; D,  $q = 0.58$ ; E,  $q = 0.88$ ; F,  $q = 1.17$

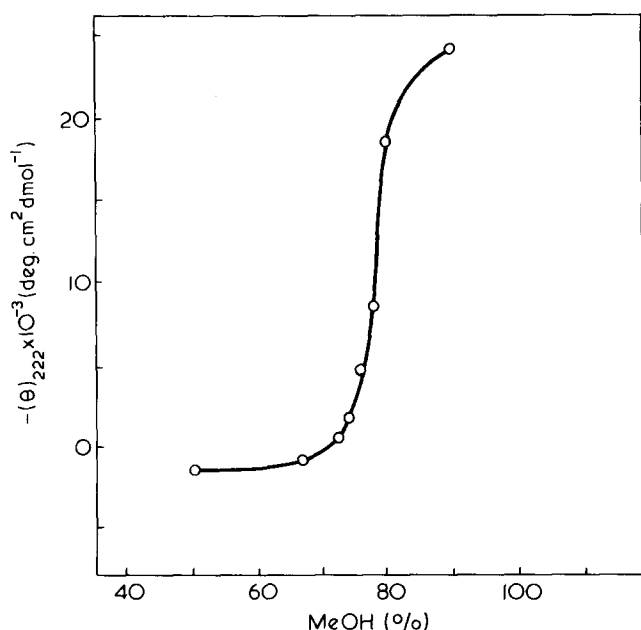


Figure 8 Dependence of  $[\theta]_{222}$  of PDCML ( $[P] = 0.012$  M) in aqueous methanol at neutral pH and 25°C on content of methanol (v/v)

dissociation from  $\text{NH}^+$  through the complex formation. In the case of  $q$  larger than 0.88, the dissociation of another proton is observed. It may correspond to the second step of the complex formation indicated in Figure 6. Analysis of the second step from the absorption data cannot be achieved and thus the pH titration analysis was carried out only for the first step of complex formation.

## DISCUSSION

PDCML is a new ampholytic homopolypeptide, which has three ionizable groups in the residue. The conformation changes as a result of the change of net charge. The pH-induced conformational change from an ordered structure such as  $\alpha$ -helix or  $\beta$ -structure to coil is not observed in a pH region of 2.5–12 at  $I$  less than 0.1 M. The polymer perhaps exists in coil form at any pH in this region, but may be in the coil with *LES* at the neutral pH.

Why does the polymer not have an ordered backbone structure? What is the main cause of the pH-induced change from *LES* to the random coil?

It should be noted that electrostatic interaction exists between the residues at any pH from 2.5 to 12. One of the possible causes for the destabilization of a conformation may be the change in the water structure around the residue.  $\text{NaClO}_4$  solution of 5–7 M changes the water structure considerably, and is found to induce the  $\alpha$ -helix structure in the polymer at the neutral pH. Methanol can induce the conformational change from the coil with *LES* to the  $\alpha$ -helix as shown in Figure 8. Thus the water structure is an important factor for determination of the PDCML conformation.

The primary carboxyl group is ionized at any pH in the region 2.5–12. The apparent pK of the secondary group in PDCML may be about 3 and that of  $\text{NH}^+$  exceeds 10 in aqueous solution without the divalent cations. Further the divalent cations enhance the proton dissociation from  $\text{NH}^+$  by forming the complexes. Although these ions destabilize the *LES*, few ordered structures can be induced by them.

An average coordination number of  $\text{Cu}^{2+}$  in the complex formed at the first step is determined by analysing the shift

of the pH titration curve for  $q$  less than 0.58 relative to that for  $q = 0$ . The following two relations are obtained using the free  $\text{Cu}^{2+}$  concentration,  $[\text{Cu}^{2+}]$ , the total  $\text{Cu}^{2+}$  concentration  $[\text{Cu}^{2+}]_t$ , the complex concentration  $[\text{LCu}^{2+}]$ , concentration of  $\text{NH}^+$   $[\text{LH}^+]$  and that of tertiary amine  $\text{N}[\text{L}]$ :

$$[\text{Cu}^{2+}]_t = [\text{Cu}^{2+}] + [\text{LCu}^{2+}], \quad (1)$$

$$[\text{P}] = [\text{L}] + [\text{LH}^+] + [\text{LCu}^{2+}] \quad (2)$$

The electroneutrality requirement and the above relations (1) and (2) lead to (3), where  $[\text{Na}^+]'$  is the effective concentration of  $\text{Na}^+$  for the complex formation and differs from the total  $[\text{Na}^+]$  ( $[\text{Na}^+]' = [\text{Na}^+] - [\text{P}]$ ):

$$[\text{LH}^+] = [\text{P}] - ([\text{Na}^+]' + [\text{H}^+] - [\text{OH}^-]) \quad (3)$$

$[\text{L}]$  was estimated from the reference plot, which was made from the pH titration data for  $q = 0$  according to Mandel and Leyte<sup>16</sup> as shown in Figure 9. The average number of coordination  $\bar{j}$  was calculated from  $[\text{L}]$ ,  $[\text{LH}^+]$ ,  $[\text{P}]$  and  $[\text{Cu}^{2+}]_t$ .  $\bar{j}$  is plotted against pH for various  $q$  values in Figure 10,

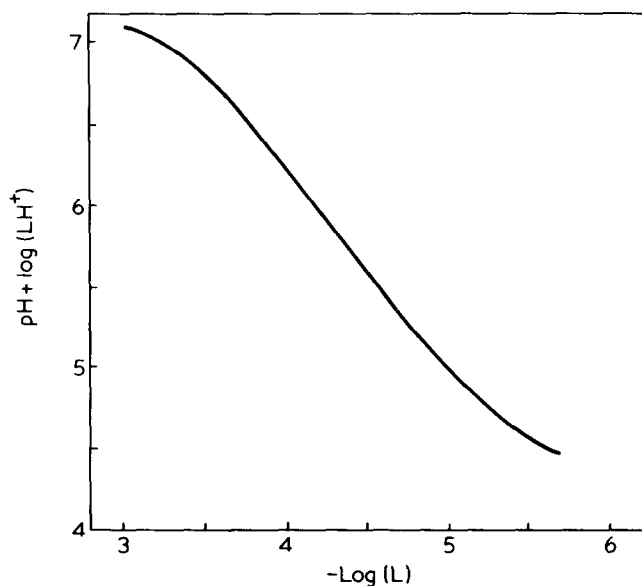


Figure 9 Reference plot made from the data in Figure 7

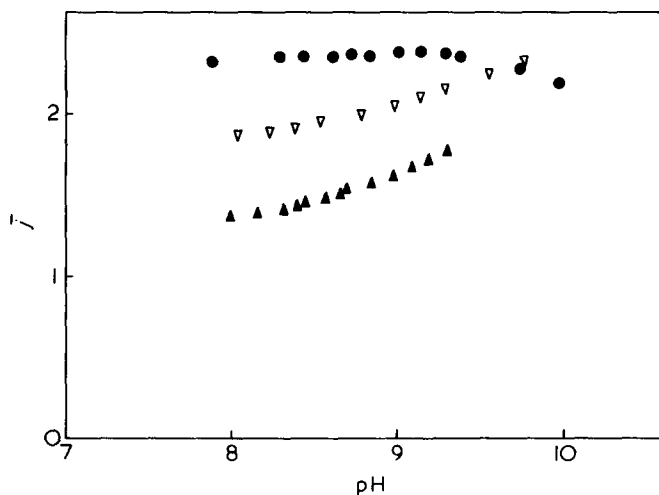


Figure 10 pH dependence of an average number  $\bar{j}$  of coordination of  $\text{Cu}^{2+}$  in the  $\text{Cu}^{2+}$ -PDCML complex ( $[P] = 0.0017$  M,  $I = 0.2$  M ( $\text{CuCl}_2$ - $\text{NaCl}$ ) and 25°C). ●,  $q = 0.29$ ; ▽,  $q = 0.44$ ; ▲,  $q = 0.58$

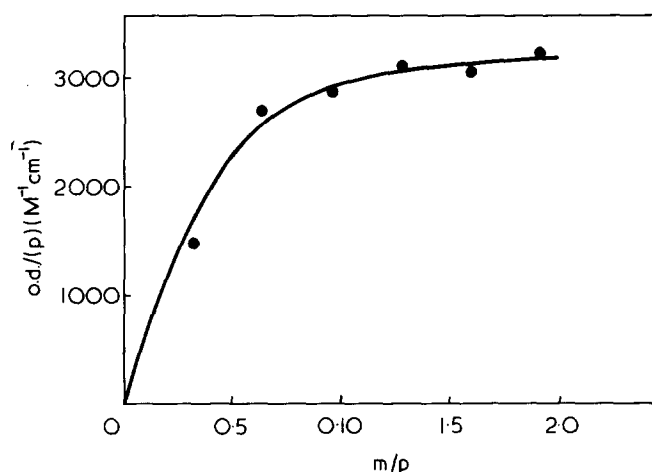


Figure 11 Plot of o.d. of the Cu<sup>2+</sup>-PDCML complex at 240 nm against *q* at pH = 5.0

which shows that Cu<sup>2+</sup> coordinated to the two nitrogen atoms behaves similarly to the Cu<sup>2+</sup>-EDTA complex.

The stability of the complex was estimated from the absorption data. Cu<sup>2+</sup> is coordinated to two residues, which are indicated as L<sub>2</sub>. The following reaction is assumed:



The association constant *K* is given as [CuL<sub>2</sub><sup>2+</sup>]/[Cu<sup>2+</sup>][L<sub>2</sub>], and the two equations (5) and (6) are obtained:

$$[\text{Cu}^{2+}]_t = [\text{Cu}^{2+}] + [\text{CuL}_2^{2+}] = m \quad (5)$$

$$[\text{L}_2]_t = [\text{L}_2] + [\text{CuL}_2^{2+}] = [\text{P}]/2 \quad (6)$$

The optical density o.d. of the complex solution in the Cu-PDCML system at 240 nm is given as:

$$\text{o.d.}/2\epsilon = K(m - \text{o.d.}/2\epsilon) ([\text{P}]/2 - \text{o.d.}/2\epsilon) \quad (7)$$

where  $\epsilon = 3340 \text{ M}^{-1} \text{ cm}^{-1}$ . From equation (7), we can derive:

$$\text{o.d.}/[\text{P}] = \epsilon \left\{ \left( \frac{m}{[\text{P}]} + \frac{1}{2} + [\text{P}]K \right) - \left\{ \left( \frac{m}{[\text{P}]} + \frac{1}{2} + \frac{1}{[\text{P}]K} \right)^2 - \frac{2m}{[\text{P}]} \right\}^{1/2} \right\} \quad (8)$$

In order to obtain the best-fitted curve to a plot of o.d. against  $q = m/[\text{P}]$  at the first step of the formation at pH = 5.0, [P]*K* must be assumed to be 14 as shown in Figure 11, from which we estimated the association constant of the reaction (4) as  $9 \times 10^{-4} \text{ M}^{-1}$ . Such a value is very similar to the equilibrium constant for the Cu<sup>2+</sup>-iminoacetic acid complex formation<sup>19</sup>, but is far smaller than that for the Cu<sup>2+</sup>-EDTA complex<sup>20</sup>.

#### ACKNOWLEDGEMENT

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