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Calcium and 1-hydroxyethylidene-1,1-bisphosphonic acid: polynuclear complex formation in the physiological range of pH

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Summary

1-Hydroxyethylidene-1,1-bisphosphonic acid (HEDP) is used in a variety of diagnostic, research and therapeutic applications at in vivo concentrations of from < 1 μM to > 1 mM. Previous studies have shown that calcium and HEDP form polynuclear aggregate complexes at high pH. Calcium ion titrations at constant pH 8.0, and pH titrations at 1 : 1 and 2 : 1 ratios of total calcium to total diphosphonate, provide evidence for the occurrence of aggregation of calcium and HEDP under physiological conditions. A robust feature of several polynuclear models examined is a dramatic pH dependence of polynuclear species concentrations between pH 5.5 and 7.0.

Introduction

HEDP has a variety of research, industrial and medical uses (Francis and Centner, 1978). Therapeutic use may involve either unlabeled or radiolabeled compounds and all medical applications are related in one way or another to the physiologic role of calcium. Paget's disease and other bone disorders have been treated with unlabeled HEDP administered orally over periods on the order of months (Fleisch, 1980), while parenteral administration has been used for treating

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malignant hypercalcemia (Jung et al., 1981). [^{32}P]-labeled HEDP has been used in a radiotherapeutic mode in advanced cases of diffuse metastatic disease of bone (Potsaid et al., 1976).

Diagnostic uses of HEDP were a result of experience with its use in treatment of metabolic bone disorders. In the early 1970s the primary radiopharmaceutical for bone scanning was Sr^{85}, a metabolic analog of calcium which has a relatively long half-life and delivers undesirably large radiation doses during diagnostic studies (Moon, 1969). The advent of ^{99m}technetium-labeled polyphosphates and phosphonates (Castronovo and Callahan, 1972; Yano et al., 1973) greatly increased efficacy of diagnostic bone scanning and the rate of studies done with the Tc-labeled phosphorous compounds increased by an order of magnitude over that with ^{85}Sr (Lamson, 1982). Technetium-phosphonates are now the agents of choice in bone imaging; they are also useful in localization of calcium in soft tissue, as in breast carcinoma and myocardial and cerebral infarcts (Berg et al., 1973), and as a measure of skeletal metabolism in 24-h whole-body retention studies (Smith et al., 1983).

Other important areas of medical uses of HEDP are those of dental research (Wu et al., 1976) and research involving kidney stones (Blomen, 1982). In the cases of both hydroxyapatite and calcium oxalate crystals, surface adsorption of HEDP causes an inhibition of dissolution, an effect which may shed further light on dissolution kinetics and lead to effective means of preventing dissolution in the case of HAP or enhancing dissolution in the case of kidney stones.

A first step toward understanding the physiologic behavior of HEDP is an examination of calcium–HEDP complex formation under physiologic conditions. A number of studies have appeared in the literature over the past several years regarding formation of Ca–HEDP complexes. While some authors have reported the presence of only simple calcium diphosphonate species in aqueous solution (Kabachnik et al., 1967; Wada and Fernando, 1972), others who have studied complex formation at pH 11 have reported the occurrence of aggregation and polynuclear complex formation (Grabenstetter and Cilley, 1971; Wiers, 1971; Callis et al., 1969). The formation of polynuclear complexes may be due to the presence of several coordinating oxygen atoms on the HEDP molecule which results in an ability to form multiple ring structures with calcium and the hydroxyl group; the hydroxyl group may be involved even if un-ionized (Uchtman, 1972a and b). The purpose of this study is to determine whether this aggregation phenomenon may be expected to occur in the physiological range of pH (Wiedmer et al., 1983).

Experimental

HEDP used in all experiments was supplied by Proctor and Gamble Co. as the disodium salt. Pure acid HEDP was prepared by eluting stock solutions of the diphosphonate through a Dowex cation exchange resin which had been prepared by eluting with 1 M HCl and deionized water. Stock solutions were then standardized by titrating with tetramethyl ammonium hydroxide (TMAOH); amounts of base

added to the 2nd and 3rd equivalence points indicated the yield from the cation exchanger to be 97.0 ± 0.8%. Solutions were checked for metal complex impurities by UV-visible spectroscopy; aqueous solutions of acid HEDP have no UV-visible absorption.

Base solutions used in all studies were prepared from Eastman reagent grade 10% aqueous solutions of TMAOH, and standardized by titrating to a phenolphthalein end point with potassium hydrogen phthalate. Precautions were taken to exclude dissolved atmospheric carbon dioxide from the base solutions. Tetramethylammonium chloride (TMACl) was used in all experiments to maintain ionic strength at 0.100 M; aqueous stock solutions were prepared by dissolving Baker reagent grade TMACl in reagent grade deionized water and all solutions were bubbled for 30 min with CO_2-free nitrogen gas. Calcium standard solutions were obtained from Radiometer as 0.100 M aqueous $CaCl_2$, shipped in plastic containers in evacuated metal cans.

Water used for preparing all solutions and for rinsing glassware was reagent grade (18 MΩ resistance, organic impurities ≈ ppb), produced from a 4 cartridge Millipore water purification unit. Since significant amounts of sodium and calcium ions will leach out of borosilicate glass storage containers over periods of a week or more, water was kept no longer than 2–3 days in Ascarite-vented storage containers. Precautions were taken throughout reagent preparations and experiments to exclude atmospheric CO_2, which may precipitate as calcium carbonate, by bubbling with nitrogen gas; the effectiveness of removing and excluding CO_2 from a closed but unsealed reaction vessel by N_2 bubbling is shown in Fig. 1. The nitrogen used had been passed through three gas washing bottles, the first containing concentrated NaOH to remove any traces of CO_2 and the second and third containing deionized water. This washing provided nitrogen gas free of any trace of CO_2 and saturated with water vapor.

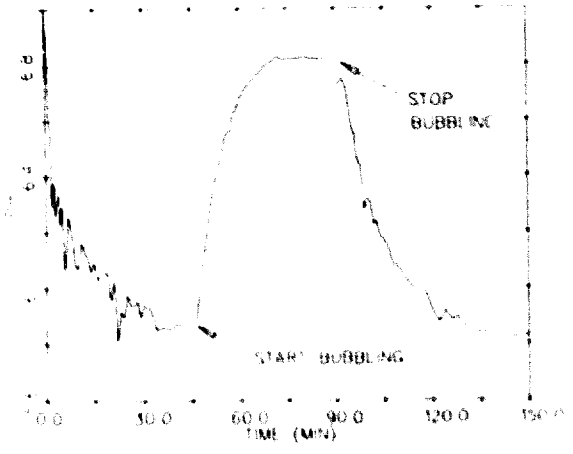

Fig. 1. Removal of CO_2 from deionized water by bubbling with nitrogen gas, as demonstrated by pH measurement. pH monitoring started immediately after deionization; bubbling started at 45 min, stopped at 90 min

All experiments were done in a water-jacketed 250 ml beaker closed at the top and with inlet ports for electrodes, a mechanical stirrer, the line supplying nitrogen gas and buret delivery tips. After solutions were added to the reaction vessel thermal equilibrium was achieved at 25 °C in approximately 10 min and bubbling was continued for another 10 min before starting any titration. Temperature was maintained at 25.0 ± 0.1 °C during the course of the experiments. Radiometer equipment was used throughout, including pH meters, autoburets and electrodes; the calcium electrode was found to produce stable and rapid potential readings down to the micromolar level, where response time became somewhat slower. Calcium standard curves were obtained before and after each run by titrating a 0.100 M solution of TMACl at the desired pH with 0.100 M calcium chloride standard. These standard runs were generally within 1–2 tenths of a millivolt down to the micromolar level and non-linearity in electrode response at the lowest concentrations was accommodated by fitting the curves to a least-squares cubic spline; the before and after standard splines were averaged by the computer program which processed the raw data. It was noted that calcium electrode response was somewhat dependent on pH and exhibited a hysteresis effect when titrating a solution of constant calcium ion concentration with a base followed by an acid. Therefore, all standards and samples were titrated in exactly the same manner.

Results

Experimental data are shown in Figs. 2–4. Figs. 2 and 3 are unsmoothed plots of pH titrations at 1 : 1 and 2 : 1 ratios of total calcium to total diphosphonate,

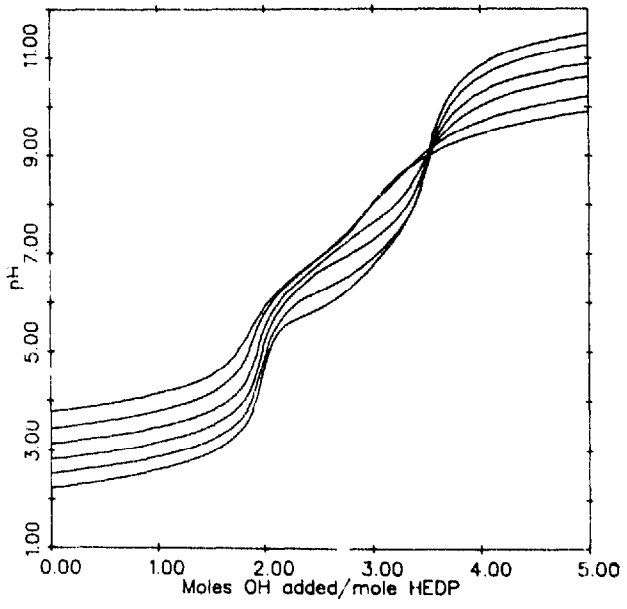

Fig. 2 Unsmoothed pH titration data at 1 : 1 ratio of CATOT : DPTOT. Diphosphonate concentrations, top to bottom on left hand side of plot, are 0.100, 0.220, 0.480, 1.100, 2.30 and 5.00 mM.

TABLE 1

HEDP ACID DISSOCIATION CONSTANTS

μ/salt/°C	pK_1	pK_2	pK_3	pK_4	pK_5	Ref.
0.0/TMACl/25	-	3.03	7.31	11.52	-	Grabenstetter et al., 1967
0.1/TMACl/25	-	2.80	7.00	11.16	-	Grabenstetter et al., 1967
0.1/KCl/25	1.7	2.47	7.28	10.29	11.13	Kabachnik et al., 1967
0.5/TMACl/25	-	2.54	6.97	11.41	-	Carroll and Irani, 1967
0.1/$TMAClO_4$	-	2.31	6.99	10.93	-	Wada and Fernando, 1971
0.0//20	2.01	3.08	7.60	11.96	13.63	Collins and Perkins, 1977
0.1/KCl/25	< 2	2.5	6.89	10.60	-	Dietsch et al., 1976

respectively. Titration curves are shifted downward at the higher ratio of total calcium to total diphosphonate in the region between the second and fourth equivalence points, more so than would be expected on the basis of mononuclear complex formation. At the higher concentrations of HEDP on both plots it is noted that there are slight kinks in the data just before the plateau region beyond the second equivalence point. Further analysis shows that this is consistent with the onset of polynuclear complex formation. Previously reported values of HEDP acid dissociation constants and mononuclear calcium–HEDP complex formation constants are shown in Tables 1 and 2. Although not consistently reported in the literature it has been demonstrated in previous work that the protonated calcium diphosphonate complex CaHDP is important in the region of the second equivalence point (Lamson et al., 1979). Reported values for formation of the Ca_2DP complex

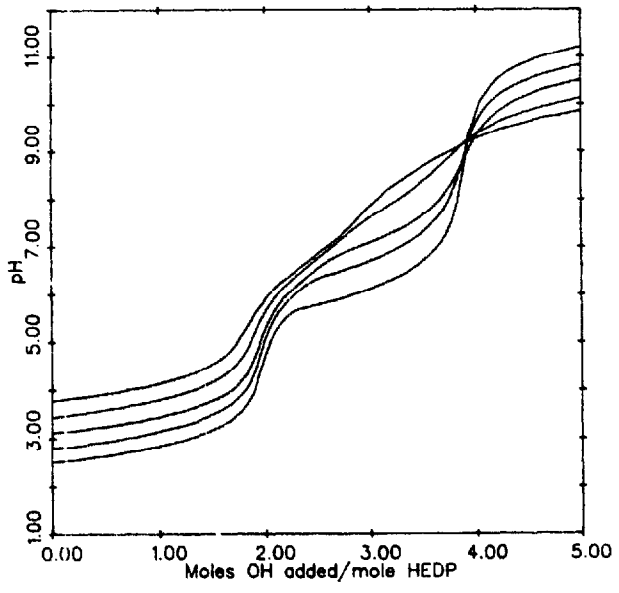

Fig. 3. Unsmoothed pH titration data at 2:1 ratio of CATOT:DPTOT. Diphosphonate concentrations, top to bottom on left side of plot, are 0.100, 0.220, 0.480, 1.10 and 2.30 mM.

TABLE 2

FORMATION CONSTANTS FOR SIMPLE Ca-HEDP COMPLEXES

μ/salt/°C	log of formation K			
	$K_{M\cdot HL}$	$K_{M\cdot L}$	$K_{M\cdot M\cdot L}$	Ref.
0.5/TMACl/25	3.58	5.74	–	Carroll and Irani, 1968
0.1/KCl/25	–	6.04	15.59	Kabachnik et al., 1967
0.1/TMABr/25	–	7.09	–	Callis et al., 1969
0.1/TMABr/25	–	5.53	–	Callis et al., 1969
0.1/TMACl/25	3.55	6.84	–	Wada and Fernando, 1972
0.1/TMACl/25	3.53	6.40	12.2	Wada and Fernando, 1972
0.1/TMACl/25	–	5.52	–	Grabenstetter and Cilley, 1971
0.1/KCl/25	3.0	6.0	–	Dietsch et al., 1976

probably represent an apparent constant for formation of Ca_2DP and other larger aggregates (Lamson, 1982).

Fig. 4 is an unsmoothed plot of formation number, (CATOT-Ca^{2+})/DPTOT, versus calcium ion concentration at pH 8.0. This set of curves is shifted to the right, i.e. toward higher levels of log[Ca^{2+}], in relation to calcium ion titrations done at pH 11 (Grabenstetter and Cilley, 1971), indicating a lower degree of complex formation at the lower pH. However, it is also noted that the curves are still well separated at pH 8.0, i.e. formation number is a function of both calcium and diphosphonate

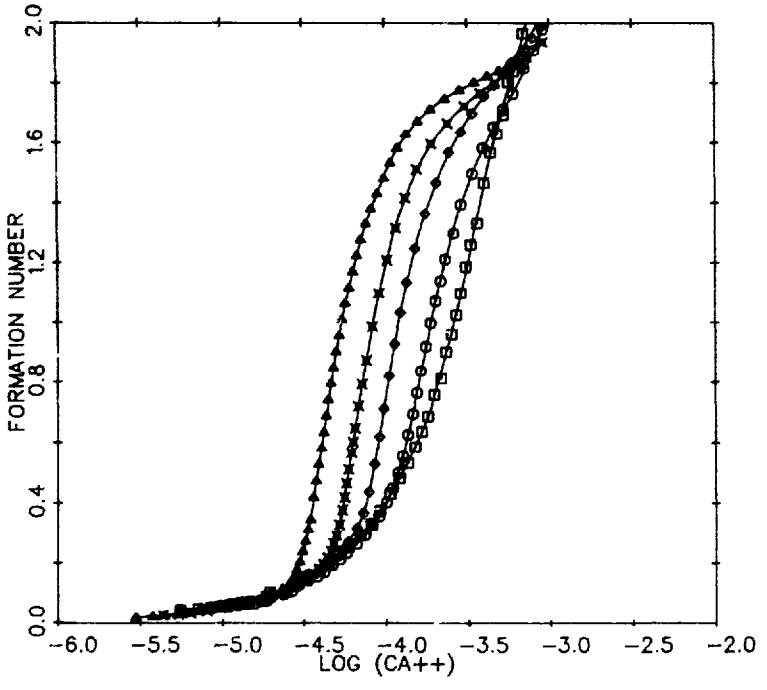

Fig. 4. Calcium ion titrations at constant pH 8.0. Unsmoothed calcium complex formation data at total diphosphonate concentrations of 2.29, 1.03, 0.478, 0.219 and 0.100 mM (left to right).

concentration. For the general reaction:

$$pCa + qDP \leftrightarrow Ca_pDP_q \qquad \beta_{pq} = \frac{[Ca_pDP_q]}{[Ca]^p[DP]^q} \tag{1}$$

the expressions for the analytical concentrations of Ca and DP in solution are:

$$CATOT = [Ca] + \sum_{p=1}^{p_{max}} \sum_{q=1}^{q_{max}} p[Ca_pDP_q] = [Ca] + \sum_{p=1}^{p_{max}} \sum_{q=1}^{q_{max}} p\beta_{pq}[Ca]^p[DP]^q \tag{2}$$

$$DPTOT = [DP] + \sum_{p=1}^{p_{max}} \sum_{q=1}^{q_{max}} q[Ca_pDP_q] = [DP] + \sum_{p=1}^{p_{max}} \sum_{q=1}^{q_{max}} q\beta_{pq}[Ca]^p[DP]^q \tag{3}$$

Substituting Eqns. 2 and 3 into the expression for formation number:

$$Z = \frac{\sum_{p=1}^{p_{max}} \sum_{q=1}^{q_{max}} p\beta_{pq}[Ca]^p[DP]^q}{[DP] + \sum_{p=1}^{p_{max}} \sum_{q=1}^{q_{max}} q\beta_{pq}[Ca]^p[DP]^q} \tag{4}$$

It is seen in Eqn. 4 that if $q_{max} = 1$ then the DP concentration terms cancel and Z is a function only of calcium ion concentration; formation curves will coincide regardless of the level of total diphosphonate concentration. If $q_{max} > 1$ then formation number is a function of both calcium and diphosphonate concentration. Separation of the curves at different levels of total diphosphonate concentration in Fig. 4 indicates that polynuclear complex formation is occurring at pH 8.

Discussion

On the basis of a model-independent PQ analysis (Sillen, 1961), i.e. model-independent determination of the numbers of calcium ions and diphosphonate molecules in aggregate species, it was shown that the most appropriate model for formation of Ca–DP aggregate species is that described by a 'core-links' model having a formation constant representing addition of links to an aggregate (Lamson et al., 1984). This is distinguished from models employing constants which represent overall complex formation. Specifically, this link-addition model is represented in the following way.

For the series of core-links complexes A, AB, AB_2, AB_3, $AB_4 \ldots AB_\infty$, where A = core = CaDP and B = link = Ca_2DP, the equilibrium constant for addition of a link to the previous aggregate species is represented by $k_n = k_0k^n$. Constants for association of all aggregates in the infinite series are determined by two parameters

k_0 and k, and are $k_1 = k_0 k$, $k_2 = k_0 k^2, \ldots, k_n = k_0 k^n$. Therefore

$$A + B \leftrightarrow AB \qquad AB = k_0 k \cdot A \cdot B$$

$$AB + B \leftrightarrow AB_2 \qquad AB_2 = k_0 k^2 \cdot AB \cdot B$$

$$= k_0 k^2 \cdot k_0 k \cdot A \cdot B \cdot B = k_0^2 k^3 \cdot A \cdot B^2$$

$$AB_2 + B \leftrightarrow AB_3 \qquad AB_3 = k_0 k^3 \cdot AB_2 \cdot B$$

$$= k_0^3 k^6 \cdot A \cdot B^3$$

$$AB_3 + B \leftrightarrow AB_4 \qquad AB_4 = k_0^4 k^{10} \cdot A \cdot B^4$$

Then the total amount of core species present in all aggregates is given by

$$ATOT = A + AB + AB_2 + \ldots + AB_\infty$$

$$= A + A \sum_{n=1}^{\infty} \left(k^{n(n+1)/2} k_0^n B^n\right)$$

Similarly the total amount of link species in all aggregates is expressed as:

$$BTOT = B + A \sum_{n=1}^{\infty} \left(n k^{n(n+1)/2} k_0^n B^n\right)$$

These series are not expressible in closed form but do converge provided the absolute value of k is less than 1.0. The computer program used to solve the equilibrium system evaluates the expression term by term with an appropriate criterion for termination. The total calcium present in aggregate species equals ATOT + 2*BTOT; total diphosphonate in aggregates is ATOT + BTOT. The equilibrium system can then be represented by 3 mass balance expressions, including all mono- and polynuclear species, describing HTOT, CATOT and DPTOT. This system of 3 non-linear equations is solved by finding roots of the mass balance expressions using a non-linear system solver described by Powell (1970), and the system solution is incorporated in a function evaluation routine, which uses parameters passed to it for equilibrium constants, in order to determine best values of constants by minimizing error square sums from experimental data. Best fitting constants for the pH 8 formation data, which are also consistent with pH titration data, are presented in Table 3; these constants were fitted to approximately the lower two-thirds of the formation data, as shown in Fig. 5, in order to be sure that concentration regions where precipitation occurs were avoided (Wiers, 1971). Curve-fitting was done by providing parameter estimates interactively at a Tektronix graphics terminal; visual observation of changes in the computed curves was found to be equally as useful as least-squares statistics in evaluating various models and goodness of fit (Lamson et al., 1979).

Based on the best fitting constants presented in Table 3 for the link-addition stepwise model, species concentration diagrams as a function of pH were computed

TABLE 3

PARAMETERS USED IN OR ESTIMATED FROM LINK-ADDITION MODEL FITTED TO pH 8 FORMATION DATA

Formation constant	log K (or pK_a)
K_1	1.7
K_2	2.59
K_3	7.02
K_4	10.78
$K_{Ca \cdot OH}$	1.4
$K_{Ca \cdot OH \cdot OH}$	3.8
$K_{Ca \cdot DP}$	5.51 *
$K_{Ca \cdot Ca \cdot DP}$	7.52 *
$K_{Ca \cdot H \cdot DP}$	14.49 *
K_0	−7.02 *
K_{STEP}	0.0253 *

* Fitted parameters noted by asterisk.

as shown in Figs. 6 and 7. Two important features of these diagrams which may be noted are the importance of the protonated species CaHDP in the slightly acid to neutral region, and the extreme pH dependence of aggregate species in the pH range 6–7. The polynuclear complexes become important at this pH and remain relatively constant throughout the basic range of pH. Furthermore, the pH dependence and general characteristics of polynuclear species concentration shown in Figs. 6 and 7

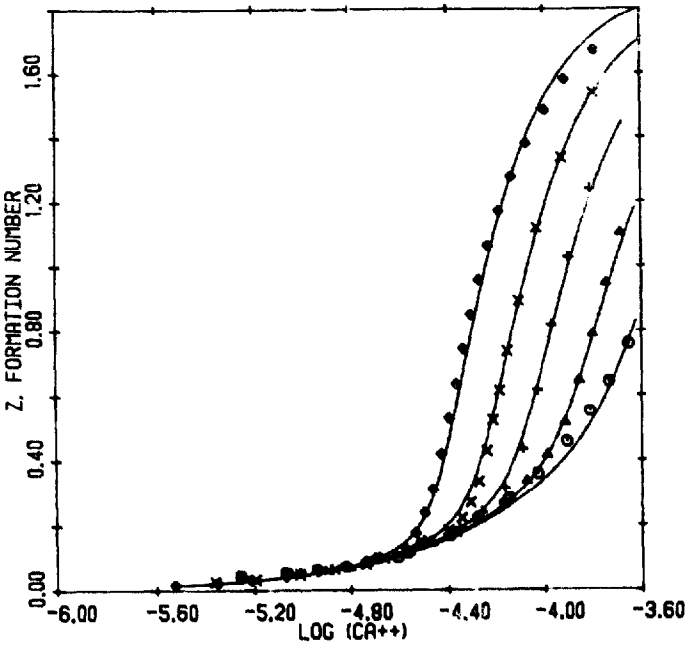

Fig. 5. Best fit of link-addition stepwise complex formation model to pH 8.0 formation curve data. Total diphosphonate concentrations (left to right) are 2.29, 1.03, 0.478, 0.219 and 0.100 mM.

are not unique to this particular link addition model; similar behavior is noted for several of the best fitting polynuclear models (Lamson, 1982).

Formation curves for Ca–HEDP complexes are bivariate at pH 8, base titration curves exhibit a subtle but noticeable dislocation in the region where species

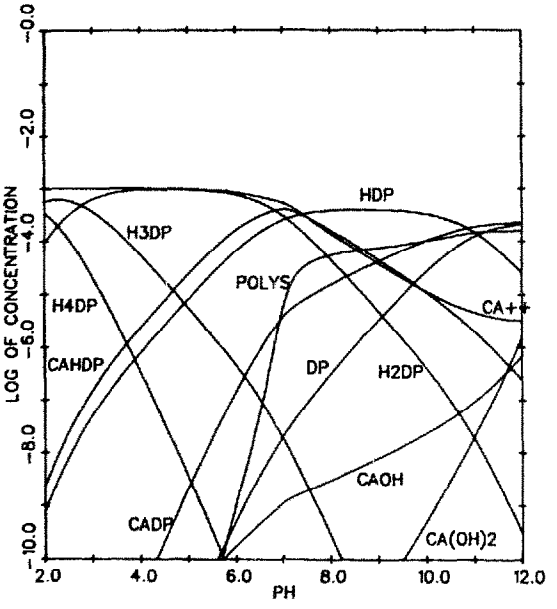

Fig. 6. Computed species concentrations as a function of pH for 1 : 1 ratio of CATOT : DPTOT based on best fit model.

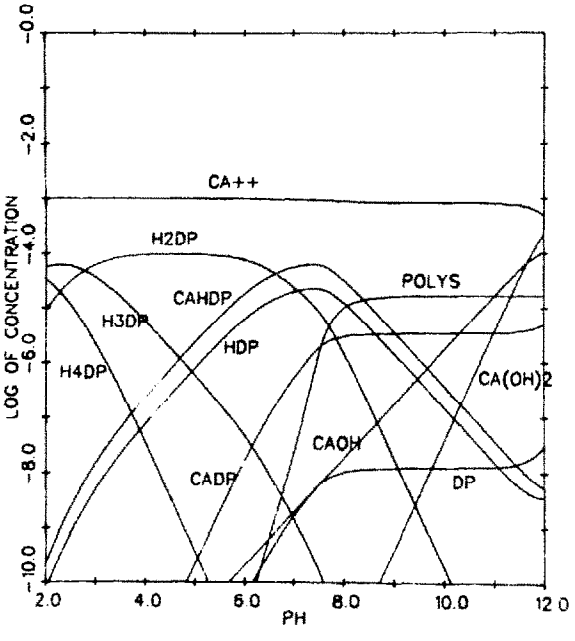

Fig. 7. Computed species concentrations as a function of pH for 10 : 1 ratio of CATOT : DPTOT based on best fit model.

concentration diagrams indicate rapid growth of polynuclear species, and all of the several models examined exhibit a similar strong pH dependence in the neutral region. Although physiological environments are considerably more complicated than these experimental conditions, they are similar with regard to ionic strength, calcium ion concentration and pH. Therefore it is concluded that in the physiological range of pH, and probably under actual physiological conditions, polynuclear aggregation occurs between calcium ion and HEDP.

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