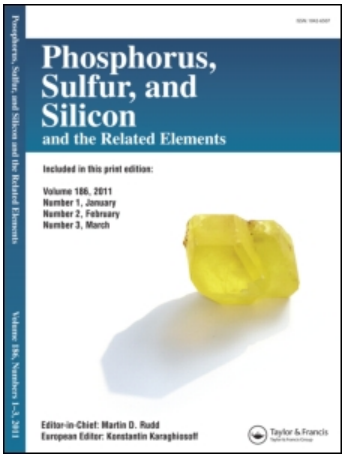

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THE CONDENSED PHOSPHATES AS INHIBITORS OF THE ZINC-ENZYME CARBONIC ANHYDRASE II

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The mobilization of the zinc ion from the zinc enzyme BCA II by the condensed phosphates pyrophosphate (PP), tripolyphosphate (TPP), and higher polyphosphate (HPP) induces the inhibition of the enzymatic activity. The chelating capacity increases with the ligand concentration and with the length of the polyphosphatic chain. The activity is restored when the polyphosphate is hydrolyzed, becoming incapable of chelating the zinc ion, which is able to reform the zinc enzyme.

Keywords: Carbonic anhydrase; higher polyphosphate (HPP); pyrophosphate (PP); tripolyphosphate (TPP)

Carbonic anhydrase (Carbonate hydro-lyase, EC 4.2.1.1) is a zinc enzyme widespread in animals and plants. The enzyme is most highly concentrated in erythrocytes, renal cortex, and gastric mucosa of mammals.

Its various physiological roles are focused on facilitating the diffusion of carbon dioxide and the transport of hydrogen ion, bicarbonate ion, and (through physiological ion-exchange reactions) the sodium ion.

All the highly purified carbonic anhydrases have a molecular weight of approximately 30,000 and contain one zinc atom per molecule. The major activity of the enzyme is the reversible hydration of CO_2 according to the following reaction:

$$CO_2 + H_2O \rightleftharpoons H^+ + HCO_3^-$$

The zinc ion forms tetrahedral complexes with the enzyme atoms. The coordination of the zinc ion involves three histidine residues (His-94, His-96, and His-119).[1–3] The fourth coordination site is occupied by

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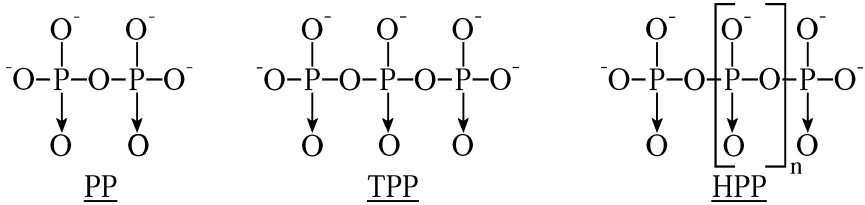
a water molecule in the low pH form of the enzyme (pH < 7), while in the high-pH form the site is occupied by a hydroxide ion (pH > 7). The molecular dynamics simulations have located a "high-affinity" binding pocket for CO_2 in close proximity to the zinc ion and its associated ligands.[4] The metal is firmly bound since it is not removed by extensive electrodialysis.[5] Zn^{2+} is released in the presence of chelating agents with a concomitant loss of the catalytic activity.

The erythrocyte bovine carbonic anhydrase (BCA II) isoenzymes catalyze the reversible hydration of CO_2 with an extraordinary efficiency, so that most methods for the determination of the reaction rate are based on measuring CO_2 or H^+.

The mobilization mechanism of the metal ion is SN2, which implies the rapid formation of an intermediary ternary complex, enzyme-Zn-ligand:[6]

$$EZn + L \rightleftharpoons (EZnL) \rightleftharpoons E + ZnL$$

The purpose of this study was the mobilization of the zinc ion by the condensed sodium phosphates: pyrophosphate (PP), tripolyphosphate (TPP), and higher polyphosphate (HPP) (Scheme 1).



SCHEME 1

RESULTS AND DISCUSSION

BCA II was isolated and purified from bovine erythrocyte. Regarding the amount of protein in erythrocytes, carbonic anhydrase takes the second place compared to hemoglobin.

The most efficient removal of hemoglobin was achieved by chromatography on DEAE-Sephadex A 50 (100%). The separation of the two forms (A and B) of BCA II was achieved by ion exchange chromatography on DEAE-Cellulose and with higher resolution on DEAE-Sephadex A 50. The enzyme (form B) was incubated with the phosphate solution at 4°C, and the enzymatic activity was determined at different time periods. The carbonic anhydrase activity was determined with a method

TABLE I The Enzymatic Activity of CA in the Presence of Condensed Phosphates

	t = 1 h		t = 2 h		t = 8 h		t = 24 h	
Conc. (mM)	[a]T (s)	[b]UE/ml	T (s)	UE/ml	T (s)	UE/ml	T (s)	UE/ml
PP								
0.909	60	256.66	80	137.50	88	105	80	137.50
1.818	64	226.87	86	115.55	88	105	80	137.50
2.727	67	206.87	88	105	97	74.84	82	128.78
TPP								
0.909	70	188.57	95	81.05	102	60.39	72	177.22
1.818	85	116.47	88	105	87	108.73	85	116.47
2.727	90	97.77	87	108.73	89	101.34	88	105
HPP								
0.909	90	97.77	95	81.05	103	57.66	97	74.84
1.818	100	66	102	60.39	103	57.66	98	71.83
2.727	108	44.81	110	40	110	40	94	84.25

[a]T = the time in seconds corresponding to the variation of the pH at the sample.
[b]1 UE = 52.87×10^{-6} moles CO_2/l/s.

based on that of Wilbur and Anderson.[7] The control activity, measured in the absence of the ligand, is $a_0 = 574.44$ UE/ml.

The catalytic activity in the presence of the condensed phosphates is presented in Table I. The effect of the chelating agents on the catalytic activity is better observed from the data shown in Table II. The restoring of activity (more obvious at HPP) in these experiments might be explained by the hydrolysis of the polyphosphates. This renewal of

TABLE II The Residual Activity (a/a_o) in % in the Presence of Condensed Phosphates

Conc. [L]		t = 1 h		t = 2 h		t = 8 h		t = 24 h	
mM	log	a/a_0	log	a/a_o	log	a/a_0	log	a/a_0	log
PP									
0.909	−3.04	44.68	1.650	23.93	1.379	18.27	1.262	23.93	1.379
1.818	−2.74	39.49	1.596	19.59	1.292	18.27	1.262	23.93	1.379
2.727	−2.56	36.01	1.556	18.27	1.262	13.03	1.115	22.42	1.350
TPP									
0.909	−3.04	32.82	1.516	14.10	1.149	10.51	1.021	30.85	1.489
1.818	−2.74	20.27	1.306	18.92	1.277	18.92	1.277	20.27	1.307
2.727	−2.56	17.02	1.231	18.92	1.277	17.64	1.246	18.27	1.262
HPP									
0.909	−3.04	17.02	1.231	14.10	1.149	10.03	1.001	13.02	1.114
1.818	−2.74	11.49	1.060	10.51	1.021	10.03	1.001	12.50	1.097
2.727	−2.56	7.80	0.892	6.96	0.842	6.96	0.842	14.66	1.166

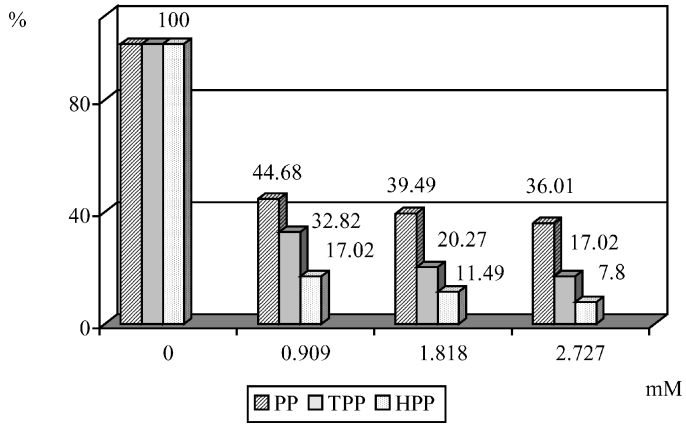


FIGURE 1 The relative activity variation with the condensed phosphates concentration.

activity points to a catalytic effect either by Zn^{2+} (through chelate formation) or by the enzyme on the hydrolysis of P—O—P groups.

Figures 1 and 2 indicate an immediate decrease of the activity following the addition of the ligands. This is explained by the rapid formation of an intermediary ternary complex: enzyme-Zn-ligand.

The inhibition increases with the ligand concentration, as illustrated in Figure 1.

A logarithmic plot of the variation in the relative activity against [L] (Figure 2) shows a linear inhibition curve, with the slope $m < 0$. The slope differs only with the polyphosphatic ligand but remains negative. We computed the residual activity "a" ($t = 1$ h) as a percentage of the control activity "a_0" and plotted the logarithms of these values.

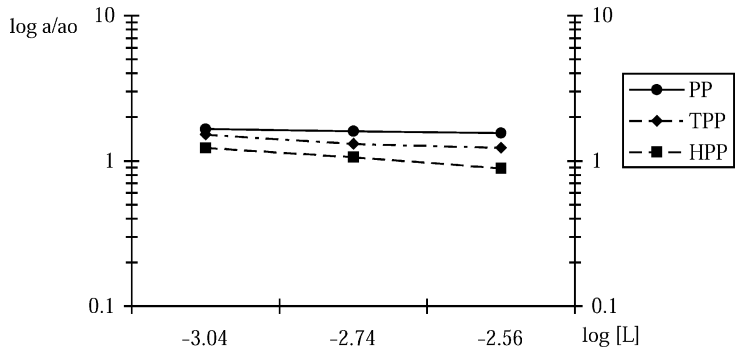
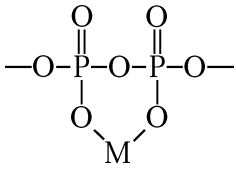


FIGURE 2 The variation of the inhibitory effect of the chelating agents with the concentration, in logarithmic diagram.

The linear regression analysis reveals an intense negative correlation between the ligand concentration and the relative activity of the enzyme. The correlation coefficients are: $r_{PP} = -0.996$, $r_{TPP} = -0.9645$, and $r_{HPP} = -1$.

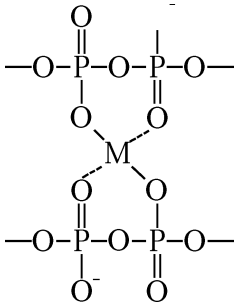
The immediate inhibition may be due to the increased ionic force (nonspecific effect) and the mobilization of Zn (specific effect).[8] The specific effect seems to be more important because at the same ionic force the inhibition decreases in the order HPP > TPP > PP.

The inorganic polyphosphates contain a great number of available donor oxygen atoms in such a way that the metal chelate rings may be formed. It has been proposed that, with suitable orientation, the polyphosphate chain may coordinate as a bidentate (Scheme 2):[9]



SCHEME 2

The stability of the metal–polyphosphate complex increases with the number of the polyphosphate molecules coordinated to a metal ion, the metal ions being chelated between two adjacent molecules (Scheme 3).



SCHEME 3

The chelating capacity increases with increasing the chain length.[10]

Besides studying the influence of the condensed sodium phosphates on the isolated and purified enzyme, we analyzed the effect of the polyphosphates on the native enzyme from the bovine blood. These in

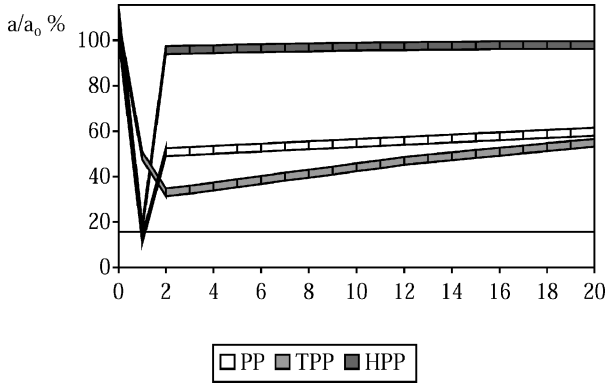


FIGURE 3 The variation of the residual activity (a/a_o) with the incubation time, for 1×10^{-3} M condensed phosphates.

vivo experiments (1×10^{-3} M polyphosphates) also revealed an immediate inhibition (Figure 3).

The inhibition is reversible in time: after 20 incubation hours, the relative activity is 84.84% of the initial activity for HPP. This might be explained by the hydrolysis of the polyphosphates in the presence of the blood polyphosphatases when phosphate is formed,[11] which acts as an enzymatic activator. At double concentrations of ligands, the inhibition is accentuated and is still reversible, but the activity restoration time is longer (Figure 4).

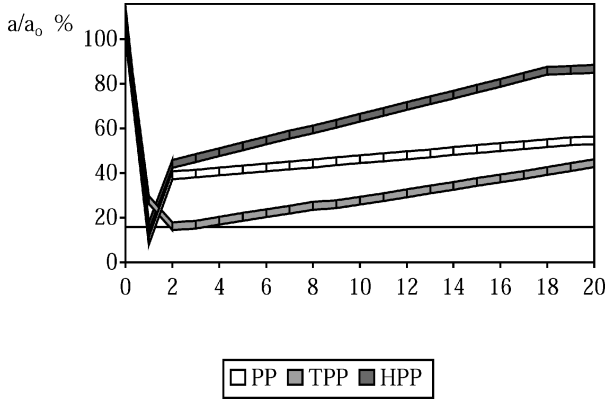


FIGURE 4 The variation of the residual activity (a/a_o) with the incubation time, for 2×10^{-3} M condensed phosphates.

EXPERIMENTAL

BCA II was isolated and purified from bovine erythrocyte. The starting material was bovine blood collected on anticoagulant. The cells were centrifuged down at 3000 rpm 30 min and washed in 0.9% NaCl. The packed cells were lysed by the addition of once their volume of distilled water. This gave a hemolysate with a hemoglobin concentration of 11.02 g/100 ml.

Regarding the amount of protein in erythrocytes, carbonic anhydrase takes the second place as compared to hemoglobin. Hemoglobin represents 98% of the total erythrocytes proteins, and therefore its removal is a key step in BCA separation and purification. After a comparative study between the most-used methods, namely "in batch" adsorption, column chromatography, and fractionating with organic solvents and with neutral salts, we concluded that the most efficient removal of hemoglobin was achieved by chromatography on DEAE-Sephadex A 50 (100%).

A column measuring 2.8×17 cm (100 cm^3) was packed with the resin, which had been equilibrated with 0.01M TRIS-HCl buffer, pH 8.0. The enzyme solution was filtered through the column at a flow rate of 130 ml/h.

The separation of the two forms (A and B) of BCA II was achieved by ion exchange chromatography on DEAE-Cellulose and with higher resolution on DEAE-Sephadex A 50 (Figure 5).

The isoenzymes were eluted from the column with 0.01M TRIS-HCl pH = 8 buffer and 0.08M TRIS-HCl. We obtained a higher resolution

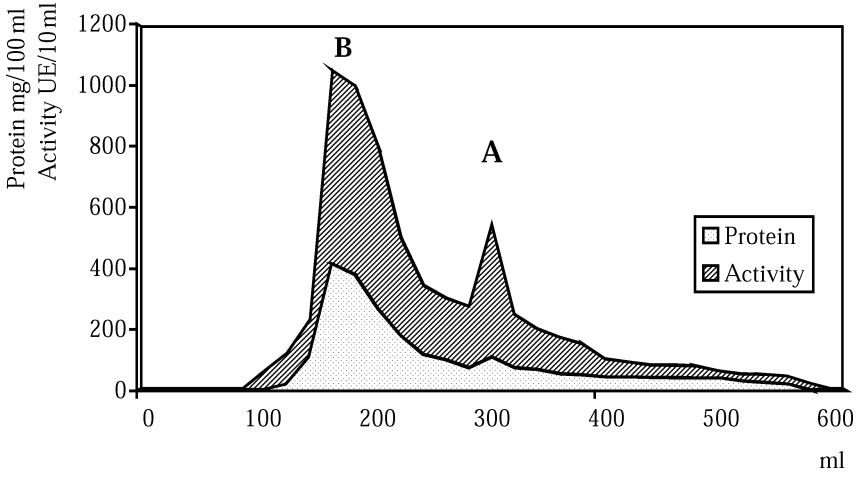


FIGURE 5 Carbonic anhydrase chromatography on DEAE-Sephadex A 50.

in the separation of the two components (A and B) of the enzyme using DEAE-Sephadex A 50. The first letter of the alphabet was given to the most acidic form, which emerges last on DEAE-Sephadex chromatography.

The enzyme (form B) was incubated with the phosphate solution at 4°C, pH = 7, and the enzymatic activity was determined at different time periods. The carbonic anhydrase activity was determined with a method based on that of Wilbur and Anderson, consisting of measuring the decreasing rate of the pH from 8.7 to 6.7 due to the transformation of CO_2 into H_2CO_3. The substrate was a saturated CO_2 solution. The enzyme activity was expressed in UE/ml. One unit (calculated as $(T_0 - T)/T$, where T_0 = the time in seconds corresponding to the variation of pH at the control sample, and T = the time in seconds corresponding to the sample) is defined as the enzyme amount necessary to reduce the reaction time at half of its value:

$$1 \text{ UE} = 52.87 \times 10^{-6} \text{ moles } CO_2/\text{l/s}$$

The experiments were performed with chelating agents at three different concentrations (0.909×10^{-3} M, 1.818×10^{-3} M, and 2.727×10^{-3} M). The residual activity (a) in the presence of the ligand, compared with the control activity (a_o) measured in the absence of the ligand, was calculated as percentage.

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