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Equilibrium Study of Iron(II) and Manganese(II) Complexes with Citrate Ion in Aqueous Solution: Relevance to Coordination of Citrate to the Active Site of Aconitase and to Gastrointestinal Absorption of some Essential Metal Ions*

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Equilibrium study of iron(II) and manganese(II) complexes with citrate ion in aqueous solution at 37 °C and I = 0.15 mol dm^{-3} (KNO_3) is reported. New species, relevant to coordination of citrate to the active site of aconitase, are evidenced. On the basis of stability constants values of dimeric species $Fe_2cit_2H_{-2}$ and $Mn_2cit_2H_{-2}$, an hypothesis is proposed on the inhibition of enzymatic activity by manganous ion. Considerations on the possible effects of the existence of the new species found on the gastrointestinal absorption of some metal ions are also reported.

Introduction

The enzyme aconitase (citrate (isocitrate) hydrolase EC 4.2.1.3) catalyzes the dehydration of citrate or 2R, 3S-(+)-isocitrate to form *cis*-aconitate, the reverse reaction and the interconversion of citrate and 2R, 3S-(+)-isocitrate. It is well known that this enzyme requires ferrous ion and a reducing agent for its maximum activity [1].

Some authors emphasize [2, 3] the formation of a ternary complex by a direct coordination among Fe^{2+} ion, citrate and aconitase, and the competitive inhibition of aconitrase catalytic activity by Mn^{2+} ion, because of formation of a mixed complex on the active site. There is, however, some disagreement about the existence of a single [4] or a double [5] catalytic center on the enzyme and about the structure of citrate in these complexes [3, 6]. Further, recent NMR investigations on Fe(II) citrate system suggests that tetraionized citrate forms tetrameric species [6].

Since this hypothesis has not been supported in literature (only monomeric species of tetraionized citrate have been evidenced [7]), our aim was to investigate the formation and the stability of complexes in the Fe(II)–citrate system, and we took particular care of what complexes form in the pH range optimum for the enzyme activity. Similar investigations were carried out for the system Mn(II)–citrate too, because of its competitive inhibition.

Since citrate is a ligand that sometimes is associated to the treatment with Fe(II) ion of human iron deficiency to favour gastrointestinal absorption of this element, the knowledge of the species present in solution and of their stability constants is quite important. By such data it is possible to show both the competition of different metal ions (Fe(II), Mn(II), Zn(II), *etc.*) for citrate ion and the competition of different ligands (citrate, succinate, folate, gluconate, *etc.*) in their bonding to metal ions, so indicating what ligand may favour the absorption of a given metal ion.

Experimental

Citric acid (C. Erba RPE) was used with no further purification. Fe(II) perchlorate was prepared according to Perrin [8]. Before measurements, the solutions were treated with purified hydrogen in the presence of platinum black to reduce possibly formed Fe(III) ions, and titrated by $KMnO_4$ standard solution. Mn(II) nitrate was a Fluka (*p.a.*) product. Stock solutions of Mn(II) were titrated by EDTA. Fe(II) and Mn(II) stock solutions were kept acid by small amounts of $HClO_4$ or HNO_3 (this excess was determined by the 'GRAN plots' method [9] or by the ACBA least squares program [10]).

The calibration of glass electrode was achieved by titrating dilute HNO_3 solutions (3–6 mmol dm^{-3}) by standard solutions of KOH.

By means of the E° values obtained in acidic solution a constant value of $-\log K_w = 13.34 \pm 0.03$

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TABLE I. Experimental Details of Alkalimetric Titrations.[a]

	C_M	C_{cit}	C_H	Acid Range	No. Points	Neutral–Alkaline Range	No. Points
Iron(II)	1.1	2.0	7.3	2.8–6.1	32	6.5–8.2	12
	1.1	2.5	8.8	2.8–6.1	31	6.5–8.2	6
	10.6	50.0	162.2	2.0–6.1	52	6.5–8.2	4
	1.1	5.1	16.4	2.6–6.1	68	6.5–8.2	17
Manganese(II)	4.9	5.0	15.1	2.8–5.7	20	7.5–8.5	9
	3.8	4.0	12.0	3.0–5.5	14	7.5–8.5	7
	3.0	3.0	9.0	2.9–5.6	19	7.5–8.5	8
	5.7	6.0	18.0	2.7–5.5	22	7.5–8.5	7
	3.0	9.0	27.0	3.0–6.0	25	7.5–8.5	8

[a] C in mmol dm^{-3}, t = 37 °C and I = 0.15 mol dm^{-3} (KNO_3).

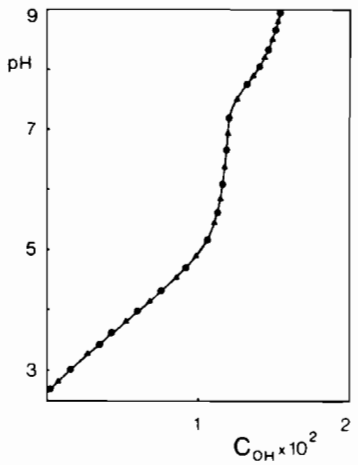

Fig. 1. Alkalimetric titration curve for Mn(II)–citrate system. Experimental points for C_{Mn} = 3.84; C_{cit} = 3.98; C_H = 11.95 mmol dm^{-3}. Full line: calculated from stability constants of Table II.

was found. The data of these titrations in the pH range 2.8–10 were also treated with the ACBA program, obtaining in this way both E° and $-\log K_w$ values: the results obtained with these two methods were in good agreement.

Other experimental details and instrumentation are reported in previous papers [11–14].

Acidimetric back titrations were carried out in order to check equilibrium conditions.

The stability constants, expressed as (charges omitted):

$$\beta_{p,q,r} = [M_p cit_q H_r]\,[M]^{-p}[cit]^{-q}[H]^{-r}$$

where M = Fe(II) or Mn(II); cit = triionized citrate; H = hydrogen ion; p, q, r are, respectively, the indices of metal ion, citrate and hydrogen ion, were first evaluated by graphical methods [15, 16] and then refined by two different least squares computer programs, the former based on the minimization of error squares sum of function $\bar{z}$ ($\bar{z} = (C_H - C_{OH} - [H^+] + [OH^-])\,C_M^{-1}$)* [16, 17], the latter, MINIQUAD [18], based on minimization of error squares sum of analytical concentrations. Calculations were also carried out on the experimental points of all the titration curves. The experimental conditions of alkalimetric titrations are reported in Table I.

Results

Experimental titration curves for Mn(II)–citrate system, as shown in Fig. 1, exhibit two sharp buffer regions, the former in the acidic range of pH values and the latter in the alkaline range. A similar trend is observed for Fe(II)–citrate system.

Acidimetric back titrations show that there are equilibrium conditions up to pH 8.5 for Fe(II)–citrate system and up to pH 9.0 for Mn(II)–citrate system. First we considered the acidic buffer region and evaluated, for each pH–C_{OH} pair, the function Y [15, 16] formally corresponding to $\beta_{1,1,0}$:

$$Y = (C_{cit} - \sum_{i=0}^{3} [H_i cit])[M]^{-1}[cit]^{-1}$$

(C_{cit} = total concentration of citric acid)

The trend of these values *vs.* $1/[H^+]$ (Fig. 2a) suggests the presence, for Mn(II)–citrate system, of Mn(cit)$^-$ and of protonated complexes.

To investigate whether only the monoprotonated complex was present, we also evaluated the function $Y' = Y/[H^+]$, formally corresponding to $\beta_{1,1,1}$. The values of Y′ plotted *vs.* $1/[H^+]$ (Fig. 2b) suggest that the diprotonated complex is present in solution as well.

*C_H, C_{OH} and C_M are, respectively, total concentrations of free and dissociable hydrogen ion, base added for each point of alkalimetric titration curve and metal ion.

TABLE II. Stability Constants for H^+–Fe(II)–Mn(II)–Citrate Systems.

	T °C	I mol dm^{-3}	$\log\beta_{110}$	$\log\beta_{111}$	$\log\beta_{112}$	$\log\beta_{121}$	$\log\beta_{11-1}$	$\log\beta_{22-2}$	Ref.
Iron(II)	25	1 ($NaClO_4$)	3.1	8.2	11.4				23
	20	0.1 ($NaClO_4$)	4.4	8.3					24
	25	0.1 (KNO_3)	4.8	8.6					25
	37	0.15 (KNO_3)	4.56 ± 0.05	8.72 ± 0.05	11.0 ± 0.1	11.91 ± 0.06		−5.4 ± 0.1	this work
	37	0.15[a]	4.70	8.94	11.2	12.2		−5.3	this work
Manganese(II)	25	0.15		9.29					26
	25	0.15		9.2					27
	25	0.15 (NaCl)	3.72						28
	33	0.25 ($NaClO_4$)		7.8					29
	25	0.1 (Me_4NCl)	4.15	8.0					30
	37	0.15 ($NaClO_4$)	3.82				−3.64		31
	37	0.15 (KNO_3)	3.79 ± 0.02	7.84 ± 0.03	11.37 ± 0.07			−5.73 ± 0.05	this work
	37	0.15[a]	3.93	8.06	11.6			−5.59	this work
Proton			$\log\beta_{011}$	$\log\beta_{012}$	$\log\beta_{013}$				
	37	0.15 (KNO_3)	5.62 ± 0.02	9.91 ± 0.03	12.78 ± 0.05				this work
	37	0.15[a]	5.75 ± 0.03	10.12 ± 0.04	12.99 ± 0.06				this work

[a]Corrected for potassium–citrate complex formation.

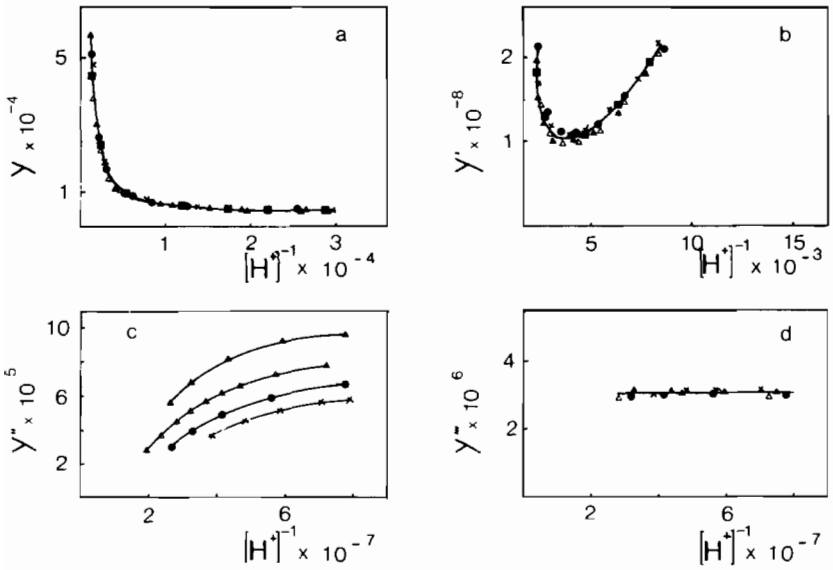

Fig. 2. Mn(II)–citrate system. × C_{Mn} = 3.00; C_{cit} = 2.98; C_H = 8.95; • C_{Mn} = 3.84; C_{cit} = 3.98; C_H = 11.95; △ C_{Mn} = 4.85; C_{cit} = 5.03; C_H = 15.11; ▲ C_{Mn} = 5.65; C_{cit} = 6.00; C_H = 18.02. ■ C_{Mn} = 3.00; C_{cit} = 9.00; C_H = 27.01 (concentrations in mmol dm^{-3}). a) values of Y *vs.* $[H^+]^{-1}$; b) values of Y′ *vs.* $[H^+]^{-1}$; c) values of Y″ *vs.* $[H^+]^{-1}$; d) values of Y‴ *vs.* $[H^+]^{-1}$.

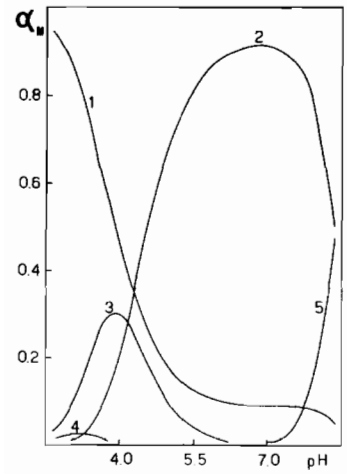

Fig. 3. Fe(II)–citrate system. Distribution of the species as α_{Fe} *vs.* pH. C_{Fe} = C_{cit} = 4.00 mmol dm^{-3}. 1: Fe^{++}; 2: Fe-cit^-; 3: $FecitH^\circ$; 4: $FecitH_2^+$; 5: $Fe_2cit_2H_{-2}^{4-}$

In the same way we could evidence, in the Fe(II)–citrate system, the species $Fe(cit)^-$, $Fe(cit)H^\circ$, $Fe(cit)H_2^+$ and $Fe(cit)_2H^{3-}$, in the acidic buffer region (up to pH = 6.0). Refinements by least squares methods confirmed the presence of these species for both the systems.

The statistical analysis of residuals by R factor [19, 20] for example, confirmed the presence of the species $Mn(cit)H_2^+$ although it forms in small amounts in solution, since $R(\beta_{1,1,0}, \beta_{1,1,1})/R(\beta_{1,1,0}, \beta_{1,1,1}, \beta_{1,1,2}) = 2.4$ (statistically significant value).

Then the neutral alkaline buffer region was examined, neglecting the species $Mn(OH)^+$ and Fe-$(OH)^+$; the function Y″, formally corresponding to $\beta_{1,1,-1}$, was evaluated for each pH–C_{OH} pair:

$$Y'' = (C_{cit} - \sum_{i=0}^{3} [H_icit] - \sum_{j=0}^{2} [MH_jcit])[M]^{-1}[cit]^{-1}[H]^{-1}$$

The values of Y″ plotted *vs.* $1/[H^+]$ (Fig. 2c) for Mn(II)–citrate system indicate the presence of polymeric species; therefore we evaluated another function $Y''' = Y''/[Mn][cit][H]^{-1}$, formally corresponding to $2\beta_{2,2,-2}$: Y‴ values plotted *vs.* $1/[H^+]$ (Fig. 2d) indicate that only a dimeric complex is probably present in solution. Both the systems Fe(II)–citrate and Mn(II)–citrate showed only the formation of dimeric species $M_2(cit)_2H_{-2}$.

Also in this case the refinement by least squares methods confirms very well the results obtained by the graphical methods.

In Table II our values of $\log\beta_{p,q,r}$ are collected and compared with some literature data. The stability constants of Table II are conditional, since citrate ion forms weak complexes with K^+ ion; corrections can be made by the expression:

$$K = K'(1 + K_K[K^+])$$

where K is the corrected formation stability constant; K′ the conditional one and K_K the stability constant of K–citrate complex.

In Table II we also collected the values of $\log\beta_{p,q,r}$ after correction by the literature data on K^+– citrate complexes [22].

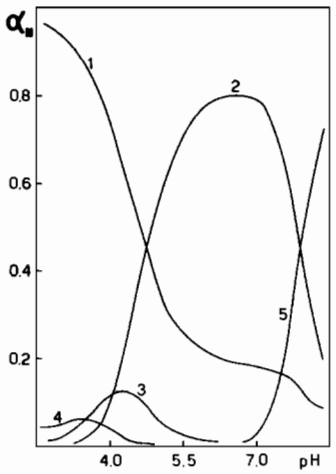

Fig. 4. Mn(II)–citrate system. Distribution of the species as α_{Mn} *vs.* pH. $C_{Mn} = C_{cit} = 4.00$ mmol dm^{-3}. 1: Mn^{++}; 2: $Mncit^{-}$; 3: $MncitH^{\circ}$; 4: $MncitH_2^{+}$; 5: $Mncit_2H_{-2}^{4-}$.

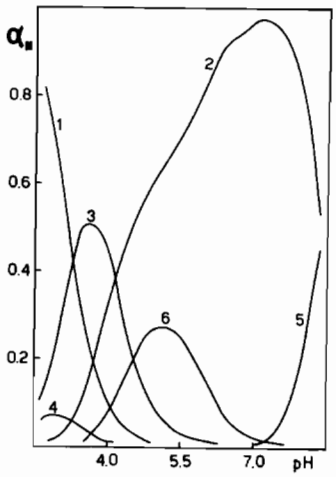

Fig. 5. Fe(II)–citrate system. Distribution of the species as α_{Fe} *vs.* pH. $C_{Fe} = 4.00$; $C_{cit} = 15.00$ mmol dm^{-3}. 1: Fe^{++}; 2: $Fecit^{-}$; 3: $FecitH^{\circ}$; 4: $FecitH_2^{+}$; 5: $Fe_2cit_2H_{-2}^{4-}$; 6: $Fecit_2H^{3-}$.

In Figs. 3 and 4 species distributions are reported for Fe(II)–citrate and Mn(II)–citrate systems respectively, as α_M (fraction of metal ion present in each complex) *vs.* pH. In Fig. 5 there is a species distribution in which the excess of citrate emphasizes the formation of $Fe(cit)_2H$ complex. These plots moreover show the importance of $M_2cit_2H_{-2}$ species for pH values higher than 7.

Discussion

The values of stability constants of the complexes $M(cit)^{-}$ and M(cit)H are in good agreement with those previously reported (see Table II) while the species $M_2cit_2H_{-2}$, for the first time brought to light for Fe(II) and Mn(II) in this study, are the same as previously found for citrate complexes with Cu(II) [11], Ni(II) [12] and Zn(II) [13].

Also the species $Fe(cit)_2H$ was not yet reported in literature. No evidence was found for the presence in solution of the species $M_4(cit)_4H_{-4}$.

NMR spectra [6] evidenced several forms of the ferrous species with citrate in alkaline solution. However, the spectrum at −8 °C indicated that a single form predominates near the freezing point of the solution. Spectra carried out at pH 8.15 and 9.25 were superimposable with those at pH 7.6 at the same temperature.

On the basis of the X-ray crystallographic investigation of a nickel–citrate complex crystallized from a solution of pH 9.2 [32] (which was considered an excellent model for the configurations and conformations of transition metal–citrate complexes in alkaline solution) and on the basis of molecular weight determination, it was proposed that the predominant form of the ferrous complex in cold alkaline solution is a tetrameric cluster. On the contrary, from species distributions of Figs. 3 and 4, calculated by our potentiometric results at 37 °C, it can be seen that the species Fe(cit) and $Fe_2(cit)_2H_{-2}$ are predominant in the pH range where aconitase exhibits its maximum activity.

Equilibrium studies first confirm the existence in aqueous solution of species of Fe(II) bridge-linked through hydroxyl group of citrate (its pK^{H} value is quite lowered by the presence of metal ion). Therefore, we may suppose that the aconitase–Fe(II)–citrate ternary complex involves a bridge coordination of tetraionized complex with two Fe(II) ions at the aconitase active site.

Further, the log of constants for the equilibrium:

$$2M\,cit \rightleftarrows M_2cit_2H_{-2} + 2H^{+}$$

are −14.52 for Fe(II) and −13.31 for Mn(II) (they can be calculated from data of Table II) and therefore the tendency of Mn(II) to form dimeric complex is greater than for Fe(II) and this suggests an explanation of the inhibition by Mn(II).

Therefore, on the basis of this thermodynamic investigation *in vitro* it can be observed that:

i) two Fe^{2+} ions are involved in coordination with citrate in slightly alkaline solution and perhaps in the ternary complex with aconitase too.

ii) the inhibition by manganous ion may be due to the fact that the complexes of Mn(II) and Fe(II) with citrate have a similar structure and that these complexes have a stability not very different.

*In the refinement by the least squares methods the species $Mn(OH)^{+}$ and $Fe(OH)^{+}$ were also taken into account ($\log\beta_{1,0,-1} = -10.3$ and −9.3 respectively [21]).

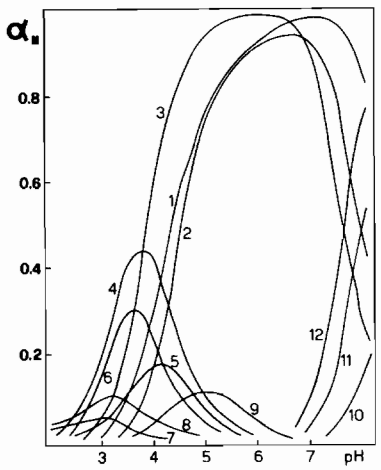

Fig. 6. Fe(II), Mn(II), Zn(II)–citrate system. Distribution of the species as α_M *vs.* pH. $C_{Fe} = C_{Mn} = C_{Zn} = 2.00$; $C_{cit} = 9.00$ mmol dm^{-3}. 1: Fecit$^-$; 2: Mncit$^-$; 3: Zncit$^-$; 4: FecitH°; 5: MncitH°; 6: ZncitH°; 7: FecitH$_2^+$; 8: MncitH$_2^+$; 9: Fecit$_2$H^{3-}; 10: Fe$_2$cit$_2$H$_{-2}^{4-}$; 11: Mn$_2$cit$_2$H$_{-2}^{4-}$; 12: Zn$_2$cit$_2$H$_{-2}^{4-}$.

From many years the ingestion of ferrous salts is recommended in the therapy of iron-deficiency diseases [33]. Initially for this purpose inorganic salts were used, but, successively, both to increase the percent of absorbed metal (generally not more than 10% of the ingested amount) and to decrease the negative side effects, organic iron(II) salts have been proposed. The metal ion absorption is particularly important for the homeostasis of iron metabolism because the excretion is not relevant [34]. Nevertheless, the mechanism responsible for iron absorption is still unknown, though different hypotheses have been proposed [35–37] and some conclusions attained both on the characteristics of organic ligands and on the active species that are able to work as carrier of ferrous ion [38]. In particular, it is definitely acquired that the formation of iron(II) neutral complexes with organic ligands having a low molecular weight increases the gastrointestinal absorption of metal ions.

Citrate sometimes is given to favour human gastrointestinal absorption of Fe(II); it may be interesting to represent the species distribution of a multicomponent system, including other metal ions of biological interest (these metal ions might be associated in the treatment with Fe(II)).

In Fig. 6 a distribution of the species is drawn as α_M *vs.* pH for the system Fe(II), Mn(II), Zn(II) citrate obtained by SCOLM program [39] (the stability constants relative to the system Zn(II)–citrate were previously reported [13] at 25 °C, I = 0.1 mol dm^{-3}, but the difference in the experimental conditions would not distort the results of our simulation).

It can be observed that there is no pH range in which a predominant neutral complex (liposoluble) exists and therefore citrate does not seem to be suitable to favour the absorption of these three metal ions: only near pH = 4 are there present in relevant amount the neutral species M(cit)H^0.

Previous investigations [31, 40], which neglected the formation of dimeric complexes, resulted in a different distribution of the species. The corresponding indications about the ligands of choice to be used as iron(II) carriers, must be revised on the basis of the new data on metal–citrate systems.

Further investigations in this field are needed in order to eventually substitute the presently suggested ligands.

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