Cadmium-Isocitrate Complex: Its Stability as a Function of Ionic Strength *

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Isocitrate Dehydrogenase, Isocitrate, Cadmium, Complexes, Ionic Strength

The complexation of cadmium by isocitrate has been studied at 25 °C and pH 7.5 in a range of ionic strength from 0.01 to 0.16. The formation constant of the complex between cadmium and tribasic isocitrate varies from 860 M^{-1} at $\mu=0.16$ to approximately 24,500 M^{-1} at infinite dilution. These data allow the distribution of the chemical forms of cadmium added to the incubation mixtures for the assay of NAD-dependent isocitrate dehydrogenase to be calculated.

A metal requirement has long been established for the reactions catalyzed by isocitrate dehydrogenases [1]. It has been shown that NAD-dependent isocitrate dehydrogenase (*threo*-D_s-isocitrate : NAD-oxidoreductase, E.C. 1.1.1.41) requires Mg^{2+}, Mn^{2+}, or Co^{2+} for activity [2]. The examination of several divalent metal ions as activators of the reactions catalyzed by native NADP-dependent isocitrate dehydrogenase (*threo*-D_s-isocitrate : NADP-oxidoreductase, E.C. 1.1.1.42) indicates that Mn^{2+}, Cd^{2+}, Zn^{2+}, Mg^{2+}, and Co^{2+} permit significant rates for the over-all oxidative decarboxylation of isocitrate [3].

More detailed studies of the NAD- and NADP-dependent enzymes, conducted respectively with magnesium an manganous ions led to the conclusion that the actual substrates are the tribasic isocitrate-metal complexes [4, 5].

It is therefore of interest to understand the function of other divalent metals in the activation process. These studies require the knowledge of the concentrations of the various ionic species present in the incubation mixtures for the kinetic assay of enzyme activity. From the values of the ionization constants for isocitrate and pyridine nucleotide, NAD or NADP, and the formation constants of the complexes between the metal and the different ionic species of isocitrate and pyridine nucleotide, the concentrations of free and complexed forms of metal, isocitrate and pyridine nucleotide can be calculated for every given set of total concentrations of the components [4, 5].

Since the association compounds between cadmium and either isocitrate, NAD or NADP are not described in literature, an investigation was undertaken to provide this information for use in successive studies directed to understand the mechanism involved in the activation of isocitrate dehydrogenase by cadmium ions.

Experimental

Complexes involving isocitric acid and alkaline earths [6-8] or manganous [7] ions are rather weak, with apparent formation constants ranging in the order $10^2 - 10^3$. Therefore, two techniques which can be applied to the study of weak complexes, such as the ion-exchange resin method [9] and the cadmium specific-ion electrode method [10], were selected for this investigation.

Method with ion-exchange resin. All solutions were freshly prepared with bidistilled water and adjusted to the required ionic strength with reagent grade sodium nitrate. DL-isocitric acid, trisodium salt, was purchased from Serva GmbH. N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid (Hepes), a BDH biochemical reagent, was employed for the preparation of the buffer solution. This zwitterionic heterocylic compound having an excellent buffer capacity at pH 7.5 exerts reduced ion effect and negligible complexing action on divalent metal ions [11]. The buffer solution was prepared by titration of Hepes to pH 7.50 with NaOH. Radioactive cadmium was obtained from the Amersham Radiochemical Centre as carrier-free $^{109}CdCl_2$ (spe-

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cific activity: > 50 mCi/mg Cd) in 0.1 M HCl. The sodium form of the synthetic cation exchanger AG 50W-8X was purchased from Bio-Rad Laboratories. ^{109}Cd radioactivity was measured with a Packard Auto-gamma scintillation spectrometer. The procedure was as follows. Hepes-NaOH buffer (10 ml) adjusted to the required concentration in Na$^+$ was added to each series of ten flasks containing a weighed amount of the resin. The buffer contained tracer levels of ^{109}Cd. A predetermined volume of isocitrate solution was added to each flask. Four different concentrations of isocitrate were employed, the concentration range chosen depended on the ionic strength. The volume in each flask was brought to 100 ml with a solution of NaNO$_3$ having the required ionic strength. Two of the ten flasks contained zero concentration of isocitrate, and an additional two flasks containing only the buffer plus sodium nitrate were run as blanks. After shaking the flasks in a thermostated water bath for six hours, an aliquot of the supernatant from each was removed for the assay of ^{109}Cd radioactivity. A typical system for $\mu = 0.16$ and the experimental data are shown in Table I.

Method with cadmium specific-ion electrode. In addition to Hepes-NaOH buffer, sodium nitrate and isocitrate, this method requires a standard solution of cadmium. This solution was prepared with reagent grade Cd(NO$_3$)$_2$ 4 H$_2$O. Measurements of cadmium ion concentration were made in a water-jacketed cell containing 50 ml of the background electrolyte (Hepes-NaOH buffer 0.02 M, pH 7.5, adjusted to the required ionic strength with NaNO$_3$). The cell contained a cadmium specific-ion electrode

(Orion 94-48A), a single junction Ag/AgCl reference electrode (Orion 90-01), and a glass electrode with internal reference connected to a Radiometer PHM-26 pH-meter with scale expander. The potential between the specific-ion and the reference electrode was measured using a digital pH/mV meter Radiometer PHM-64. The temperature was maintained at 25 ± 0.2 °C. Magnetic stirring at approximately 200 rpm. A period of 10 min was allowed for the electrode system to reach equilibrium. Afterwards, readings were taken every 30 sec for 10 min. The standard error on the average of these 20 readings was within 0.1 mV. The electrode was standardized in the range of cadmium concentration between 10^{-5} and 10^{-4} M by the addition of known quantities of cadmium nitrate to the background electrolyte. When the solution was 10^{-4} M in cadmium, the potential between the specific-ion and the reference electrode was measured in the presence of known concentrations of isocitrate. Since the calibration had been carried out in a non-complexing solution with the same total cadmium concentration and the same ionic strength, these measurements allowed the calculation of free cadmium concentration in equilibrium with cadmium-isocitrate and free isocitrate. The system employed for $\mu = 0.16$ and the experimental data are given in Table II.

Results

Apparent dissociation constants of isocitric acid at 25 °C and $\mu = 0.1$, expressed as pK$_a$, are 3.02, 4.28, and 5.75 [7]. At pH 7.50, the predominant ionic

Table I. Ion exchange resin: experimental system and results obtained for $\mu = 0.16$. Resin, sodium form of Bio-Rad AG50W-8X, 20—50 mesh, equilibrated with 0.16 M NaNO$_3$. Buffer solution, Hepes-NaOH 0.2 M, pH 7.50, containing ^{109}CdCl$_2$; sodium isocitrate solution 0.020 M; sodium nitrate solution 0.16 M. All solutions were made 0.16 M in Na$^+$ with NaNO$_3$. Temperature 25 °C; pH 7.50; shaking time 6 hours.

Flask No.	Resin [mg]	Buffer [ml]	NaNO$_3$ [ml]	Isoc. [ml]	Final isocitrate conc. [M]	^{109}Cd activity in equil. soln. [cpm/ml]	Average amount ^{109}Cd in separated phases [%] Solution	Resin	K_d	K_f
1	0	10.0	90.0	0	0	201.81				
2	0	10.0	90.0	0	0	204.19	100	0	· · ·	· · ·
3	1000	10.0	90.0	0	0	39.62				
4	1000	10.0	90.0	0	0	39.30	19.44	80.56	0.414 (K_d^0)	· · ·
5	1000	10.0	85.0	5.0	1×10^{-3}	62.43				
6	1000	10.0	85.0	5.0	1×10^{-3}	63.58	31.04	68.96	0.222	865
7	1000	10.0	80.0	10.0	2×10^{-3}	79.52				
8	1000	10.0	80.0	10.0	2×10^{-3}	81.55	39.67	60.33	0.152	862
9	1000	10.0	75.0	15.0	3×10^{-3}	95.53				
10	1000	10.0	75.0	15.0	3×10^{-3}	92.86	46.40	53.60	0.116	856
11	1000	10.0	70.0	20.0	4×10^{-3}	103.25				
12	1000	10.0	70.0	20.0	4×10^{-3}	101.73	50.49	49.51	0.098	806

Average $K_f \pm$ S.E. $= 847 \pm 14$

Standardization of the electrode

Standard solution [μl]	Cadmium conc. [μM]	Negative potential [mV ± S.E.]
0	0	273.47 ± 0.08
5	10	215.52 ± 0.06
10	20	206.67 ± 0.05
20	40	197.81 ± 0.04
30	60	192.63 ± 0.05
40	80	188.95 ± 0.04
50	100	186.10 ± 0.04

Table II. Cadmium electrode: experimental system and results obtained for $\mu = 0.16$. Cadmium specific-ion electrode Orion 94-48 A. Single junction reference electrode Orion 90-01. Digital pH/mV meter Radiometer PHM-64. Electrolyte solution: 50 ml Hepes-NaOH buffer 0.02 M, pH 7.50, adjusted to $\mu = 0.16$ with NaNO$_3$. Standardizing solution: 0.100 M Cd(NO$_3$)$_2$ 4 H$_2$O. Sodium isocitrate solution: 1.0 M. Temperature 25 °C; pH 7.50; magnetic stirring. Equilibration time 10 min, then readings every 30 sec for 10 min.

Measurements of cadmium ion concentration in the presence of isocitrate

Isocitrate solution [μl]	Isocitrate concentration [μM]	Negative potential [mV ± S.E.]	[Cd^{2+}] [μM]	[Cd-Isoc.$^-$] [μM]	[Isoc.$^{3-}$] [μM]	K_f [M^{-1}]
20	400	189.76 ± 0.05	75.12	24.88	375.12	883
40	800	192.48 ± 0.04	60.68	39.32	760.68	852
60	1200	195.25 ± 0.05	49.08	50.92	1149.08	903
80	1600	196.78 ± 0.04	43.34	56.66	1543.34	847

Average K_f ± S.E. = 871 ± 13

species, representing more than 98% of total isocitrate concentration, is the anion isocitrate^{3-}. Therefore, it may be assumed that in our experimental conditions only tribasic isocitrate ions are present in solution.

The formation constant for the complex ion (Cd-isocitrate$_n$)$^{2-3n}$, in terms of the equilibrium ion-exchange formulation [9]. is given by the expression:

$$K_f = \frac{(K_d^0/K_d) - 1}{[\text{isocitrate}]^n} \qquad (1)$$

where K_d^0 and K_d are the distribution coefficients obtained in the absence and presence, respectively, of the isocitrate. The distribution coefficient for the cadmium ion is defined as:

$$K_f = \frac{\% \text{ cadmium in the resin}}{\% \text{ cadmium in solution}}$$
$$\times \frac{\text{volume of solution [ml]}}{\text{mass of resin [mg]}} = R \frac{v}{m}$$

where R represents the percent ratio.

The Eq. (1) assumes that cadmium is complexed only by isocitrate, i. e. that complex formation by other anions in solution does not occur. This proved to be true in our experimental system. Nitrate does not complex cadmium to any appreciable extent [12]. Formation of cadmium hydroxide has been shown to be negligible at pH 7.5 [10]. Addition of increasing concentrations of Hepes buffer to a solution of cadmium nitrate in NaNO$_3$ did not modify the distribution coefficient of cadmium ion.

The term K_d^0 can be obtained experimentally or calculated from values of K_d by graphic or analytical methods. It is convenient to plot $1/K_d$ vs [isocitrate] and to extrapolate the straight line for proper values of n to zero concentration of isocitrate, as indicated by the relation:

$$\frac{1}{K_d} = \frac{1}{K_d^0} + K_f \frac{[\text{isocitrate}]^n}{K_d^0} .$$

This procedure confirmed the experimentally determined values of K_d^0 (Fig. 1). In the whole range of isocitrate concentration employed in this investigation it was found that $n = 1$. Therefore, the ion-exchange resin method can be used to study the formation of a 1:1 complex between cadmium and isocitrate, according to the reaction:

$$\text{Cd}^{2+} + \text{isocitrate}^{3-} \rightleftarrows \text{Cd-isocitrate}^- .$$
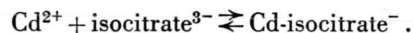
The distribution coefficient of cadmium ion in the absence of isocitrate was strongly affected by
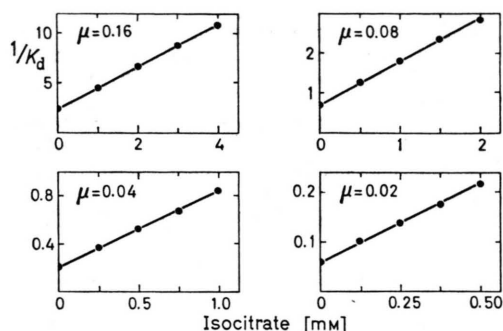
Fig. 1. Variations of the reciprocal of the distribution coefficient for Cd^{2+} as a function of isocitrate concentration.

variations in ionic strength. However, if the $K_d{}^0$ coefficient was multiplied by the square of sodium ion activity, a constant value was obtained independently of the ionic strength of the solution, as expected on the basis of the ion-exchange reaction [13].

The data relating to the formation constant of cadmium-isocitrate in the range of ionic strength between 0.16 and 0.01 are presented in Table III and will be examined together with the results obtained with the cadmium specific-ion electrode.

Table III. Interaction of cadmium ion with isocitric acid at different ionic strengths (temperature 25 °C; pH 7.50).

Ionic strength $[\mu]$	Formation constant (K_f)		$\log K_f$ (average)	Ratio of activity coefficients [a]	$\log K_f{}^0$ (inf. diln.)[b]
	Ion-exchange resin	Cadmium electrode			
0.160	847	871	2.93	29.1	4.40
0.140	924	956	2.97	25.8	4.38
0.120	1034	1075	3.02	22.2	4.37
0.100	1218	1280	3.10	18.5	4.36
0.090	1349	1402	3.14	16.8	4.36
0.080	1496	1563	3.18	15.0	4.36
0.070	1721	1792	3.24	13.2	4.37
0.060	2018	2097	3.31	11.8	4.38
0.050	2392	2547	3.39	9.83	4.39
0.040	2915	3163	3.48	8.01	4.39
0.030	3736	3975	3.59	6.47	4.40
0.025	4383	4497	3.65	5.53	4.39
0.020	5268	5583	3.73	4.81	4.42
0.015	6314	6605	3.81	4.03	4.42
0.010	7773	8065	3.90	3.35	4.42
0	23500 [c]	25000 [c]	4.38 [c]		

[a] $\gamma_{\text{Cd-isoc.}}/\gamma_{Cd^{2+}}\,\gamma_{\text{isoc.}^{3-}}$ obtained from individual ion activity coefficients calculated [14, 15] assuming that ionic radii of isoc.$^{3-}$ and Cd-isoc.$^-$ are equal to those reported by Kielland [16] for citrate^{3-} and H_2-citrate$^-$.

[b] Calculated by multiplication at a given ionic strength of the average K_f value by the activity coefficient ratio.

[c] Obtained by extrapolation of a plot of $\log K_f$ vs $\sqrt{\mu}$.

Theoretically, the potential between the cadmium specific-ion electrode and the reference electrode follows the relation:

$$E = E_a + \frac{2.3026\,RT}{2\,F} \log a_{Cd^{2+}}$$

where E_a is the potential due to reference electrode and internal solutions, $2.3026\,RT/2\,F$ is the Nernst factor, and $a_{Cd^{2+}}$ is the cadmium ion activity in the solution. Taking into account the activity coefficient $\gamma_{Cd^{2+}}$ and the concentration of cadmium ion:

$$E = A + B \log \gamma_{Cd^{2+}} + B \log[Cd^{2+}]$$

where A and B are constants. In good agreement with this expression, plots of E vs $\log[Cd^{2+}]$, with $[Cd^{2+}]$ from 10^{-5} to 10^{-4} M, where straight lines in the whole range of ionic strength taken into consideration. The average slope obtained from 15 experiments was 29.276 ± 0.178 mV for the 10-fold increase in cadmium concentration. This value is in very good agreement with the theoretical Nernst factor of 29.58 mV. Therefore, the potential E can be expressed as a linear function of cadmium concentration:

$$E = C + B \log[Cd^{2+}]$$

where $C = A + B \log \gamma_{Cd^{2+}}$ varies with the ionic strength of the solution, and also from day to day at constant ionic strength, over a range of about 1.2 mV. Therefore, in each experiment the electrode was calibrated in the interval of cadmium concentration between 10^{-5} and 10^{-4} M. Increasing amounts of isocitrate were added to the solution and the potential was measured after each addition. The results of a typical experiment are shown in Fig. 2. From the calibration curve:

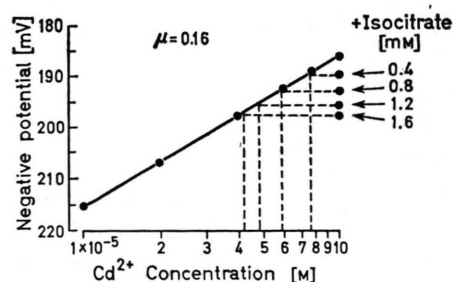$$\Delta E = 29.422\,(4 - \log[Cd^{2+}])$$

Fig. 2. Variations in potential of the cadmium specific-ion electrode with Cd^{2+} concentration and after the addition of increasing amounts of isocitrate to the solution 0.1 mM in cadmium nitrate. See text and Table II for a detailed description of the experimental procedure.

so that cadmium ion concentration in the presence of isocitrate could be calculated from the measured potentials:

$$[Cd^{2+}] = 10^{-(4+\Delta E/29.422)}.$$

Since:

$$[Cd\text{-isocitrate}^-] = [Cd_{tot.}] - [Cd^{2+}] \qquad (2)$$

$$[\text{isocitrate}^{3-}] = [\text{isocitrate}_{tot.}] - [Cd\text{-isocitrate}^-]$$

the formation constant:

$$K_f = \frac{[Cd\text{-isocitrate}^-]}{[Cd^{2+}][\text{isocitrate}^{3-}]} \qquad (3)$$

could be easily calculated.

Measurements of free cadmium ion concentration in the presence of increasing concentrations of isocitrate were carried out in the interval of ionic strength between 0.16 and 0.01. Some of these results are presented in Fig. 3. Since the highest isocitrate concentration used in each experiment modified the total ionic strength by less than 10% and
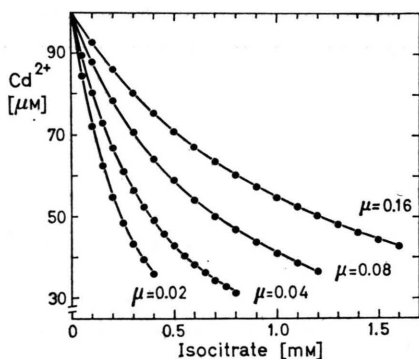
Fig. 3. Measured concentrations of free cadmium ion after the addition of increasing amounts of isocitrate to 0.1 mM solutions of cadmium nitrate at different ionic strength.

the response of the electrode is practically unaffected by such a change, the potential measured could be considered as proportional to the logarithm of free cadmium concentration. The distribution of ionic species present in solution was calculated according to Eq. (2).

Plots of the logarithm of the ratio [Cd-isocitrate$^-$]/[Cd^{2+}] against the logarithm of free isocitrate concentration [isocitrate^{3-}] were straight lines (Fig. 4), as predicted by the Eq. (3) which can be written as follows:

$$\log\frac{[Cd\text{-isocitrate}^-]}{[Cd^{2+}]} = \log K_f + \log[\text{isocitrate}^{3-}]$$
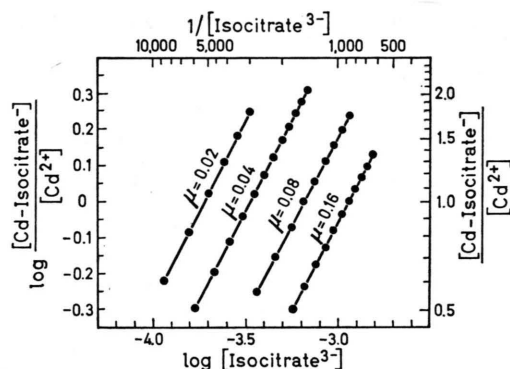
Fig. 4. Graphs of $\log[Cd\text{-isocitrate}^-]/[Cd^{2+}]$ against $\log[\text{isocitrate}^{3-}]$. Total cadmium concentration: 0.1 mM.

from which the K_f value could be obtained:

$$\log K_f = \log\frac{[Cd\text{-isocitrate}^-]}{[Cd^{2+}]} - \log[\text{isocitrate}^{3-}]$$

in particular, when [Cd-isocitrate$^-$] is equal to [Cd^{2+}]:

$$pK_f = \log[\text{isocitrate}^{3-}].$$

Table III contains the data relating to the effect of ionic strength on the formation constant of cadmium-isocitrate. It can be seen that the method with the cadmium ion-specific electrode provided results slightly higher than those obtained by the ion-exchange resin method.

The agreement between the average value of the formation constant at infinite dilution calculated from individual ion activity coefficients [14-16] and that extrapolated from a plot of $\log K_f$ against $\sqrt{\mu}$ (Fig. 5) is very satisfactory. The slope of the extrapolated region of the curve is -5.2 and the standard free energy change of the complexation reaction, calculated as $\Delta F^0 = -RT\ln K_f^0$, is approximately -6000 cal.

Discussion

As shown in Table IV, the formation constant of the complex cadmium-isocitrate is higher than those reported for the complexes of isocitrate with alkaline earths metals, but does not differ significantly from that of the complex manganese-isocitrate.

The knowledge of the formation constant (K) of the complex cadmium-isocitrate allows the concentration of free cadmium ion (Cd$_f$) in solutions containing cadmium and isocitrate to be calculated from total cadmium (Cd) and total isocitrate (I) concentrations.
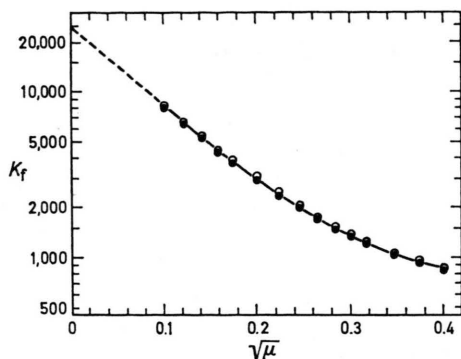
Fig. 5. Variation of the formation constant of cadmium-isocitrate with ionic strength. The symbols ● represent results obtained by the ion-exchange resin method; the symbols ○ indicate results obtained by the use of cadmium specific-ion electrode.

Table IV. Apparent formation constants of complexes between divalent metals and isocitrate.

Complex	μ	K_f	Method	Reference
Mg-isocitrate	0.10	525	pH-titration	(7)
Mg-isocitrate	0.10	476	enzymatic	(8)
Ca-isocitrate	0.16	295	ion-exchange	(6)
Sr-isocitrate	0.16	105	ion-exchange	(6)
Mn-isocitrate	0.10	1148	pH-titration	(7)
Cd-isocitrate	0.16	847	ion-exchange	this work
Cd-isocitrate	0.10	1218	ion-exchange	this work
Cd-isocitrate	0.16	871	Cd-electrode	this work
Cd-isocitrate	0.10	1280	Cd-electrode	this work

By substitution of Eq. (2) into Eq. (3):

$$K = \frac{Cd - Cd_f}{Cd_f[I - (Cd - Cd_f)]}$$

and solution of the resulting quadratic equation, we obtain:

$$Cd_f = \frac{Cd - I - K^{-1} \pm \sqrt{(I - Cd + K^{-1})^2 + 4\,Cd\,K^{-1}}}{2}$$

The concentration of cadmium-isocitrate and that of free isocitrate can be calculated from the conservation Eq. (2).

The results presented in Fig. 6 show how the distribution of the chemical forms of cadmium in a solution containing 0.25 mM cadmium nitrate and 0.50 mM isocitrate is influenced by changes of total ionic strength. It becomes clear that a comparison between different metal ions as activators of isocitrate dehydrogenases can be made only when the kinetic assays have been carried out in solutions of comparable ionic strength. However, the knowledge of the formation constants in a large interval of
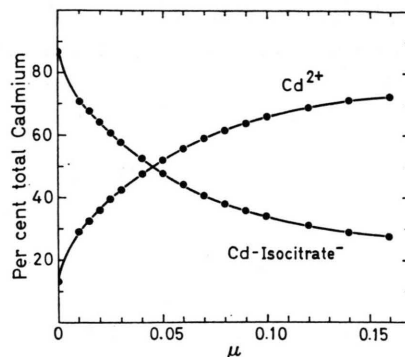

Fig. 6. Calculated distribution of the chemical forms of cadmium in solutions of various ionic strengths containing 0.50 mM isocitrate and 0.25 mM cadmium nitrate.

ionic strength allows the concentrations of free and isocitrate-bound metal to be calculated [17] in any experimental condition. This information can be utilized to evaluate enzyme kinetic parameters as a function of ionic strength.

The data presented in this paper can be applied to the reaction mixtures for the kinetic assay of NAD-dependent isocitrate dehydrogenase, since the binding of divalent ions to NAD is relatively weak [18, 19] and the concentration of NAD is generally so low that it will not influence the distribution of the chemical forms of cadmium. Modifiers of the enzymatic reaction, such as nucleoside di- and triphosphates, should be absent since they exert complexing action on divalent metal ions [19, 20].

In the case of the NADP-dependent enzyme, the distribution of the various chemical forms of cadmium is complicated by the fact that the secondary phosphate of NADP has a considerable affinity for divalent metal ions [19]. Preliminary results showed that when the 2′-phosphate is doubly ionized the value of the formation constant of the complex cadmium-NADP is approximately equal to that of the complex cadmium-isocitrate. Since the concentrations of NADP in the reaction mixtures for the assay of isocitrate dehydrogenase are generally of the same order of magnitude of those of isocitrate, a competition between the two ligands for cadmium will occur. This competition must be considered in order to evaluate properly the effects of NADP and divalent metal ions on the reaction kinetics.

At present, studies are in progress to determine the influence of ionic strength on the formation constant of the complex cadmium-NADP. These data will be utilized for the calculation [21] of the distribu-

tion of the various chemical forms of cadmium, another divalent metal, isocitrate, and NADP, present in the incubation mixtures for the assay of NADP-dependent isocitrate dehydrogenase.

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