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 μ = 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 dehydrogenases¹. It has been shown that NAD-dependent isocitrate dehydrogenase (threo-D₃-isocitrate : NAD-oxidoreductase, E.C. 1.1.1.41) requires Mg²⁺, Mn²⁺, or Co²⁺ for activity². The examination of several divalent metal ions as activators of the reactions catalyzed by native NAD-dependent isocitrate dehydrogenase (threo-D₃-isocitrate : NAD-oxidoreductase, E.C. 1.1.1.42) indicates that Mn²⁺, Cd²⁺, Zn²⁺, Mg²⁺, and Co²⁺ permit significant rates for the over-all oxidative decarboxylation of isocitrate³.

More detailed studies of the NAD- and NADP-dependent enzymes, conducted respectively with magnesium and manganous ions led to the conclusion that the actual substrates are the tribasic isocitrate-metal complexes⁴,⁵.

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 components⁴,⁵.

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 earths⁶—⁸ or manganous ions⁷ are rather weak, with apparent formation constants ranging in the order 10²—10⁵. Therefore, two techniques which can be applied to the study of weak complexes, such as the ion-exchange resin method⁹ and the cadmium specific-ion electrode method¹⁰, 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 heterocyclic compound having an excellent buffer capacity at pH 7.5 exerts reduced ion effect and negligible complexing action on divalent metal ions¹¹. 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-free¹²¹CdCl₂ (spe-
cific activity: \( >50 \text{ mCi/mg Cd} \) in 0.1 M HCl. The sodium form of the synthetic cation exchanger AG 50W-8X was purchased from Bio-Rad Laboratories. 109Cd 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 109Cd. 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 NaN03 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 109Cd 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(NO3)2·4H2O. 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 NaN03). 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 \( 10^{-4} \text{ M} \) by the addition of known quantities of cadmium nitrate to the background electrolyte. When the solution was \( 10^{-4} \text{ M} \) 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. At pH 7.50, the predominant ionic

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</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>10.0</td>
<td>90.0</td>
<td>0</td>
<td>0</td>
<td>201.81</td>
<td>100</td>
<td>0</td>
<td>...</td>
<td>...</td>
</tr>
</tbody>
</table>
| 2        | 0          | 10.0        | 90.0       | 0          | 0                         | 204.19                                 | 19.44                                    | 80.56 | 0.414 | (Kd) | ...
| 3        | 1000       | 10.0        | 90.0       | 0          | 0                         | 39.62                                  | 31.04                                    | 68.96 | 0.222 | 865  |
| 4        | 1000       | 10.0        | 90.0       | 0          | 0                         | 39.30                                  | 19.44                                    | 80.56 | 0.414 | (Kd) | ...
| 5        | 1000       | 10.0        | 85.0       | 5.0        | 1 x 10⁻³                   | 62.43                                  | 63.58                                    | 68.96 | 0.222 | 865  |
| 6        | 1000       | 10.0        | 85.0       | 5.0        | 1 x 10⁻³                   | 79.52                                  | 79.52                                    | 68.96 | 0.222 | 865  |
| 7        | 1000       | 10.0        | 80.0       | 10.0       | 2 x 10⁻³                   | 81.55                                  | 39.67                                    | 60.33 | 0.152 | 862  |
| 8        | 1000       | 10.0        | 80.0       | 10.0       | 2 x 10⁻³                   | 95.53                                  | 95.53                                    | 60.33 | 0.152 | 862  |
| 9        | 1000       | 10.0        | 75.0       | 15.0       | 3 x 10⁻³                   | 103.25                                 | 46.40                                    | 53.60 | 0.116 | 856  |
| 10       | 1000       | 10.0        | 75.0       | 15.0       | 3 x 10⁻³                   | 101.73                                 | 50.49                                    | 49.51 | 0.098 | 806  |
| 11       | 1000       | 10.0        | 70.0       | 20.0       | 4 x 10⁻³                   | 101.73                                 | 50.49                                    | 49.51 | 0.098 | 806  |
| 12       | 1000       | 10.0        | 70.0       | 20.0       | 4 x 10⁻³                   | 101.73                                 | 50.49                                    | 49.51 | 0.098 | 806  |

Average \( K_f \pm \text{S.E.} = 847 \pm 14 \).
Standardization of the electrode

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

Table II. Cadmium electrode: experimental system and results obtained for μ = 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 μ = 0.16 with NaNO₃. Standardizing solution: 0.100 M Cd(NO₃)₂ 4 H₂O. 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

<table>
<thead>
<tr>
<th>Isocitrate solution [μl]</th>
<th>Isocitrate concentration [μM]</th>
<th>Negative potential [mV ± S.E.]</th>
<th>[Cd²⁺] [μM]</th>
<th>[Cd-Isoc.⁻] [μM]</th>
<th>[Isoc.₃⁻] [μM]</th>
<th>Kᵦ [M⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>400</td>
<td>189.76 ± 0.05</td>
<td>75.12</td>
<td>24.88</td>
<td>375.12</td>
<td>883</td>
</tr>
<tr>
<td>40</td>
<td>800</td>
<td>192.48 ± 0.04</td>
<td>60.68</td>
<td>39.32</td>
<td>760.68</td>
<td>852</td>
</tr>
<tr>
<td>60</td>
<td>1200</td>
<td>195.25 ± 0.05</td>
<td>49.08</td>
<td>50.92</td>
<td>1149.08</td>
<td>903</td>
</tr>
<tr>
<td>80</td>
<td>1600</td>
<td>196.78 ± 0.04</td>
<td>43.34</td>
<td>56.66</td>
<td>1543.34</td>
<td>847</td>
</tr>
</tbody>
</table>

Average Kᵦ ± S.E. = 871 ± 13

species, representing more than 98% of total isocitrate concentration, is the anion isocitrate³⁻. 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-isocitrateₙ)²⁻ₙ, in terms of the equilibrium ion-exchange formulation, is given by the expression:

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

(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:

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

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. Formation of cadmium hydroxide has been shown to be negligible at pH 7.5. Addition of increasing concentrations of Hepes buffer to a solution of cadmium nitrate in NaNO₃ 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:

$$\frac{1}{K_d} = \frac{1}{K_d^0} + Kᵦ \frac{[\text{isocitrate}]}{K_d^0}$$

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:

$$\text{Cd}^{2+} + \text{isocitrate}³⁻ \rightleftharpoons \text{Cd-isocitrate}⁻.$$
The theoretically, the potential between the cadmium specific-ion electrode and the reference electrode follows the relation:

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

where $E_a$ is the potential due to reference electrode and internal solutions, $2.3026 \cdot RT/2F$ 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 $29.276 \pm 0.178$ 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 12 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:

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

![Fig. 1. Variations of the reciprocal of the distribution coefficient for Cd$^{2+}$ as a function of isocitrate concentration.](image1)

![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.](image2)
so that cadmium ion concentration in the presence of isocitrate could be calculated from the measured potentials:

\[ [\text{Cd}^{2+}] = 10^{-4.3 \pm 0.02} \]

Since:

\[ [\text{Cd-isocitrate}^-] = [\text{Cd}_{\text{tot}}] - [\text{Cd}^{2+}] \]  \hspace{1cm} (2)

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

the formation constant:

\[ K_f = \frac{[\text{Cd-isocitrate}^-]}{[\text{Cd}^{2+}][\text{isocitrate}^3^-]} \]  \hspace{1cm} (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 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 \([\text{Cd-isocitrate}^-]/[\text{Cd}^{2+}]\) against the logarithm of free isocitrate concentration \([\text{isocitrate}^3^-]\) were straight lines (Fig. 4), as predicted by the Eq. (3) which can be written as follows:

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

from which the \(K_f\) value could be obtained:

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

in particular, when \([\text{Cd-isocitrate}^-] \) is equal to \([\text{Cd}^{2+}]\):

\[ \text{p}K_f = \log[\text{isocitrate}^3^-] \]

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 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 \((\text{Cd})\) in solutions containing cadmium and isocitrate to be calculated from total cadmium \((\text{Cd})\) and total isocitrate \((\text{I})\) concentrations.
By substitution of Eq. (2) into Eq. (3):

\[ K = \frac{Cd - Cd_t}{Cd_t[1 - (Cd - Cd_t)]} \]

and solution of the resulting quadratic equation, we obtain:

\[ Cd_t = \frac{Cd - I - K^{-1} \pm \sqrt{(I - Cd + K^{-1})^2 + 4 Cd K^{-1}}}{2} \]

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 ionic strength allows the concentrations of free and isocitrate-bound metal to be calculated 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 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.

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


The technical assistance of Mrs. Monique Roumengous is gratefully acknowledged.