The Equilibrium of the Reaction Catalysed by Citrate Oxaloacetate-Lyase

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1. A method of preparation and purification of citrate oxaloacetate-lyase (EC 4.1.3.6) from *Aerobacter aerogenes* is described. 2. The equilibrium of this reaction has been determined at pH 8.4 and 25°. It has been shown that K , i.e. $[\text{citrate}^{3-}]/[\text{oxaloacetate}_{\text{keto}}^{2-}][\text{acetate}^{-}]$, is 3.08 ± 0.72 , but that K_{app} , i.e. $[\text{total citrate}]/[\text{total oxaloacetate}][\text{total acetate}]$, is markedly affected by the initial concentrations of the reactants and magnesium. 3. The free-energy change during the cleavage of citrate has been calculated and compared with data from other sources. 4. The free energy of hydrolysis of acetyl-CoA has been evaluated from the present data. 5. A detailed knowledge of the interactions of the reactants with metal ions has been shown to be important in the calculation of the equilibrium constant and related thermodynamic functions.

The Mg^{2+} - or Mn^{2+} -activated enzyme citrate lyase (citrate oxaloacetate-lyase; EC 4.1.3.6) catalyses the reaction:

This reaction may be compared with that catalysed by citrate synthase (EC 4.1.3.7):

and, as has been pointed out (Gunsalus, 1958), the ratio of the equilibrium constants of reactions (1) and (2) is an expression of the difference between the free-energy contents of the thiol ester, acetyl-CoA, and water on the one hand, and acetate and CoA on the other. In other words the ratio of the two equilibrium constants gives a value for the free-energy change on hydrolysis of acetyl-CoA.

Previous measurements of the equilibrium constant of reaction (1) have been of the apparent constant defined as follows:*

$$K_{\text{app}} = [\text{Cit}_t]/[\text{Oxal}_t][\text{Ac}_t] \quad (3)$$

Smith, Stamer & Gunsalus (1956), using an enzyme from *Streptococcus faecalis* at pH 7.6 with Mn^{2+} as the activating cation, found K_{app} to be $1.56 \text{ l. mole}^{-1}$ at 27°, whereas Harvey & Collins (1963) found K_{app} to be $15.7 \text{ l. mole}^{-1}$ with an enzyme from

Streptococcus diacetilactis at pH 7.0 and 30°, presumably with Mg^{2+} as the activating metal ion.

Both groups of workers used the sulphates of the activating metals, salts that themselves are incompletely dissociated (Bjerrum, Schwarzenbach & Sillén, 1958). Harvey & Collins (1963) used a phosphate buffer, which forms complexes with Mg^{2+} (Clarke, Cusworth & Datta, 1954), whereas Smith *et al.* (1956) used a tris buffer, which reacts with oxaloacetate (Mahler, 1961). For these reasons it is doubtful whether it is justifiable to use these data for thermodynamic calculations, since the equilibrium constants thus evolved are susceptible to changes in total metal ion and total substrate concentration.

In addition, the reactants themselves are very complex. Oxaloacetate presents a formidable problem since its aqueous solutions contain both keto and enol isomers, the latter behaving as a tribasic acid (Tate, Grzybowski & Datta, 1964a). Also, the dianions of both isomers form 1:1 ligand: Mg^{2+} complexes, whereas the enolic trianions form very stable 1:1 and 2:1 complexes (Tate, Grzybowski & Datta, 1964b). The keto isomer of oxaloacetate is the reactive form in reaction (1) (Tate & Datta, 1964).

The anions of citrate also form complexes with Mg^{2+} . Walser (1961) found the value for the stability constant K_{MgCit^-} , i.e., $[\text{MgCit}^-]/[\text{Mg}^{2+}][\text{Cit}^{3-}]$, to be 3.57×10^3 at 25° and 1.016, and S. S. Tate, A. K. Grzybowski & S. P. Datta (unpublished work) found K_{MgCit^-} to be 5.32×10^3 at 25° and $I \sim 0.1$.

* Abbreviations (in kinetic equations and in the Tables): Ac, acetate; Cit, citrate; Oxal, oxaloacetate; the subscripts e, k and t indicate enol, keto and total of all ionic and complex species present at equilibrium respectively.

Harvey & Collins (1963) concluded from electro-phoretic data that at pH 7.0 the complex between citrate and Mg^{2+} is a 1:1 complex bearing two negative charges; they assign the second negative charge to the ionization of a proton from the hydroxyl group of citrate. We have been unable to confirm this claim by pH titration experiments. The complex between acetate and Mg^{2+} is very weak, K_{MgAc^+} , i.e. $[MgAc^+]/[Mg^{2+}][Ac^-]$, being 3.16 at 20° and $I \sim 0.2$ (Cannan & Kibrick, 1938).

In the present paper we report the effect of changes in the concentrations of Mg^{2+} and the three reactants on K_{app} and the evaluation of the 'true' equilibrium constant, K , for the citrate-lyase reaction where:

$$K = [Cit^{3-}]/[Oxal_k^{2-}][Ac^-] \quad (4)$$

The enzyme used was prepared from *Aerobacter aerogenes*. The results reported are at 25° and $I \sim 0.1$ in triethanolamine-hydrochloric acid buffers, pH 8.4. This buffer was chosen because it does not form complexes with Mg^{2+} . As shown below the equilibrium constant, K , of eqn. (4) is unaffected by changes in concentration of the reactants and magnesium.

EXPERIMENTAL

Materials

Trisodium citrate. A.R. grade salt was recrystallized from water.

Oxaloacetic acid. The same sample was used as for the determination of the dissociation constants (Tate *et al.* 1964a).

Sodium acetate. A.R. grade salt was used without further purification. A stock solution was prepared in CO₂-free water and the acetate concentration determined after acidification and steam-distillation of the acetic acid (Friedemann, 1938).

Magnesium chloride. A stock solution was prepared from A.R. grade $MgCl_2 \cdot 5H_2O$ and the magnesium concentration determined as the pyrophosphate (Vogel, 1951).

Tetramethylammonium chloride. A commercial sample (Eastman Kodak, Rochester, N.Y., U.S.A.) was purified by precipitation from ethanolic solution by the addition of anhydrous diethyl ether.

Triethanolamine. A commercial sample (British Drug Houses Ltd., Poole, Dorset) was distilled under reduced pressure, the middle colourless fraction being retained.

Alumina C_γ gel. This was prepared by the method of Willstätter & Kraut (1923) and allowed to stand for at least 3 months before use. The dry weight of the suspension was 25 mg./ml.

Buffers. The phosphate buffers used in the enzyme preparation were made by adjusting a solution of KH_2PO_4 of the stated molarity to the stated pH with approx. 2 M-NaOH. The triethanolamine buffers used in the enzyme assay and in the equilibrium studies were prepared by mixing a known amount of constant-boiling HCl with enough of approx. 0.5 M-triethanolamine to give the desired

pH. After dilution to the desired concentration the pH was checked and adjusted if necessary.

Preparation and purification of citrate lyase

The method used was similar to that of Siva Raman (1961) in the first steps but differed somewhat in the final ones. The organism used was *Aerobacter aerogenes*, NCTC strain 418, adapted to citrate by the method of Dagley & Dawes (1953).

Assay. During the preparation the enzyme activity was assayed by the initial increase in extinction at 280 mμ caused by the oxaloacetate formed from citrate at 25° by 0.2 ml. of extract added to 2.8 ml. of a solution containing trisodium citrate (10 mM), $MgCl_2$ (2 mM) and triethanolamine-HCl buffer, pH 7.4 and $I \sim 0.1$, in an Optica CF4 recording spectrophotometer [Optica (U.K.) Ltd., Gateshead upon Tyne]. The initial straight part of the curve was extrapolated to 1 min. to obtain the apparent extinction at this time. One unit of activity was defined as the extinction at 280 mμ of 1 μmole of oxaloacetate measured under these conditions.

Crude extract. The organism was grown in the citrate-mineral salts medium of Dagley & Dawes (1953) in 10 l. bottles at 37° for 30–36 hr. without shaking or aeration. The cells were harvested in a Sharples supercentrifuge and washed with 0.03 M-phosphate buffer, pH 7.0. The washed cells (50 g. wet wt. from approx. 40 l. of culture) were suspended in 40 ml. of 0.03 M-phosphate buffer, pH 7.0, and crushed, without abrasive, in a Hughes (1951) press pre-cooled to -15°. The crushed cell mass was extracted with 160 ml. of the same cold buffer and the extract centrifuged at 13000 g at 2° for 1 hr. The supernatant (215 ml.) was preserved.

Removal of nucleic acids. The crude extract was diluted with cold 0.03 M-phosphate buffer, pH 7.0, to 400 ml. with a protein content of approx. 1%. Streptomycin sulphate (1.4 g./100 ml.) was added to the diluted extract and the precipitated nucleic acids were removed by centrifugation. The clear solution (380 ml.) contained approx. 0.8% of protein.

Fractionation with alumina C_γ gel. To the streptomycin-treated solution was added 15.2 ml. of alumina C_γ suspension (approx. 1 mg. dry wt. of alumina/ml. of extract). The gel was centrifuged down and discarded. To the supernatant was added a further 45.6 ml. of gel suspension. The mixture was kept at 2° for 30 min. with frequent stirring. At this stage the enzyme became adsorbed on the gel. The gel was removed by centrifugation and the supernatant discarded. The gel was washed with 80 ml. of 0.01 M-phosphate buffer, pH 7.4, and the enzyme was then eluted from the gel by 80 ml. of 0.075 M-phosphate buffer, pH 7.4; the supernatant after centrifugation contained approx. 0.3% of protein.

Fractionation with ammonium sulphate. Powdered ammonium sulphate was added to the eluate from the gel to bring it to 0.1 saturation and the resulting turbidity was centrifuged down and discarded. The enzyme was then precipitated by addition to the supernatant of more ammonium sulphate to bring the saturation to 0.6. The precipitate, separated by centrifugation at 17000 g for 1 hr. at 2°, was dissolved in 14 ml. of cold 0.01 M-phosphate buffer, pH 7.4, and dialysed in the cold for about 4 hr. against 3–4 l. of the same buffer. The volume of the resulting

Table 1. *Purification of citrate lyase from extracts of Aerobacter aerogenes, NCTC 418*

One unit of citrate lyase is that amount of enzyme which catalyses the cleavage of 1 μ mole of citrate./min at 25° and pH 7.4 under the conditions described in the text. Specific activity is the number of units/mg. of protein. The total protein added to the column was 132 mg. (1848 units); the protein recovered was 41.25 mg. (1450 units).

Procedure	Volume (ml.)	Total units	Protein (mg./ml.)	Specific activity (units/mg. of protein)	Yield (%)	Purification
1. Crude bacterial extract	215	4975	19.86	1.165	—	—
2. Streptomycin-treated extract	380	5139	8.32	1.63	100	1
3. Supernatant from first alumina C_γ treatment	395	4985	6.85	1.84	97	1.13
4. 0.075 M-Phosphate buffer extract after second alumina C_γ treatment	80	2650	2.95	11.23	51.57	6.91
5. 0.1–0.60 Satd. $(NH_4)_2SO_4$ fraction	17	2244	9.45	14.0	43.67	8.61
6. DEAE-cellulose column chromatography followed by $(NH_4)_2SO_4$ fractionation	15	1450	2.75	35.16	28.22 (34.27)*	21.62

* The yield has been corrected, since only 14.0 ml. of 0.1–0.6 saturated $(NH_4)_2SO_4$ fraction was added to the DEAE-cellulose column.

solution was 17 ml. and contained approx. 0.95% of protein.

Fractionation on DEAE-cellulose. A 14 ml. portion of the non-diffusible material was applied to a column (30 cm. $\times$ 2 cm.) of DEAE-cellulose that had been equilibrated against 0.01 M-phosphate buffer, pH 7.4. The protein was washed on to the column with 100 ml. of the same buffer. Gradient elution was then carried out from a closed mixing chamber (capacity 300 ml.), filled with 0.01 M-phosphate buffer, pH 7.4, to which was connected a reservoir containing a solution of KH_2PO_4 (0.01 M) and NaCl (0.5 M) adjusted to pH 7.4 with approx. 2 M-NaOH. The eluate was collected in 4 ml. fractions, the protein contents of these were determined from the extinctions at 280 $m\mu$ and their sodium contents by flame photometry. The enzyme activity of the fractions was assayed as described above. The activity was eluted as a single peak between 0.15 M- and 0.25 M- Na^+ ; these fractions were pooled (approx. 50 ml.) and dialysed for about 4 hr. against 0.01 M-phosphate buffer, pH 7.4. The non-diffusible material was made 0.9 saturated with ammonium sulphate, and the precipitated enzyme was separated by centrifugation and dissolved in 13 ml. of the same buffer. The enzyme was stored as a suspension in 0.9 saturated ammonium sulphate buffer.

The overall purification by this procedure was about 22-fold, with a yield of 34%. The preparation was completely free from oxaloacetate-decarboxylase activity. The various steps in the purification are summarized in Table 1.

Determination of the equilibrium concentrations of the reactants

Citrate cleavage. All experiments were done at 25° in the cuvette of an Optica CF4 recording spectrophotometer fitted with a thermostatically controlled cell holder. Since the enzyme is inhibited by the oxaloacetate produced (Dagley & Dawes, 1955; Wheat & Ajl, 1955; Bowen & Rogers, 1963), experiments were done in the presence of

acetate, excess of $MgCl_2$, and citrate in limiting concentrations; the reaction was started by the addition of an excess of enzyme. The total volume of each solution was 3.0 ml., containing 1.5 ml. of triethanolamine-HCl buffer, pH 8.4, $MgCl_2$, citrate, acetate, enzyme and sufficient tetramethylammonium chloride to make $I \sim 0.1$. The increase in extinction at 280 $m\mu$ was observed until equilibrium was reached, and then 2.5 ml. of the reaction mixture was pipetted into 0.5 ml. of cold trichloroacetic acid and the protein removed by centrifugation.

Determination of oxaloacetate. Oxaloacetic acid and its anions exist in aqueous solution as equilibrium mixtures of the keto and enol isomers. The molar extinction coefficient of the enol isomers ($\epsilon_{280m\mu}$ 3600) is about 140 times as great as that of the keto isomers, the observed extinction at 280 $m\mu$ being almost completely due to the enol isomer content (Tate *et al.* 1964a). The enol isomers are stabilized by Mg^{2+} , particularly above pH 7 (Tate *et al.* 1964b), and so the extinction at 280 $m\mu$ cannot be used to determine the total oxaloacetate concentration in a solution containing Mg^{2+} . The method of Greenwood & Greenbaum (1953), which depends on the borate complex of enolic oxaloacetate and which is unaffected by Mg^{2+} , was therefore used.

Determination of citrate. The total citrate concentration in the deproteinized solutions was determined by the pentabromacetone method of Stern (1957).

Acetate was not determined in the equilibrium mixture, its concentration being calculated from the quantity originally present and the amount of citrate cleaved.

Citrate synthesis. Because of the high extinction at 280 $m\mu$ of these solutions, due to their relatively high oxaloacetate content, it was not possible to follow the reaction spectrophotometrically. Experiments were therefore carried out in tubes in a water bath at 25°. These contained triethanolamine-HCl buffer, pH 8.4, acetate, enzyme and tetramethylammonium chloride. The reaction was started by the addition of oxaloacetate and the incubation was continued for 10 min.

Expt. no.	Initial concn. (mm)		$[Mg_t]$ (mm)	Equilibrium concn. (mm)						$K_{app.}$	K
	[Cit]	[Ac]		[Cit _t]	[Oxal _t]	[Ac ⁻]	$[Mg^{2+}]$	[Cit ³⁻]	[Oxal _t ²⁻]		
Enzyme: 3.0 units/ml.											
1	2.0	25.0	2.5	0.72	1.25	26.25	1.65	0.0737	0.89	21.90	3.15
2	2.0	25.0	5.0	0.93	1.05	26.05	3.78	0.0440	0.61	34.10	2.75
3	2.0	25.0	7.5	1.07	0.90	25.90	6.09	0.0320	0.44	45.82	2.81
4	2.0	25.0	10.0	1.06	0.74	25.74	8.58	0.0227	0.31	56.07	2.86
5	2.0	25.0	12.5	1.12	0.75	25.75	10.97	0.0189	0.27	54.30	2.68
6	2.0	12.5	0.5	0.18	1.81	14.31	0.32	0.0668	1.50	7.25	3.12
7	2.0	12.5	1.0	0.36	1.62	14.12	0.61	0.0847	1.29	16.89	4.64
Enzyme: 2.0 units/ml.											
8	2.0	12.5	0.5	0.21	1.78	14.28	0.31	0.0800	1.47	8.47	3.80
9	2.0	12.5	1.0	0.33	1.68	14.18	0.63	0.0763	1.34	13.53	4.01
10	2.0	12.5	1.5	0.34	1.65	14.15	1.01	0.0534	1.26	14.99	3.00
11	2.0	12.5	2.0	0.48	1.51	14.01	1.36	0.0582	1.11	23.23	3.74
12	2.0	6.25	1.0	0.21	1.78	8.03	0.69	0.0448	1.41	15.63	3.96
13	2.0	18.75	1.0	0.32	1.66	20.01	0.63	0.0733	1.32	10.14	2.72
14	2.0	25.0	1.0	0.35	1.64	26.64	0.62	0.0819	1.31	8.15	2.35
15	0.5	12.5	10.0	0.16	0.33	12.83	9.66	0.0031	0.13	39.79	1.84
16	1.0	12.5	10.0	0.34	0.65	13.15	9.32	0.0067	0.26	41.13	1.97
17	1.5	12.5	10.0	0.65	0.85	13.35	8.92	0.0134	0.35	56.80	2.89
18	2.0	12.5	10.0	0.92	1.08	13.58	8.55	0.0198	0.45	62.86	3.23
									Mean + s.d. 3.08 + 0.72

Fig. 1. Effect of initial concentrations of citrate, acetate and magnesium on the apparent equilibrium constant, K_{app} , i.e. $[Cit_t]/[Oxal_t][Ac_t]$, at 25° and pH 8.4. The values of the 'true' equilibrium constant, K ,

$$\text{i.e. } [Cit^3-]/[Oxal_k^{2-}][Ac^-]$$

for all the points lie in the shaded area. The numbers refer to the experiments shown in Table 2. Initial concentrations:

○, Citrate, 2.0 mM; acetate, 25.0 mM; enzyme, 3.0 units/ml.; Mg^{2+} varied. ●, Citrate, 2.0 mM; acetate, 12.5 mM; enzyme, 2.0 units/ml.; Mg^{2+} varied. ×, Citrate, 2.0 mM; Mg^{2+} , 1.0 mM; enzyme, 2.0 units/ml.; acetate varied. △, Acetate, 12.5 mM; Mg^{2+} , 10.0 mM; enzyme, 2.0 units/ml.; citrate varied.

From eqns. (5), (6) and (8), together with the equilibria shown in Table 3, it is possible to derive the following cubic expression for the free Mg^{2+} concentration at equilibrium, $[Mg^{2+}]$:

$$\begin{aligned} & [Mg^{2+}]^3 \{K_{MgCit^-} - K_{MgOxal} ([H^+] + K'_{a(Oxal)}) \\ & + [Mg^{2+}]^2 \{K_{MgCit^-} ([H^+] + K'_{a(Oxal)}) + K_{MgOxal} ([H^+] \\ & + K'_{a(MgOxal)}) + K_{MgCit^-} - K_{MgOxal} ([H^+] \\ & + K'_{a(MgOxal)}) ([Cit_t] + [Oxal_t] - [Mg_t])\} \\ & + [Mg^{2+}] \{([H^+] + K'_{a(Oxal)}) (1 + K_{MgCit^-} [Cit_t] \\ & - K_{MgCit^-} [Mg_t]) + K_{MgOxal} ([H^+] \\ & + K'_{a(MgOxal)}) ([Oxal_t] - [Mg_t])\} \\ & - [Mg_t] ([H^+] + K'_{a(Oxal)}) = 0 \end{aligned} \quad (9)$$

Eqn. (9) was solved graphically for each set of equilibrium concentrations, $[Cit_t]$, $[Oxal_t]$ and $[Mg_t]$. The values of $[Mg^{2+}]$ found are shown in Table 2.

From eqn. (5) and the expression for K_{MgCit^-} , the following relation gave $[Cit^3-]$:

$$[Cit^3-] = [Cit_t] / (1 + K_{MgCit^-} [Mg^{2+}]) \quad (10)$$

The values of $[Cit^3-]$ obtained are shown in Table 2.

The sum of the concentrations of the Mg^{2+} ion complexes of oxaloacetate dianions was found from eqn. (11), which was derived from eqn. (6) and the expressions for K_{MgOxal} , $K'_{a(Oxal)}$ and $K'_{a(MgOxal)}$:

$$\begin{aligned} & ([MgOxal_k] + [MgHOxal_e]) \\ & = [Oxal_t] K_{MgOxal} [H^+] [Mg^{2+}] / \{([H^+] + K'_{a(Oxal)}) \\ & + K_{MgOxal} [Mg^{2+}] ([H^+] + K'_{a(MgOxal)})\} \end{aligned} \quad (11)$$

By using the expressions for K_{MgOxal} and $K_{T(Oxal)}$, the tautomeric equilibrium constant, the following expression for $[Oxal_k^{2-}]$ was obtained:

$$[Oxal_k^{2-}] = \frac{[MgOxal_k] + [MgHOxal_e]}{K_{MgOxal} [Mg^{2+}] \left(1 + \frac{1}{K_{T(Oxal)}}\right)} \quad (12)$$

Table 3. Values of the stability constants, the acid dissociation constants and the tautomeric equilibrium constants at $I \sim 0.1$ and 25° used in the evaluation of the equilibrium constant of the reaction catalysed by citrate lyase

The data on citrate were obtained by S. S. Tate, A. K. Grzybowski & S. P. Datta (unpublished work), and those on oxaloacetate are from Tate *et al.* (1964a,b).

(a) Stability constants

$$\begin{aligned} MgCit^- & K_{MgCit^-} = [MgCit^-] / [Mg^{2+}] [Cit^3-] = 5.32 \times 10^3 \\ (MgOxal_k + MgHOxal_e) & K_{MgOxal} = ([MgOxal_k] + [MgHOxal_e]) / [Mg^{2+}] ([Oxal_k^{2-}] + [HOxal_e^{2-}]) = 91.2 \\ MgOxal_k & K_{MgOxal_k} = [MgOxal_k] / [Mg^{2+}] [Oxal_k^{2-}] = 81.3 \\ MgHOxal_e & K_{MgHOxal_e} = [MgHOxal_e] / [Mg^{2+}] [HOxal_e^{2-}] = 158.5 \\ MgOxal_e^- & K_{MgOxal_e^-} = [MgOxal_e^-] / [Mg^{2+}] [Oxal_e^{3-}] = 1.862 \times 10^6 \end{aligned}$$

(b) Acid dissociation constants

$$\begin{aligned} K'_{a(Oxal)} & = [H^+] [Oxal_e^{3-}] / ([Oxal_k^{2-}] + [HOxal_e^{2-}]) = 8.71 \times 10^{-14} \\ K'_{a(MgOxal)} & = [H^+] [MgOxal_e^-] / ([MgOxal_k] + [MgHOxal_e]) = 1.78 \times 10^{-9} \end{aligned}$$

(c) Tautomeric equilibrium constants

$$\begin{aligned} K_{T(Oxal)} & = [Oxal_k^{2-}] / [HOxal_e^{2-}] = 6.09 \\ K_{T(MgOxal)} & = [MgOxal_k] / [MgHOxal_e] = 3.14 \end{aligned}$$

Table 4. Attempts to determine the equilibrium of the citrate-lyase reaction at 25° and pH 8.4 in the direction of citrate synthesis

The conditions were the same as those given in the legend to Table 2, except that incubation was continued for 10 min. without extinction measurements. The reasons for failure to reach true equilibrium are described in the text.

Expt. no.	Concn. of enzyme (units/ml.)	[Mg _t] (mm)	'Equilibrium' concn. (mm)						K _{app.}	(K)
			[Cit _t]	[Oxal _t]	[Ac ⁻]	[Mg ²⁺]	[Cit ³⁻]	[Oxal _k ²⁻]		
Initial concentrations: oxaloacetate, approx. 10 mm; acetate, 25.0 mm										
19	0.5	2.0	0.21	8.03	24.79	0.99	0.0336	6.15	1.05	0.22
20	1.0	2.0	0.31	7.76	24.69	0.94	0.0518	5.97	1.60	0.35
21	1.5	2.0	0.37	8.19	24.63	0.89	0.0648	6.34	1.71	0.41
22	2.0	2.0	0.40	7.65	24.60	0.90	0.0685	5.91	2.13	0.47
23	3.0	2.0	0.43	7.32	24.57	0.91	0.0739	5.65	2.37	0.53
24	4.0	2.0	0.45	7.65	24.55	0.88	0.0793	5.93	2.39	0.54
Initial concentrations: oxaloacetate, approx. 20 mm; acetate, 50.0 mm										
25	5.0	4.0	0.44	16.89	49.56	1.29	0.0559	12.51	0.52	0.09
26	5.0	5.0	0.46	16.92	49.54	1.68	0.0463	12.04	0.54	0.08
27	5.0	10.0	0.46	15.29	49.54	4.27	0.0194	8.59	0.61	0.046
28	5.0	20.0	0.39	12.56	49.61	12.09	0.0060	4.32	0.63	0.03

The values of [Oxal_k²⁻] thus obtained are also given in Table 2.

From the values of [Cit³⁻], [Oxal_k²⁻] and [Ac⁻] the equilibrium constant *K* of eqn. (4) was calculated. The values given in Table 2 show that *K* does not vary very much and is independent of the initial concentrations of the reactants and magnesium. This constancy is also illustrated in Fig. 1.

Citrate synthesis

The results of ten experiments in the direction of citrate synthesis are shown in Table 4. The concentrations of the various species at equilibrium were calculated in the same way as for the experiments on citrate cleavage. Expts. 19–24 show the effect of enzyme concentration on the position of equilibrium, and Expts. 25–28 show the effects of magnesium concentration.

DISCUSSION

Citrate cleavage

The results given in Table 2 demonstrate the necessity for considering a number of factors that seem to have escaped the notice of previous workers. The apparent equilibrium constant of the reaction, *K*_{app.}, of eqn. (3) is manifestly not a constant and varies very considerably with changes in the total magnesium concentration (Fig. 1). This effect on *K*_{app.} is not mentioned by Harvey & Collins (1963), and, further, these workers do not state what metal ion was used, or its concentration, in their equilibrium studies. It is thermodynamically impossible

that the effect of metal ions on the equilibrium constant is different with the enzyme from *Streptococcus diacetylactis* used by Harvey & Collins (1963).

Oxaloacetate is a powerful inhibitor of citrate lyase from *A. aerogenes* and *Escherichia coli* (Dagley & Dawes, 1955; Wheat & Ajl, 1955), though this is not remarked on by Harvey & Collins (1963) with the *S. diacetylactis* enzyme. Bowen & Rogers (1963) have reported that no equilibrium constant could be calculated from their data, since the amount of oxaloacetate formed in their conditions was dependent on the amount of enzyme present. These workers, however, used high citrate concentrations (about 67 mm) where the amount of oxaloacetate formed is enough to inhibit the enzyme. We have also observed this phenomenon, but the present work shows that, by using limiting concentrations of citrate (about 2 mm), an excess of magnesium chloride and of enzyme, and acetate, true equilibrium conditions can be obtained in the direction of citrate cleavage.

Since the keto isomer of oxaloacetate is the one produced during the cleavage of citrate by the *A. aerogenes* enzyme (Tate & Datta, 1964), it is possible that the inhibition by oxaloacetate is due to the enol isomer. This view is supported by the findings of Bowen & Rogers (1963), which have been confirmed by us, that malate is also inhibitory. Harvey & Collins (1963) believe the keto isomer of oxaloacetate to be the isomer produced from citrate by the enzyme from *S. diacetylactis*. These authors' belief that the discrepancy between their equilibrium results and those of Smith *et al.* (1956) is due to the failure of commercial oxaloacetate, which is

enolic in the solid (Meyer, 1912; Banks, 1961), to reach tautomeric equilibrium in solution cannot be sustained because, even at 2°, equilibrium, near neutral pH, is reached in about 2 min., and at 25° it is virtually instantaneous (Tate *et al.* 1964a).

Free-energy changes. The 'true' equilibrium constant, K , where:

$$K = [\text{Cit}^{3-}]/[\text{Oxal}_k^{2-}][\text{Ac}^-] \quad (4)$$

is, as shown in Table 2, remarkably constant for reactions, in the direction of citrate cleavage, carried out at a variety of reactant and metal ion concentrations. Unfortunately, this constancy gives no information as to whether these ionic species are indeed the reactive ones. If it is assumed that the reaction is between the Mg^{2+} complexes of citrate and oxaloacetate and free acetate, another equilibrium constant, K' , is obtained, where:

$$K' = [\text{MgCit}^-]/[\text{MgOxal}_k][\text{Ac}^-] \quad (13)$$

It is at once apparent from eqn. (4) and (13), together with the expressions for K_{MgCit^-} and K_{MgOxal_k} in Table 3, that:

$$K' = K \cdot K_{\text{MgCit}^-}/K_{\text{MgOxal}_k}$$

and it follows that K' will also be constant for the experiments reported above.

From the K value 3.08 ± 0.72 (Table 2) the value of $\Delta G'_{(4)}$ is $-668 \text{ cal. mole}^{-1}$ (range of variation -509 to -793) for the reaction shown in eqn. (4), exergonic in the direction of citrate synthesis. This may be compared with the value calculated from the data of Burton (1961) for the reaction:

for which the equilibrium constant is given by:

$$K'' = [\text{Cit}^{3-}]/([\text{Oxal}_k^{2-}] + [\text{HOxal}_e^{2-}][\text{Ac}^-]) \quad (15)$$

Burton (1961) gives $\Delta G'_f$, the free energy of formation in aqueous solution at 25°, of the following ions: citrate³⁻, $-278.83 \text{ kcal. mole}^{-1}$; oxaloacetate²⁻ (presumably an equilibrium mixture of keto and enol tautomers), $-190.53 \text{ kcal. mole}^{-1}$; acetate⁻, $-88.24 \text{ kcal. mole}^{-1}$. Thus $\Delta G'_{(14)}$ for reaction (14) is $-60 \text{ cal. mole}^{-1}$, exergonic in the direction of citrate synthesis.

Since from eqn. (15) the free-energy change for reaction (14) is:

$$\Delta G'_{(14)} = -RT \cdot \ln 10 \cdot \log K'' = -60 \text{ cal. mole}^{-1}$$

and from eqns. (4) and (15) and the expression for $K_{\text{T(Oxal)}}$ (Table 3):

$$K'' = K \left(1 + \frac{1}{K_{\text{T(Oxal)}}} \right)$$

then the free-energy change for reaction (14):

$$\Delta G'_{(14)} = \Delta G'_{(4)} + RT \cdot \ln 10 \cdot \log \left(1 + \frac{1}{K_{\text{T(Oxal)}}} \right)$$

Table 5. *Effect of magnesium concentration on the extinction due to oxaloacetate at 280 mμ*

The data are for Expts. 1–5 of Table 2. The total oxaloacetate concentration was determined by the method of Greenwood & Greenbaum (1953).

Expt. no.	[Mg _i](mM)	At equilibrium	
		[Oxal _i] (mM)	$E_{280\text{m}\mu}$
1	2.5	1.25	0.957
2	5.0	1.05	0.965
3	7.5	0.90	0.955
4	10.0	0.74	0.860
5	12.5	0.75	0.922

By using the $K_{\text{T(Oxal)}}$ value 6.09 and the $\Delta G'_{(4)}$ value $-668 \text{ cal. mole}^{-1}$, $\Delta G'_{(14)}$ is $-578 \text{ cal. mole}^{-1}$, which is to be compared with the $\Delta G'_{(14)}$ value $-60 \text{ cal. mole}^{-1}$ from Burton's (1961) data.

Since these two values have been obtained from different data, the agreement is satisfactory. The value found in the present work is probably more reliable, since it depends on the direct measurement of the equilibrium concentrations. The value calculated from Burton's (1961) data may be in error simply from one small error in one of the values of $\Delta G'_f$.

From our $\Delta G'_{(14)}$ value $-578 \text{ cal. mole}^{-1}$, together with the $\Delta G'_{(2)}$ value $-7.72 \text{ kcal. mole}^{-1}$ at pH 7.2 and 22° for the citrate-synthase-catalysed reaction (Stern, Ochoa & Lynen, 1952), the free energy of hydrolysis of acetyl-CoA, $\Delta G'_{\text{Ac-CoA}}$, may be calculated as follows. $\Delta G'_{(14)}$, which defines the free-energy change of reaction (14), is independent of pH. However, to compare $\Delta G'_{(14)}$ with $\Delta G'_{(2)}$ at pH 7.2, allowance must be made for the fact that all the citrate will not be in the trianionic form. Since pK'_a of citrate at 25° and $I \sim 0.1$ is 5.84 (S. S. Tate, A. K. Grzybowski & S. P. Datta, unpublished work), at pH 7.2, 0.042 of the total citrate will be in the citrate²⁻ form. Then, by ignoring the difference in temperature between 25° and 22°, we have:

$$\Delta G'_{(2)} = -7.72 \text{ kcal. mole}^{-1}$$

$$-\Delta G'_{(14)} = +0.58 \text{ kcal. mole}^{-1}$$

$$\Delta G' = +0.042 RT \cdot pK'_a = +0.33 \text{ kcal. mole}^{-1}$$

Adding these equations we obtain:

$$\Delta G'_{\text{Ac-CoA, pH } 7.2} = -6.81 \text{ kcal.mole}^{-1}$$

Extinction at 280 m μ . Although the amount of oxaloacetate formed at equilibrium by citrate cleavage diminishes with increasing concentration of total magnesium in the system, the extinction at 280 m μ reaches approximately the same value; this is shown for Expts. 1-5 in Table 5. These results are due to the stabilization of the enolic form of oxaloacetate by Mg²⁺. It follows therefore that the equilibrium extinction at 280 m μ cannot be used to determine the extent of the reaction.

Citrate synthesis

The results given in Table 4 of experiments in the direction of citrate synthesis demonstrate that in the conditions used true equilibrium was not reached. The K_{app} values are reasonably consistent and are near the values obtained by Smith *et al.* (1956) for experiments in the same direction. The K values are very variable and are markedly influenced by the concentrations of magnesium (Expts. 25-28) and enzyme (Expts. 19-24).

The failure to reach equilibrium was no doubt due to the inhibition of the enzyme by the high concentrations of oxaloacetate present in these experiments, coupled with the slowness of the reaction. There are considerable experimental difficulties in these experiments. Thus, if the concentration of enzyme had been increased, it would have been difficult to estimate small amounts of reactants in the presence of relatively large amounts of protein. The concentration of oxaloacetate could have been decreased, but this leads to great difficulties because of its decarboxylation. If the concentration of acetate had been greatly increased, it would no longer have been possible to ignore the formation of the complex MgA⁺, and the calculation of K would have been greatly complicated.

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