The Equilibrium Constants of the Fumarase and Aconitase Systems

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This paper is the first of a programme of investigations designed to obtain accurate data for the free-energy changes associated with the individual stages of the tricarboxylic acid cycle and related reactions. There is only one stage of the cycle, namely the reaction succinate → fumarate + 2H, for which data of satisfactory accuracy are available. In other cases, such as those dealt with in the present paper, the reported data are either not very accurate or they do not refer to the standard temperature of 25° now commonly used in thermodynamic measurements. There are also several stages for which no reliable data have as yet been reported.

Borsook & Schott (1931*a*) estimated the value of the equilibrium ratio L-malate/fumarate at 25° to be 3·2. This figure was based on electrometric measurements of the equilibrium potential of the succinate-fumarate system in the absence and presence of fumarase, and no great accuracy was claimed. Jacobsohn (1934) reported a ratio of 5·1 (25°, barbitone buffer of pH 6·8). Krebs, Smyth & Evans (1940) found values of 2·65 at 50°; 3·17 at 40°; 3·54 at 30° and 4·57 at 20°. Interpolation gives a value of 4·0 at 25°. Scott & Powell (1948) found a ratio of 4·05 at 25°.

All previous measurements of the equilibrium between citric, *iso*citric and *cis*-aconitic acids in the presence of aconitase refer to blood temperature (Krebs & Eggleston, 1943; Martius & Leonhardt, 1943).

EXPERIMENTAL AND RESULTS

Determination of the equilibrium constant in the fumarase system

Dialysed heart-muscle suspension (homogenate) served as a fumarase preparation. This was prepared in a stainless-steel homogenizer from 1 part of minced sheep heart muscle and 2 parts of water and was dialysed for 3 hr. against running tap water at room temperature. In most experiments 50 ml. 0·100 M-fumarate, 20 ml. heart-muscle suspension, 3 ml. M-$NaHCO_3$ 10 drops *sec.*-octanol and water to make up 100 ml. were gassed with 5% CO_2 in N_2, and incubated at 25°. Under these conditions pH was 7·4. At intervals samples were removed for the determination of malic and fumaric acids. A control sample containing no fumarate was incubated at the same time to supply blank values for the analyses. Phosphate buffer was avoided because it interferes with the polarimetric determination of malic acid.

Polarimetric determination of L-*malic acid.* Into a 20 ml. measuring flask were pipetted, in the following sequence, 1 ml. glacial acetic acid, 4 ml. test solution, 4 ml. water, 2 ml. 1 M-sodium citrate and 29% ammonium molybdate (w/v) to the 20 ml. mark. After thorough shaking, the mixture was filtered and the clear filtrate was poured into a 2 dm. polarimeter tube. The temperature of the solution was noted; it was usually 22°. Under these conditions pure malic acid gave the following rotations: 24·0 mg. L-malic acid in the total sample of 20 ml.: $\alpha_D^{22°}$ 3·325°; $[\alpha]_D$ 1385°; 7·11 mg. L-malic acid in 20 ml.: $\alpha_D^{22°}$ 1·05°: $[\alpha]_D$ 1475° (see Krebs & Eggleston, 1943).

Manometric determination of fumaric acid and malic acid. 10 ml. of the test solution were pipetted into a test tube containing 1 ml. 20% (w/v) metaphosphoric acid. After thorough shaking and a few minutes' standing the mixture was filtered. Fumaric acid was determined by measuring the amount of H_2 required for quantitative catalytic hydrogenation, under the conditions used by Johnson (1939) for the determination of aconitic acid. The palladium catalyst of Köppen (1932)* (10 mg.) was ground up in a mortar and with 3 ml. 2% metaphosphoric acid was placed in the main compartment of a conical manometer vessel. The side arm contained 1 ml. of the test solution, the gas space H_2. The manometer vessel was provided with a valve stopper so that the gassing could be carried out in the bath, whilst the manometers were being shaken. Commercial cylinder H_2 washed with 10% pyrogallol in 10% NaOH and acid dichromate (5% potassium dichromate in 10% (w/v) H_2SO_4) was used (Johnson, 1939). The bath temperature was 40°. When equilibrium was established the contents of side arm and main compartment were mixed. The H_2 uptake was usually completed within 30–60 min.

At the end of the experiment the contents of the cups were poured on a filter and 2 ml. of the filtrates were used for the determination of malic acid with the malic decarboxylase of *Lactobacillus arabinosus* (Korkes & Ochoa, 1948; Nossal, 1952). The main compartment of the manometer vessels contained, in addition to 2 ml. filtrate, sufficient N-KOH to bring pH to approximately 5 (usually 0·3 ml.), 0·5 ml. 0·2 M-acetate buffer (pH 5·0), 0·1 ml. 0·1 M-glucose, 0·1 ml. 2% (w/v) $MnCl_2$ and water to make up 4 ml. The side arm contained 0·2 ml. of a stock suspension of *Lb. arabinosus* (20 mg. dry weight of cells per ml.) and 0·1 ml. 0·2 M-acetate buffer, pH 5·0. The gas space contained air. Bath temperature was 35°. Cells of *Lb. arabinosus* contain small quantities of fumarase and therefore decarboxylate fumaric acid as well as malic acid. This source of error is eliminated by the prior removal of fumaric acid through catalytic hydrogenation.

* Obtained from Dr R. Köppen, Norden, Ostfriesland, Germany; also obtainable from Baker Platinum Ltd., 52 High Holborn, London, W.C. 1.

As the total volume of test solutions analysed in this procedure was 1 ml. the method requires a relatively high concentration of fumarate and malate, say, above 0·02 M. A few experiments were carried out at lower concentrations and in these fumarate was extracted from 10 ml. of the solution acidified with H_2SO_4, as described for aconitic acid by Johnson (1939). The extracted material was dissolved in a small volume of 2% metaphosphoric acid, usually 1·2 ml., and 1 ml. was analysed. Quantitative ethereal extraction of malic acid proved difficult and the polarimetric method was therefore used.

It should be mentioned that the palladium catalyst can be inactivated by substances present in tissue extracts. These were not encountered in dilute extracts of heart and muscle, but were present in less dilute extracts and in baker's yeast. If inhibitory substances are present, larger amounts of catalyst are required.

Results. Data from three separate experiments, carried out after the methods had been perfected, are shown in Table 1. The concentration of fumarate plus malate was 0·05 M. In considering the equilibrium values the first three measurements of Exp. 3 have to be omitted because when the samples were taken equilibrium had not been established. The other eight values for the ratios L-malate/fumarate vary between 4·34 and 4·52, the average being 4·42.

In other experiments the concentration of fumarate plus malate was 0·01 M, and the buffer was 0·01 M-$NaHCO_3$ with 1% CO_2 in N_2. The value for the equilibrium constant was the same within the limits of error.

Determination of the equilibrium constants in the aconitase system

Sheep heart muscle was coarsely minced in a kitchen meat mincer and suspended in about 30 vol. of distilled water. After about 5 min. the tissue was centrifuged off. The washed muscle was stored in the frozen state in a deep freeze. In order to reduce blank values in the aconitase determination, arising from the dehydrogenation of unsaturated fatty acids, the frozen material was mixed with 8 vol. of ice-cold ethanol in a Waring Blendor and filtered. The insoluble material was dried in a desiccator over H_2SO_4 and then triturated with ether. It was stored in a desiccator. Part of this material (3 g.) was suspended in a total volume of 100 ml. incubated solution. In an alternative enzyme preparation the ethanol was replaced by ice-cold acetone. Both preparations were about equally active. In all experiments the substrate was 0·200 M-sodium citrate. $NaHCO_3$ (0·03 M) and CO_2 (5%) served as buffer.

Aconitic acid was determined manometrically by quantitative catalytic hydrogenation (Johnson, 1939). 8 ml. of the incubated materials were deproteinized with 2 ml. 20% (w/v) metaphosphoric acid and a measured amount of the filtrate (7–8 ml.) was extracted with ether in a continuous extractor. Extraction was found to be completed within 2 hr. The ether-soluble material was collected in a total volume of 1·5 ml. 2% metaphosphoric acid and 1 ml. of this solution was placed in the side arm of a manometer vessel. Otherwise the procedure was as described for the determination of fumaric acid. *iso*Citric acid was determined polarimetrically under the conditions described by Eggleston & Krebs (1949).

Results. The results shown in Table 2 are reasonably consistent. A possible source of error is the gradual formation of *trans*-aconitic from *cis*-aconitic acid. The fact that analytical results after 6 and 24 hr. showed no change indicates that no significant amounts of *trans*-aconitic acid were formed under the test conditions.

The average value for the *iso*citric acid formed was 178 μl./ml. and of *cis*-aconitic acid 130 μl./ml. The most reliable determination of citric acid is by difference and the equilibrium mixture at 25° thus contained 6·20% *iso*citric acid, 2·90% *cis*-aconitic acid and 90·9% citric acid. The corresponding figures at 38° are 6·6% *iso*citric acid, 4·3% *cis*-aconitic acid, 89·1% citric acid (Krebs & Eggleston, 1943; Eggleston & Krebs, 1949).

Table 1. *Equilibrium concentrations of fumarate and malate in the presence of fumarase at* pH 7·4 (25°)

(For procedure see text. The manometric results of the malate determinations were used for the calculation of the malate/fumarate ratio. In Exp. 3 a dialysed fumarase solution which had been stored about 6 days in the refrigerator and which was less active was used.)

Exp. no.	Period of incubation (hr.)	L-Malate		Fumarate (μl./ml.)	Ratio: L-malate / fumarate
		Manometrically (μl./ml.)	Polarimetrically (μl./ml.)		
1	2	900	—	207	4·34
	4	886	—	201	4·41
	5	884	—	202	4·37
	7	910	—	201	4·52
2	2·5	902	924	205	4·42
	5	904	905	204	4·43
	24	920	922	204	4·52
3	2	791	778	310	2·55
	4	843	852	250	3·37
	6	894	902	210	4·29
		909	919	207	4·38

Table 2. *Equilibrium concentrations of* isocitrate *and* cis-aconitate *in the presence of aconitase at* pH 7·4

(For procedure see text. Temp., 25°. All data corrected for blank. Total tricarboxylic acid concentration 0·200 M (4480 μl./ml.).)

Exp. no.	Enzyme preparation	Period of incubation (hr.)	*iso*Citrate			*cis*-Aconitic acid	
			$\alpha_D^{22°}$	(μl./ml.)	% of total tricarboxylic acid	(μl./ml.)	% of total tricarboxylic acid
1	Ethanol	24	−1·69	276	6·16	133	2·97
						128	2·86
						126	2·81
						133	2·97
2	Ethanol	18	−1·71	280	6·26	129	2·88
						130	2·90
						133	2·97
						135	3·01
3	Ethanol	6	−1·72	281	6·21	129	2·58
		24	−1·70	278	6·21	131	2·93
4	Acetone	6	−1·60	262	—	122	—
		24	−1·70	278	6·21	127	2·84
5	Ethanol	24	−1·70	278	6·21	—	—
6	Washed sheep heart	6	−1·70	278	6·21	127	2·84
		24	−1·69	276	6·16	129	2·88
				Av.	6·20		2·90

NOTE ON THE EFFECT OF THE HYDROGEN-ION CONCENTRATION ON THE EQUILIBRIUM RATIOS

If, at a given temperature, the equilibrium ratio for one of the three forms in which the two dibasic acids occur in aqueous solutions (undissociated, univalent anion, divalent anion), and the ionization constants of the acids are known, the value of the equilibrium ratio L-malic acid (all forms)/fumaric acid (all forms) can be calculated for any hydrogen-ion concentration.

Let H_2A, HA^- and A^{2-} be the undissociated form and the uni- and bi-valent anions respectively of a dibasic acid and T the sum of H_2A, HA^- and A^{2-}. It follows from the equations defining the ionization constants that

$$[A^{2-}]=\frac{[T]\,K_1K_2}{K_1K_2+[H^+]^2+[H^+]\,K_1}, \qquad (1)$$

where $[H^+]$ is the H-ion concentration and K_1 and K_2 are the two ionization constants (see Michaelis, 1922). Hence,

$$\frac{[A^{M2-}]}{[A^{F2-}]}=\frac{[T^M]}{[T^F]}\times\frac{K_1^MK_2^M[K_1^FK_2^F+[H^+]^2+[H^+]K_1^F]}{K_1^FK_2^F[K_1^MK_2^M+[H^+]^2+[H^+]K_1^M]}, \qquad (2)$$

where the superscripts M and F refer to malic and fumaric acids. At pH 7·4 $[A^{M2-}]/[A^{F2-}]$ is practically the same as $[T^M]/[T^F]$, i.e. equal to the ratio measured in the experiments recorded in Table 1, henceforth referred to as r. Substituting r for $[A^{M2-}]/[A^{F2-}]$ in (2):

$$\frac{[T^M]}{[T^F]}=r\times\frac{K_1^FK_2^F[K_1^MK_2^M+[H^+]^2+[H^+]K_1^M]}{K_1^MK_2^M[K_1^FK_2^F+[H^+]^2+[H^+]K_1^F]}. \qquad (3)$$

This formula may be used to calculate the equilibrium concentrations of total malic and fumaric acids at any H-ion concentration. If $[H^+]$ is small compared with K_1 and K_2 (below 10^{-7}) all terms containing $[H^+]$ become negligible and $[T^M]/[T^F]=r$. As $[H^+]$ rises the numerator of the fraction increases more rapidly than the denominator because K_1^F and K_2^F are greater than K_1^M and K_2^M. $[T^M]/[T^F]$ rises towards a maximum value of $r\times K_1^FK_2^F/K_1^MK_2^M$. Assuming $r^{25°}=4·42$ and

$K_1^F=9·6\times10^{-4}$; $K_2^F=4·0\times10^{-5}$ (German & Vogel, 1936; Sihvonen, 1930),

$K_1^M=3·3\times10^{-4}$; $K_2^M=7·7\times10^{-6}$ (Clark, 1928),

the following values for $[T^M]/[T^F]$ are obtained

pH	$[T^M]/[T^F]$
7·4	4·42
7·0	4·46
6·0	4·86
5·0	8·26
4·0	22·4
3·0	44·6
2·0	62·0
1·0	66·4

A graphical presentation of the effect of pH on the equilibrium ratio is given in Fig. 1.

To test the theory the equilibrium was measured at pH 4·85. As fumarase loses its activity at lower pH ranges the test cannot be extended over the whole range. Owing to the weak activity of the enzyme at pH 4·85 relatively large amounts of fumarase have to be used. Sheep heart was minced in the Latapie mill and stored at −12°. To prepare an extract the frozen material was allowed to thaw and homogenized in the Waring Blendor with 2 vol. of water. The homogenate was centrifuged and the supernatant, after 2 hr. dialysis against running tap water, served as a source of fumarase. 20 ml. extract, 1 ml. 0·5 M-fumarate, 2·5 ml. M-acetate buffer, pH 4·7, 1·5 ml. water and a few drops *sec.*-octanol were incubated at 25°, together with a control containing no fumarate. At intervals 5 ml. samples were removed and mixed immediately with 1 ml. 40% (w/v) metaphosphoric acid. The filtrate was used for the determination of fumarate as described before except that 50 mg.

catalyst instead of 10 mg. were used. This modification proved necessary because the tissue extract contained substances which reduced the activity of the catalyst. pH was measured at the end of the incubation period; the value of 4·85 was somewhat higher than that of the buffer owing to the neutralizing effects of substrate and enzyme solution.

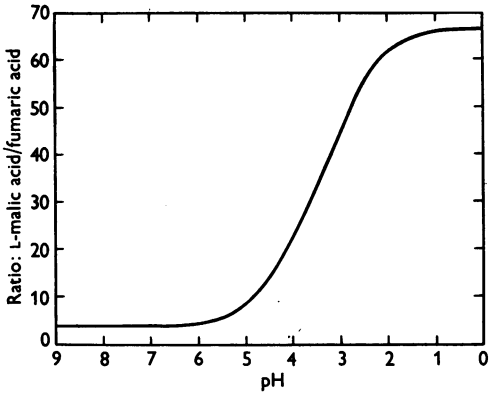

Fig. 1. Effect of pH on the equilibrium ratio malic acid (all forms)/fumaric acid (all forms) calculated according to eqn (3).

The results were as follows:

	4 hr.	6 hr.
L-Malate (μl./ml.)	398	404
Fumarate (μl./ml.)	49	50

The ratios malate/fumarate were thus 8·1. This is somewhat lower than calculated, but the deviation is not unexpected as the ionization constants used for Fig. 1 are the thermodynamic ionization constants. At the ionic strength of the incubated solutions the ionization constants would be greater which would result in a shift of the S-shaped curve of Fig. 1 to the right.

The theoretical treatment of this problem is analogous to that of the effect of pH on oxido-reduction potentials (Borsook & Schott, 1931*b*). Corresponding equations can be derived for mono- or tri-carboxylic acids. In the case of a monobasic acid (1) becomes

$$[A^-]=\frac{[T]K}{[H^+]+K},$$

and (3)
$$\frac{[T_I]}{[T_{II}]}=\frac{[A_I^-]}{[A_{II}]}\times\frac{K^{II}([H^+]+K^I)}{K^I([H^+]+K^{II})}. \qquad (4)$$

If the ionization constants of di- and poly-basic acids are very different, such acids may be treated, within a limited pH range, as monobasic acids. This applies, for example, to the equilibria in various phosphatase systems, where it is sufficient, within the pH range of enzyme activity, say pH 5–9, to consider only the second ionization constant.

Examples illustrating the validity of equation (4) are provided by data published in papers of Cori, Cori & Green (1943) and of Hanes (1940*a*, *b*). In the phosphorylase system

glycogen + phosphate ⇌ glucose 1-phosphate

the equilibrium ratio total inorganic phosphate/total glucose phosphate was found to vary between 2·45 and 10 when pH varied between 5 and 7·3,

Table 3. *Equilibrium ratio total inorganic* P*/total glucose* 1-P *in the presence of phosphorylase*

(The observed ratios in series 1 are taken from Cori *et al.* (1943), Table 3, in series 2 from Hanes (1940*b*) Table 7. For the calculation according to equation (4) the equilibrium ratio of the bivalent anions was assumed to be 2·0 (Cori *et al.* 1943); K_2 of inorganic phosphate = $1{\cdot}51\times10^{-7}$; K_2 of glucose 1-phosphate = $7{\cdot}76\times10^{-7}$ (Cori, Colowick & Cori, 1937.)

Series	$[H^+]$ (M)	Observed ratio	Calculated ratio
1	$0{\cdot}47\times10^{-7}$	2·45	2·48
	$0{\cdot}10\times10^{-7}$	3·11	3·03
	$3{\cdot}09\times10^{-7}$	4·08	4·36
	$8{\cdot}51\times10^{-7}$	5·90; 6·81	6·44
2	1×10^{-7}	3·1	2·9
	$3{\cdot}2\times10^{-7}$	4·7	4·4
	10×10^{-7}	6·7	6·7
	32×10^{-7}	9·0	8·4
	100×10^{-7}	10·8	9·7

whilst the ratios of the bivalent and univalent anions were constant. Table 3 shows that the above ratio can be satisfactorily calculated for different pH values if the ratio for the bivalent anions is known.

SUMMARY

1. The concentrations of fumarate and L-malate, and of *iso*citrate, aconitate and citrate have been measured in the aqueous equilibrium mixtures in the presence of fumarase and aconitase.

2. At equilibrium, the fumarase system was found to contain 18·45 % fumarate and 81·55 % L-malate, the aconitase system 6·20 % *iso*citrate, 2·90 % *cis*-aconitate and 90·9 % citrate (pH 7·4; 25°).

3. A theory is given for the calculation of the effect of pH on the equilibrium concentrations of systems containing acids or bases. The experimental test of the theory in the fumarase and phosphorylase systems proved satisfactory.

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Equilibria in Transamination Systems

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Preliminary determinations of the equilibrium constants in the glutamic-aspartic and the glutamic-alanine system by Cohen (1940) gave the following values at 38°:

$$\frac{[\alpha\text{-ketoglutarate}] \times [\text{L-aspartate}]}{[\text{L-glutamate}] \times [\text{oxaloacetate}]} = 3{\cdot}5, \quad (1)$$

$$\frac{[\alpha\text{-ketoglutarate}] \times [\text{L-alanine}]}{[\text{L-glutamate}] \times [\text{pyruvate}]} = 1. \quad (2)$$

Improved techniques employed by Darling (1945, 1947) gave 5·44 for (1) and 2·25 for (2) whilst Lénárd & Straub (1942) reported a value of 1·43 for (2). In the present work the two constants were redetermined with the help of the newer more accurate manometric methods for the determinations of glutamic, α-ketoglutaric, aspartic, pyruvic and oxaloacetic acids. Under optimum conditions the probable error of a single estimation of any of these substances is not greater than 1%, but a complication arises in the determination of the equilibrium constant of (1) from the instability of oxaloacetate; its slow conversion into pyruvate and bicarbonate is unavoidable and no perfect equilibrium can be reached. However, any error due to this side reaction is negligible if the rate of the side reaction is slow in relation to that of transamination. The presence of additional substances formed by side reactions does not cause errors if the concentrations of all four reactants in the transamination systems are determined directly.

EXPERIMENTAL AND RESULTS

Measurements in the glutamic-aspartic system

Enzyme preparation. Finely minced horse heart muscle was extracted with 2 vol. 0·5% NaCl solution for 60 min. and then centrifuged. The supernatant was dialysed for at least 4 hr. against running tap water to remove preformed substrates. The dialysed extract was stored in the refrigerator after addition of, and thorough mixing with, *sec.*-octanol. The extract contained a powerful glutamic-aspartic transaminase and showed weak alanine-glutamic transaminase and oxaloacetic decarboxylase activity. That the rate of side reactions under the test conditions was very small can be judged from data given in Table 1. In the absence of the side reactions the sum of aspartic and glutamic acids should remain constant and should equal the amount of glutamate added, any deficit being due to the formation of alanine. Furthermore, the sum of oxaloacetate and aspartate should be constant and equal the amount of aspartate added, any loss being due to the decarboxylation of oxaloacetate.

Reaction mixture and sampling for analysis. Equal volumes (10–15 ml.) of about 0·08 M-α-ketoglutarate, about 0·08 M-L-aspartate, enzyme solution and 0·1 M-phosphate buffer, pH 7·4, and a few drops of *sec.*-octanol were mixed and placed in a water bath at 25·0°. The actual initial concentrations of the α-ketoglutarate and L-aspartate solutions were determined in each experiment. A control mixture contained buffer and enzyme but no substrate. At specified times samples were removed for analysis. For the determination of oxaloacetic acid 3 ml. were placed in the main compartment of an ice-cooled conical manometer vessel containing 1 ml. 2 N-HCl. For the determination of the three other reactants 9 ml. were pipetted into a graduated