THE FREE ENERGY, HEAT, AND ENTROPY OF FORMATION OF *l*-MALIC ACID

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In the previous communication (1), it was shown that the free energy of the bivalent fumarate ion at 25° is —144,630 calories. In the present communication the measurement of the equilibrium at 25° between fumaric acid and malic acid in the presence of fumarase is reported, and from the values obtained computations are made of the free energy and heat of formation of the bivalent *l*-malate ion and of the free energy and entropy of solid *l*-malic acid.

It has been known for many years that a reversible hydrolysis of fumaric acid to *l*-malic acid occurs in the presence of minced muscle or of extracts made from it. The values obtained for the equilibrium constant have varied with different workers. Recently Lehmann (2) has described a potentiometric measurement of this equilibrium constant.

In order to obtain the value of ΔH for this reaction and thereby the means of computing the equilibrium constants at various temperatures, the authors carried out a number of such potential measurements at 25°.

In his employment of this potentiometric method the assumption was made by Lehmann that the only reactions taking place, even when the enzyme preparation contained fumarase, were: fumaric acid + $H_2O \rightarrow$ *l*-malic acid; and succinic acid + methylene blue $\rightarrow$ fumaric acid + leucomethylene blue. The observations described below, of the authors, show that this is not generally true, and that the value of the equilibrium constant obtained from potentiometric measurements may not be accepted unreservedly. The measurements of Lehmann were made on either initially equi-

molar mixtures of succinic acid and fumaric acid, or on equimolar mixtures of these acids to which a quantity of *l*-malic acid was added corresponding to the estimated amount of *l*-malic acid which would be in equilibrium with the fumaric acid initially added.

The authors' measurements were made with initially 1:9, 5:5, and 9:1 ratios of succinic acid to fumaric acid, and at two different hydrogen ion concentrations. It was found that whereas with fumarase-free enzyme preparations the experimental value of n, *i.e.* the number of electrochemical equivalents involved in the electrode reaction, was 2, when fumarase was present the value of n was always greater than 2, varying from 2.17 to 2.26, giving a mean value of 2.22. Our interpretation of this fractional value for n is that some of the malic acid formed from the fumaric acid is oxidized and that the resulting leucomethylene blue reacts with some of the fumaric acid to form oxidized methylene blue and succinic acid. In support of this interpretation is the observation that in the presence of even fumarase-free enzyme preparations *l*-malic acid is oxidized with the reduction of methylene blue. In the absence of any other metabolite than malic acid complete reduction of the dye occurs. When fumaric acid is also added, the reduction of the methylene blue stops at a potential corresponding to a low ratio of succinic acid to fumaric acid. This cessation of the reaction suggests that when a small amount of the malic acid is oxidized, with the formation of a corresponding amount of succinic acid, an equilibrium is attained. That only a small amount of malic acid is oxidized is indicated also by the value of n being close to 2. These results show the danger of measuring reduction potentials with only one metabolite ratio. As Table I shows, the values for the equilibrium constant at the two different hydrogen ion concentrations for each metabolite ratio agree well with each other, yet the values with different metabolite ratios vary systematically.

In the absence of interfering secondary reactions the potential difference between the initially 9:1 and 1:9 mixtures of succinic acid to fumaric acid would have been 56 millivolts; the difference found experimentally was 51 millivolts. We have based our calculation of the value of the equilibrium constant on the potentials obtained with the initially 5:5 mixtures of succinic acid and fu-

maric acid. The possible error is approximately $\pm$ 2.5 millivolts. Though this uncertainty of 2.5 millivolts introduces a relatively large uncertainty into the value of the equilibrium constant, for the purpose of estimating the free energy of formation of l-malic acid from fumaric acid the error is quite small, amounting to not more than 150 calories.

The technique employed in these determinations was identical with that described in the previous communication (1), except that the enzyme preparation was modified so as to preserve the

TABLE I

Equilibrium Potentials of Succinic Acid-Fumaric Acid Mixtures in Presence of Fumarase-Containing Enzyme

Initial ratio of succinic acid to fumaric acid	pH	Potential, if no fumarase had been present E'_h	Potential observed E''_h	$-\left(E'_h - E''_h\right)$	K for $\dfrac{\text{(malic)}}{\text{(fumaric)}} = K$
		volt	*volt*	*volt*	
9:1	6.81	-0.0054	$+0.0104$	0.0158	2.42
9:1	6.81	-0.0054	$+0.0097$	0.0151	2.24
9:1	7.12	$+0.0128$	$+0.0280$	0.0152	2.27
5:5	6.81	-0.0336	-0.0158	0.0178	2.99
5:5	6.81	-0.0336	-0.0156	0.0180	3.06
5:5	7.12	-0.0154	$+0.0032$	0.0186	3.25
5:5	7.12	-0.0154	$+0.0027$	0.0181	3.09
1:9	6.81	-0.0618	-0.0406	0.0212	4.20
1:9	6.81	-0.0618	-0.0415	0.0203	3.85
1:9	7.12	-0.0438	-0.0236	0.0200	3.74
1:9	7.12	-0.0438	-0.0231	0.0205	3.93

fumarase activity. Beef heart was finely minced, triturated, and washed five times with 0.25 per cent NaCl, once with distilled water, and then extracted, after grinding with powdered glass, as in the preparation of the fumarase-free enzyme suspension.

The results obtained are collected in Table I.

The equilibrium constant for the reaction

$$\text{Fumaric acid} + H_2O \rightleftarrows l\text{-malic acid}$$
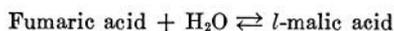
$$\frac{\text{(Malic)}}{\text{(Fumaric)}} = K$$

was calculated from the potentials (the secondary reaction being disregarded) as follows:

E'_h = potential observed when fumarase is absent from enzyme preparation

E''_h = potential observed in presence of fumarase

(Fum) = equilibrium concentration of fumaric acid in presence of fumarase

$\therefore$ (Fum) $(1 + K)$ = initial concentration of fumaric acid

$$\therefore E''_h = \tilde{E} + \frac{RT}{F}\, \text{pH} + \frac{RT}{nF} \ln \frac{\text{(Succ.)}}{\text{(Fum)}} + \text{correction for ionization}$$

$$\therefore E'_h - E''_h = \frac{RT}{nF} \ln \frac{\dfrac{\text{(Succ.)}}{\text{(Fum)} (1 + K)}}{\dfrac{\text{(Succ.)}}{\text{(Fum)}}}$$

$$= \frac{RT}{nF} \ln \frac{1}{(1 + K)}$$

$$\therefore -(E'_h - E''_h) = \frac{RT}{nF} \ln (1 + K)$$

In the calculation of K in Table I we have taken the value of n as 2 in spite of the fact that the value calculated from the experimental results is 2.21. This value for n of 2.21 is based upon the assumption of a value of −0.437 volt for $\tilde{E}$ which must be erroneous since it does not take into account the secondary reaction discussed above. A second erroneous assumption is made also in considering that the various ratios of succinic acid to fumaric acid maintain their initial relationships to each other after the attainment of equilibrium in the presence of the fumarase-containing preparation. This assumption would have been valid if, apart from the hydrolysis of the fumaric acid to *l*-malic acid, the reduction of the methylene blue had been the only significant reaction occurring here. On account of our inability to measure this secondary reaction it seemed preferable for the time being to accept only those values of the equilibrium constant calculated from the potentials of the initially 5:5 mixtures of succinic acid to fumaric

acid, and to take the value of n as 2. The mean of these values is 3.1. As Table I shows, these are intermediate between the diverging extremes: the final value of the ratio of succinic acid to fumaric acid is least divergent in this range from that calculated on the assumption that the fumaric acid is converted only to l-malic acid, and the rate of the change of the potentials with varying ratios of reductant to oxidant is least here. The maximum error in this approximation is probably much less than 2.5 millivolts, which corresponds to a change in the value of K of 0.75 and to differences in the free energy change of less than 150 calories.

Lehmann, at 37°, also calculating from the potentials of initially 5:5 ratios, and taking $n = 2$, obtained a mean value of 3.0. It seems probable that the enzyme preparations employed by Lehmann would have given divergences similar to those shown in Table I. Woolf measured this equilibrium with *Bacillus coli* as catalyst, in the presence of l-aspartate (3) at 37°. The equilibrium ratios of l-malate to fumarate were found to be 3.1 and 3.2. These values provide an independent check on the values for the equilibrium constant since the l-malic acid was measured polarimetrically.

The coincidence of the values for the equilibrium constant obtained with *Bacillus coli* and with enzyme preparations from minced horse and skeletal muscle shows, as in the case of the succinate-enzyme-fumarate equilibrium, that the equilibrium position is practically independent of the source of the enzyme.

In view of the uncertainty regarding the precise values of the equilibrium constants no reliance could be placed on a value of ΔH calculated from the equilibrium constants at 37° and 25°. Less error is likely to be incurred by assuming ΔH to be the same as ΔF, *i.e.* about 700 calories. The heat of formation of the bivalent l-malate ion may then be estimated from the more accurately determined values of the heats of formation of the bivalent fumarate ion and of water, which are respectively, $-189,120$ calories (1) and $-68,310$ calories, yielding for the heat of formation of bivalent l-malate ion a value of $-258,100$ calories.

Computation of the Free Energy and Entropy of Formation of
l-Malic Acid

The fraction of the total malic acid in the bivalent form at pH 6.81 is 96.8 per cent and at pH 7.12, 97.8 per cent, when pK_1

= 3.48, and pK_2 = 5.11 (4) for the dissociation constants of *l*-malic acid. On account of the uncertainty of the value for the equilibrium constant we shall consider the malic acid as completely dissociated, and not introduce the trifling correction into the remaining fumaric acid for the undissociated malic acid. We shall write therefore

$$\text{Fumarate}^= (1 \text{ M}) + H_2O \rightleftarrows l\text{-malate} (1 \text{ M})$$

$$\Delta F_{l\text{-malate}^=} - 144{,}630 - 56{,}560 - 670 = - 201{,}860 \text{ calories}$$

TABLE II

Activity Coefficients of Aqueous d-Tartaric Acid Solutions at High Concentrations

Molality	Activity coefficients from	
	Vapor pressure at 100°	Depression of freezing point
1.5		1.11
2.0	1.27	
3.0	1.49	1.24
4.0	1.73	1.35
5.0		1.48
5.3	2.06	
6.5	2.12	
10.0	2.74	

The free energy of ionization of *l*-malic acid

$$- \Delta F_{\text{ionization}} = RT \ln K_1K_2 = - 11{,}720 \text{ calories}$$

where K_1 and K_2 are respectively the first and second hydrogen ion dissociation constants. Therefore the free energy of formation of undissociated *l*-malic acid at 1 molal activity is

$$\Delta F_{(l\text{-malic, 1 M})} = - 201{,}860 - 11{,}720 = - 212{,}580 \text{ calories}$$

The solubility of *l*-malic acid was found to be 100 gm. in 129 gm. of solution, corresponding to a mol fraction of 0.317. The mol fraction of molal malic acid in the undissociated state is 1:56.51 = 0.0177. Since no data are available for the estimation of the activity coefficient of *l*-malic acid in its saturated solution, we have assumed that it is not very different in this respect from

d-tartaric acid for which vapor pressure and freezing point lowering data up to 10 and 5 molal solutions respectively are given in the International Critical Tables. By means of the h and j functions of Lewis and Randall (5) the activity coefficients of d-tartaric acid were computed from these data. These are set out in Table II.

It seems hardly probable that the discrepancies between the activity coefficients for d-tartaric acid calculated from vapor pressure and freezing point data can be due to experimental errors. More probably the differences are due to the large difference in temperature at which the two measurements were made. The accuracy of the measurements would not warrant taking into account the temperature coefficient of the partial molal specific heat content of d-tartaric acid, even if the data were available; especially as in any case an extrapolation from a 10 molal to a 22 molal solution (the molality of the saturated l-malic acid solution) is necessary. We have, therefore, taken the activity coefficient of l-malic acid in its saturated solution to be 2. We feel that this approximation does not incur an error in the estimation of the free energy of l-malic acid of more than 200 or 300 calories.

The ionization of malic acid in its saturated solution, which is negligible here, being disregarded the free energy of transfer at 25° from the solution in which its activity is molal to the saturated solution is $RT \ln \dfrac{0.0177}{0.317 \times 2} = -2120$ calories. The free energy of formation of solid undissociated malic acid at 25° is $-212,580 + 2120 = -210,460$ calories.

The heat of combustion of l-malic acid (solid) at 19° is 320,100 calories (6) which we may take as 320,000 calories at 25°. The heat of formation of l-malic acid in the reaction $C_4H_6O_5 + 3O_2 \rightarrow 4CO_2 + 3H_2O$ is therefore,

$$\Delta H = 320,000 - 4(94,240) - 3(68,310) = -261,890 \text{ calories}$$

From the relationship $\Delta S = \dfrac{\Delta H - \Delta F}{T}$, the entropy of formation of solid l-malic acid at 25°,

$$\Delta S = \frac{-261,890 + 210,460}{298} = -172.5 \text{ E.U.}$$

The entropy of solid *l*-malic acid since

$$S_{\text{malic}} - S_{(4\,\text{C})} - S_{(3\text{H}_2)} - S_{(2\frac{1}{2}\text{O}_2)} = -172.5 \text{ E.U.}$$

is

$$S(\text{solid})_{298°} = -172.5 + 4(1.3) + 3(31.23) + 2.5(49.03)$$

$$= 49 \text{ E.U.}$$

It is obvious that the value obtained for the free energy of *l*-malic acid cannot be considered as final. On the other hand the approximations cannot, it seems, have introduced a large error. The uncertainty regarding the equilibrium constants corresponds to 150 calories. The employment of titration constants at 18° and 25° instead of the dissociation constants at 25° and at infinite dilution, if we judge from the values for fumaric acid, does not amount to more than 100 calories, and the uncertainty regarding the activity coefficient of *l*-malic acid in its saturated solution to another 250 calories. Even if these errors were all in the same direction the total would be not more than 500 calories, which is negligible for most energy calculations in intermediary metabolism.

The entropy value depends also on the reliability of the heat of combustion. It is planned to obtain an independent determination of the entropy and free energy value of *l*-malic acid by specific heat measurements.

SUMMARY

1. The equilibrium constant for the reaction

$$\text{Fumarate}^- + \text{H}_2\text{O} \rightleftarrows l\text{-malate}^-; \ \frac{(l\text{-malate}^-)}{(\text{fumarate}^-)} = K$$
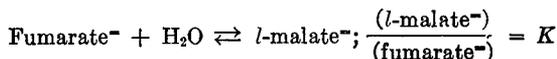
was estimated from the electrometric measurements at 25° to be approximately 3.1.

2. One of the possible errors of the potentiometric method of measuring the equilibrium constant is demonstrated.

3. The value of the free energy of formation of *l*-malic acid (solid) at 25° was estimated at −210,450 calories, with an error not greater than ± 500 calories. The value of the free energy of bi-

valent l-malate ion at 1 molal activity was estimated at $-201,940$ $\pm$ 150 calories; and the heat content at $-258,100$ calories.

4. The value of the entropy of solid l-malic acid at 25° was estimated at 49 E.U.

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