THE RÔLE OF THE ENZYME IN THE SUCCINATE-ENZYME-FUMARATE EQUILIBRIUM

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The following is an account of an investigation into the rôle of the enzyme in the succinate-enzyme-fumarate equilibrium. The method consisted in the comparison of the value of the free energy change in this reaction obtained from oxidation-reduction potentials, with that calculated from the entropies and other physicochemical properties of succinic acid and fumaric acid.

The findings of Wishart (1), Quastel and Whetham (2), Thunberg (3), and Lehmann (4) show that the oxidation of succinic acid to fumaric acid by means of a dehydrogenase, in the presence of a reversible hydrogen acceptor such as methylene blue, satisfies the necessary criteria for thermodynamic reversibility. The equilibrium constant has been measured colorimetrically by Quastel and Whetham, with resting *Bacillus coli* as catalyst, by Thunberg, colorimetrically and electrometrically, and by Lehmann, electrometrically with an enzyme derived from horse skeletal muscle. The values obtained by the latter two workers are not concordant, and further there is some uncertainty regarding the agreement or disagreement of the potentials with the equilibrium value obtained by Quastel and Whetham.

A priori, it seemed possible, apart from experimental errors, that these discrepancies might be due to variations in the nature of the enzyme employed. In other words, the enzyme is not a "perfect" catalyst, and therefore the value of the equilibrium constant (or of the oxidation-reduction potential) depends to some extent upon the nature of the enzyme employed. In this event it would be expected that the value for the standard free energy change calculated from the entropies and other physicochemical

properties of succinic acid and fumaric acid would not be the same as the experimental values, the differences being attributable to the participation of the enzymes, and varying with each enzyme.

This possibility is definitely excluded, it seems, by the remarkable agreement between the calculated values and all except one of the experimental values, shown in Tables IV and V, obtained by Quastel and Whetham with "resting" *Bacillus coli* as catalyst, by Thunberg and by Lehmann with horse skeletal muscle, and by the authors with beef diaphragm and with beef heart.

This coincidence of the calculated and observed values may be taken as indicating that the enzyme promoting the oxidation of succinic acid to fumaric acid probably operates as a "perfect" catalyst, wherever it occurs, *in vivo* as well as *in vitro*.

One proviso must be made, that the reduction of the oxidizing agent, *e.g.* methylene blue, must also be perfectly reversible. It is possible that the reduction of oxygen *in vivo* is irreversible, in which case the whole reaction

$$\text{Succinic acid} + \tfrac{1}{2}O_2 \xrightarrow{\text{(enzyme)}} \text{fumaric acid} + H_2O$$

is, of course, irreversible. Nevertheless it is convenient to consider the reaction,

$$\text{Succinic acid} + \xrightarrow{\text{(enzyme)}} \text{fumaric acid} + 2H^+ + 2e$$

analogous to a half-cell, which operates reversibly even *in vivo*.

This coincidence between calculated and observed values suggests one general application of the second law of thermodynamics to biological systems and is additional confirmation of the validity of the third law.

Experimental Technique and Procedure

The electrode potential measurements, yielding the results set out in Table I, were carried out with a vacuum technique, in a modified Thunberg tube depicted in Fig. 1. The principal modification consists in the attachment of a capillary tube of 0.75 mm. bore and approximately 10 cm. long, containing an agar-saturated potassium chloride bridge. When the electrode vessel is in use the end of the capillary tube dips into a vessel containing a saturated solution of potassium chloride, in communication with the

reference electrode, here a saturated calomel cell. With these vessels it is possible to carry out a number of determinations simultaneously, only one reference electrode being employed. The technical difficulty overcome was the preparation of an agar-potassium chloride bridge in glass, capable of withstanding a difference in pressure of 1 atmosphere between its two ends. After a number of trials, the following method of preparation was found to be satisfactory. Potassium chloride solution and sufficient

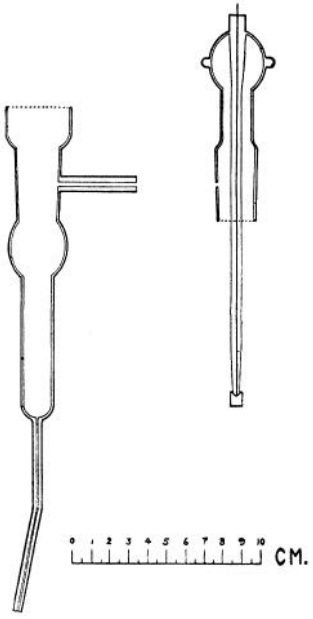

FIG. 1. Modified Thunberg tube for electrode potential measurements with a vacuum technique.

dry agar to give a final concentration of 3 per cent are weighed separately, after which the agar is dissolved by heating nearly to boiling, distilled water being added from time to time so that the weight of the solution remains equal to the sum of the initial weights of the agar and salt solution. When as much as possible of the agar is dissolved, the beaker is covered and set away in an oven at 98° in which the electrode vessel is also placed. After the agar solution has become clear and free of suspended particles

of undissolved agar and of air bubbles, it is poured into a narrow test-tube which is a little taller than the length of the capillary tube of the electrode vessel. The capillary tube is then immersed in the agar solution and by slight suction quickly drawn up through the capillary, filling 1 or 2 cc. in the bottom of the vessel. The electrode vessel is then stoppered and sufficient agar solution is added to the test-tube so that the height of the solution is the same outside and inside the electrode vessel. For about an hour the temperature of the oven is maintained at 95° in order to allow the solution to wet the wall of the capillary tube. The heater of the oven is then turned off. As a rule about 4 hours elapse before the temperature falls to the jellying point of the agar. After the oven has cooled to room temperature, the outside test-tube is removed and replaced by a small vial filled with saturated potassium chloride. The agar at the bottom of the electrode vessel is scraped out with a glass stirring rod and a few cc. of salt solution are added. With both ends of the capillary tube protected in this way with salt solution when the vessel is not in use, bridges so prepared withstand a difference in pressure of 1 atmosphere even at 45°, and at 25° were used repeatedly for weeks.

The electrodes employed were mainly gold-plated platinum foil. In some of the earlier experiments platinum foil was used. The results obtained were the same with either type of electrode.

The procedure employed in the potential determinations was as follows: The specified amounts of succinate, fumarate, dye, and buffer solutions were pipetted into the electrode vessel in a cold room maintained at 2°, where the stock solutions were kept. The vessel was then stoppered and evacuated at room temperature for 1 minute, after which it was transferred to a water bath at 37°, the evacuation being continued until the solutions had been boiling for 2 minutes. After the termination of the evacuation the stopper was turned, closing the vessel, the side arm and water seal above were filled with water, and these in turn were enclosed by a layer of vaseline. The vaseline prevented any loss of water even after many hours of shaking. The evacuated vessels were transferred to an air bath maintained at 25°, at which temperature the determinations were made. It was found during the course of the investigation that more concordant results were obtained when the vessels were continually shaken throughout

the period of observation.[1] The tubes were so clamped in the shaking device that the ends of the capillary tubes containing the agar bridges dipped intermittently in and out of the saturated potassium chloride solution, in which was immersed also the end of a saturated calomel electrode.

The hydrogen ion activity determinations were made with a Moloney electrode (5)[2] in an air bath maintained at 25°. The reproducibility obtained with different electrodes was within ±0.25 millivolt. The reference electrode employed for the pH determinations was also a saturated calomel half-cell, checked against the similar half-cell employed in the potential determinations. In this manner any errors due to differences between the reference cells or to liquid junctions were eliminated.

The fumarase-free enzyme solution was prepared by a modification of the method described by Lehmann. The heart and diaphragm muscle were obtained, as a rule, 2 or 3 days after the death of the animal. After removal of fat and connective tissue, the meat was passed through the finest cutter of a meat grinder, suspended in water (½ pound of meat in 800 cc. of suspension) and

[1] The authors are indebted to Mr. G. L. Keighley for the design and construction of the shaker employed, as well as for assistance in the working out of the method of preparing the agar bridges described above.

[2] The serviceability and accuracy of this type of hydrogen electrode depends upon the method of preparation of the electrode. The technique finally settled upon by the authors is the result of experience gained over a number of years by Professor H. Wasteneys and Mr. D. A. MacFadyen of the University of Toronto, and the authors. The Moloney electrode consists of 6 or 7 mm. of 1 mm. platinum wire projecting from a glass tube, and surrounded by a thread of glass forming a narrow loop extending a few mm. past the end of the wire. The electrode is first cleaned by heating in boiling aqua regia, washed with distilled water, and then heated again in boiling cleaning fluid, or immersed in hot alcoholic soda for a short time. After being washed again with distilled water it is electrolyzed in 10 per cent H_2SO_4 for 5 minutes, washed again, and then plated for 5 to 10 seconds in a 1 per cent solution of platinic chloride in 0.3 per cent HCl. The plating current employed here is 0.025 ampere and 4.0 volts, giving a current density of 0.181 ampere per sq. cm. After being plated it is washed and then electrolyzed again in 10 per cent H_2SO_4 for 30 seconds. We have found that electrodes so prepared could be used for some time, if the electrodes were reelectrolyzed for 30 seconds in 10 per cent H_2SO_4, before being used on a new solution. The great advantage of this electrode is that equilibrium is attained in 1 to 2 minutes.

heated, with constant stirring, for 15 minutes at 50°, after which it was squeezed to dryness through closely woven muslin. The dry residue was triturated with 200 to 500 cc. of cold water and again squeezed to dryness. This procedure, the heating and subsequent trituration, was repeated twice more. The resulting dry residue was then ground to a paste with an equal volume of powdered glass and with 2 cc. of M/15 K_2HPO_4 per gm. of meat. After standing for ½ hour at room temperature the mass was centrifuged, the supernatant suspension (the enzyme solution) passed through cloth and set away preserved with toluene in a cold room at 2°. On several occasions when the first extract was found to be inactive, a second grinding with a solution of phosphate (1 cc. of M/15 K_2HPO_4 + 0.5 cc. of M/15 KH_2PO_4 per gm. of meat) and extraction yielded an active enzyme preparation.

The activity and the residual reduction of the enzyme suspensions so obtained were tested by the methods described by Lehmann. Enzyme preparations with reduction times greater than 50 minutes at 37° were discarded.

Preliminary experiments indicated that at 25° the reduction of the dye ceases before the attainment of equilibrium if the amount of enzyme employed is too small. After a number of trials the following reaction mixture was finally settled upon: enzyme solution, 2 cc.; phosphate buffer or water, 1.5 cc.; 0.0007 M methylene blue, or other dye solution, 0.5 cc.; 0.02 M succinate or fumarate, 0.5 cc. The pH determinations were made on duplicate mixtures except that water was substituted for the dye solution. It was found that the slight concentration of the solutions occurring during the evacuation left the pH of the mixtures, within the limits of accuracy of our measurements, unchanged.

Thionine and indigo tetrasulfonate were used in the extreme acid and alkaline solutions instead of methylene blue.

The succinic acid and fumaric acid employed were twice recrystallized from Eastman preparations. These gave colorless solutions with theoretical titration values. The melting points of the solids were respectively 188° and 281° (corrected).

In the experiments of Thunberg and of Lehmann, in most cases, the potential was determined only with equal concentrations of succinate and fumarate. In our routine procedure the potentials were measured simultaneously in three mixtures containing differ-

ent ratios of succinate to fumarate, as a rule 9:1, 5:5, and 1:9. In this way it was possible to detect with some assurance the presence of other interfering enzymes, such as fumarase, or other

TABLE I

Oxidation-Reduction Potentials of Succinic Acid-Fumaric Acid System at 25°

Succinate/Fumarate = 9/1			Succinate/Fumarate = 5/5			Succinate/Fumarate = 1/9			Enzyme
pH	Phosphate concentration	$-\bar{E}$	pH	Phosphate concentration	$-\bar{E}$	pH	Phosphate concentration	$-\bar{E}$	
	M			M			M		
6.19	0.2	0.440	6.10	0.2	0.440	6.10	0.2	0.435	Beef heart
6.67	0.05	0.438	6.25	0.2	0.437	6.19	0.2	0.437	
6.67	0.05	0.437	6.67	0.05	0.441	6.25	0.2	0.437	
6.76	0.2	0.444	6.67	0.05	0.438	6.67	0.05	0.439	
7.07	0.2	0.436	6.81	0.2	0.436	6.67	0.05	0.439	
7.08	0.2	0.436	7.07	0.2	0.434	6.76	0.2	0.434	
7.08	0.2	0.436	7.08	0.2	0.437	6.81	0.2	0.434	
7.08	0.2	0.437	7.08	0.2	0.435	7.07	0.2	0.434	
7.08	0.2	0.437	7.12	0.2	0.434	7.08	0.2	0.437	
7.08	0.2	0.440	7.47	0.2	0.437*	7.12	0.2	0.434*	
7.12	0.2	0.439				7.46	0.2	0.436	
7.47	0.2	0.439				7.47	0.2	0.440	
Mean.....		0.438			0.437			0.436	
6.16	0.2	0.444	7.05	0.2	0.439	6.16	0.2	0.437	Beef diaphragm
7.05	0.2	0.439	7.12	0.03	0.435	7.05	0.2	0.437	
7.05	0.2	0.437	7.12	0.03	0.434	7.12	0.03	0.435	
7.12	0.03	0.433	7.90	0.2	0.436	7.90	0.2	0.434	
7.12	0.03	0.431							
Mean.....		0.437			0.436			0.436	

* Values were obtained after the evacuated mixtures had been set away in a water bath for ½ or 1 hour at 37°, until the methylene blue was decolorized and then set away at 25°.

oxidizing enzymes, by discrepancies between the values obtained with the different ratios and by the continuously negatively drifting potential, and in some of the earlier experiments such disturbing factors as air leaks through the agar-potassium chloride bridges.

The values recorded in Table I are those of experiments in which a final potential was steady to within 0.1 millivolt for 1 hour or more. The validity of this selection of values we feel is demonstrated by the concordance among themselves of these steady values over a wide range of hydrogen ion activities, and metabolite ratios, by the correspondence of the mean value with that obtained by other workers at various temperatures and with various enzyme preparations, and finally by the coincidence of these experimental values with that calculated from the entropies, solubilities, ionization constants, and thermal data of succinic acid and fumaric acid.

The values set out in Table I show that the molal electrode potential, $\tilde{E}$, is independent (*a*) of the hydrogen ion activity in the range of pH 6.10 to 7.90, (*b*) of the source of the enzyme, (*c*) of the metabolite ratio employed, and (*d*) of the ionic strength of the solution.

The values marked with an asterisk were obtained after the evacuated mixtures had been set away in a water bath for ½ hour or 1 hour at 37°, until the methylene blue was decolorized. The tubes were then removed to the air bath at 25°. In both tubes the color of the methylene blue was partially restored. This and the final values obtained confirm the reversibility of the reaction.

Theoretical Formulation

The term $\tilde{E}$ in Table I corresponds to the molal electrode potential against the normal hydrogen electrode for the reaction

$$\text{Succinate}^{=} \rightleftarrows \text{fumarate}^{=} + 2\text{H}^{+} + 2e$$

We have employed a somewhat different derivation of the electrode potential equation than that commonly employed, in order to obtain a clearer insight into the thermodynamic significance of the various terms. Though the mathematical relationships of the terms in the final equation, as Clark has emphasized (6), are independent of the mechanism postulated in its derivation, nevertheless the practice in the conventional derivation of assembling constants and including them in the characteristic potential obscures the thermodynamic significance of the various terms, because by including miscellaneous constants in the term E_0, the postulated mechanism, *ipso facto*, is changed. Though no error is incurred

in such a change, since the chemical mechanism postulated is conventional only, and is chosen for its convenience, yet it is desirable for clarity to maintain throughout the derivation of an equation, and in its application to experimental results, when it is possible, the mechanism initially postulated, arbitrary or conventional though it be. Maintaining this consistency it is possible, in passing from system to system, to realize the significance of the various terms in the final equation, without the labor of rederiving these equations every time, which is necessary when a number of constants are assembled into a characteristic constant E_0, whose meaning consequently may vary from system to system according to the constants included in it. We feel also that the fixing of attention on the free energy changes provides a "scaffolding" which conforms more closely to the features of the process than is obtained with the more conventional derivation of oxidation-reduction potential equations.[3]

The process of converting succinic acid to fumaric acid and hydrogen ions isothermally in any given solution may be considered as occurring in the following steps. 1 mol of the succinate ion is transferred from an infinitely large volume of solution where the total concentration of succinic acid in all its forms and the hydrogen ion activity are those of the experimental solution,

[3] The following steps yield a general derivation of oxidation-reduction potential equations: (1) calculation of concentration of that form of reductant postulated in the mechanism obtaining at the experimental hydrogen ion activity and total concentration of reductant; (2) concentration of 1 mol of this form to 1 molal activity; (3) oxidation:—reductant (1 M) → oxidant (1 M) + $n\mathrm{H}^+$ (1 M) + ne; (4) dilution of oxidant to the experimental activity and hydrogen ion activity (this step is equivalent to the sum of Steps 1 and 2); (5) dilution of n equivalents of hydrogen ions from 1 molal to experimental activity.

In this derivation the characteristic constant, $\tilde{E}$, or E_0 corresponds to Step 3. The term $\frac{RT}{nF} \ln \frac{S_r}{S_0}$ is derived from Steps 2 to 4; the free energy change due to changing dissociation constants is obtained from the difference between the corresponding values calculated in Step 1 and Step 4. The term for the dependence on the hydrogen ion activity is derived from Step 5, and is always $\frac{nRT}{nF} \ln \frac{1}{(\mathrm{H}^+)}$.

to another infinitely large volume of solution containing the succinate ion, fumarate ion, and hydrogen ion, all at molal activities. If S_s represents the total succinic acid in the initial solution, the molal free energy change in this step is

$$-\Delta F_1 = RT \ln \frac{K^s_1 K^s_2 \cdot S_s}{(H^+)^2 + K^s_1(H^+) + K^s_1 K^s_2} \tag{1}$$

For the second step

$$\text{Succinate}^= (1\text{ M}) \rightarrow \text{fumarate}^= (1\text{ M}) + 2H^+ (1\text{ M}) + 2e$$

we may designate the free energy change as $-\Delta\tilde{F}$.

The third step consists in the transfer of 1 mol of fumarate ion and of 2 mols of hydrogen ions from this hypothetical solution in which their activities are 1 molal to a solution in which the activities of the fumaric acid, and of the hydrogen ion, are those obtaining in the experimental solution. In this step the free energy change for the transfer of the fumarate ion is

$$-\Delta F_2 = RT \ln \frac{(H^+)^2 + K^f_1(H^+) + K^f_1 K^f_2}{K^f_1 K^f_2 \cdot S_f} \tag{2}$$

and for the hydrogen ions

$$-\Delta F_3 = 2RT \ln \frac{1}{(H^+)} \tag{3}$$

The total free energy change therefore is,

$$-\Delta F_{\text{obs.}} = -\Delta F_1 - \Delta\tilde{F} - \Delta F_2 - \Delta F_3$$

$$-\Delta F_{\text{obs.}} = -\Delta\tilde{F} + RT \ln \frac{S_s}{S_f} + RT \ln \frac{K^s_1 K^s_2}{K^f_1 K^f_2} \times$$

$$\frac{(H^+)^2 + K^f_1(H^+) + K^f_1 K^f_2}{(H^+)^2 + K^s_1(H^+) + K^s_1 K^s_2} + 2RT \ln \frac{1}{(H^+)} \tag{4}$$

$$-\Delta F = EnF \tag{5}$$

$$\therefore E_{\text{obs.}} = \tilde{E} + \frac{RT}{nF} \ln \frac{S_s}{S_f} + 2\frac{RT}{nF} \ln \frac{1}{(H^+)} + \frac{RT}{nF} \ln \frac{K^s_1 K^s_2}{K^f_1 K^f_2} \times$$

$$\frac{(H^+)^2 + K'_1(H^+) + K'_1K'_2}{(H^+)^2 + K^s_1(H^+) + K^s_1K^s_2} \qquad (6)$$

The employment of an intermediate solution in which all the reactants are at 1 molal activity is open to the criticism that in a solution in which the hydrogen ion activity is 1 molal, the dissociation of both succinic acid and fumaric acid is completely suppressed. This criticism could have been avoided by employing the mechanism

$$H_2 \text{ succinic acid} \rightarrow H_2 \text{ fumaric acid} + 2H^+ + 2e$$

Nevertheless we have preferred the mechanism with bivalent succinate and fumarate ions, because it corresponds more closely to the experimental conditions, and because the assumptions which must be made regarding differences in activity coefficients are less significant in the calculation of the free energy changes for the two dissociated forms than for the two undissociated forms. As Table I shows the potentials obtained show no systematic variation with the ionic strength. This we have taken as indicating that the ratio of the activity coefficients of succinate and fumarate remains constant, within the limits of accuracy of our measurements, over a range of ionic strengths varying from 0.09 to 0.6, and therefore is probably unity. The data given by Lehmann exhibit a similar independence of the potential and the ionic strength. On the other hand uncertainties regarding the values of the activity coefficients of succinic acid and fumaric acid would have introduced an uncertainty of 24 millivolts if the calculations had been based upon the mechanism involving the undissociated forms.

In the computation of the values of $\tilde{E}$ in Table I the following data reported by Sihvonen (7) were employed for the variations of the titration constants with ionic strength.

Succinic Acid + KCl

$$pK'_{18^\circ} = 4.213 - 0.998\sqrt{\mu} + 1.27\mu$$
$$pK''_{18^\circ} = 5.634 - 1.996\sqrt{\mu} + 2.74\mu$$
$$pK'_{37^\circ} = 4.182 - 1.030\sqrt{\mu} + 1.34\mu$$
$$pK''_{37^\circ} = 5.650 - 2.060\sqrt{\mu} + 2.68\mu$$

Fumaric Acid + KCl

$$pK'_{18°} = 3.031 - 0.998\sqrt{\mu} + 2.80\mu$$
$$pK''_{18°} = 4.466 - 1.996\sqrt{\mu} + 2.83\mu$$
$$pK'_{37°} = 3.042 - 1.030\sqrt{\mu} + 2.40\mu$$
$$pK''_{37°} = 4.511 - 2.060\sqrt{\mu} + 3.03\mu$$

The mean value obtained for the molal electrode potential at 25° for the succinate-enzyme-fumarate system is —0.437 volt, or —20,140 calories.

Employing the above titration data of Sihvonen and the equation of Cohn (8)

$$pH + \log \frac{KH_2PO_4}{K_2HPO_4} = 7.16 - \frac{1.5\sqrt{\mu}}{1 + 1.5\sqrt{\mu}}$$

we have recalculated the data given by Lehmann. The corrections for the variations in the ionization of succinic acid and fumaric acid with ionic strength range from 0.0135 volt at pH 5.0 to 0.0002 volt at pH 7.0; and yield a good correspondence between calculated and observed potentials over this pH range. On the other hand the corrections employed by Lehmann, 0.0180 to 0.0002 volt respectively, give calculated values which are systematically more positive than the experimental values in the acid reactions.

In this connection it may be pointed out that the marked negative aberrations of the potentials from the calculated curve at hydrogen ion activities more alkaline than pH 7.56 observed by Lehmann, seem, from our observations, to be due to the interference of another enzymatic oxidation, which is not obtained with every enzyme preparation. As Table I shows we have succeeded at times in obtaining an enzyme preparation which did not show this effect even at pH 7.9. When this secondary reaction occurred there was a persistently negative drift of the potentials even after many hours and at the same time the values of different metabolite ratios were discordant. On the other hand when this effect was absent steady potentials were obtained with different metabolite ratios which were in good agreement with each other, and with the values obtained at other hydrogen ion activities.

The data supplied by Lehmann, after the introduction of the corrections discussed above yield mean values for $\tilde{E}$ at 37° and at

18° of −0.430 and −0.443 volt respectively. These values are the same as those deduced by Lehmann from the potentials obtained in the neighborhood of pH 7.0 where the error due to the employment of faulty titration constants is negligible. Employing a linear temperature coefficient, the values of $\tilde{E}$ and $-\Delta F$ at 25° from the above are −0.438 volt and −20,180 calories, respectively.

The close correspondence between the values obtained by Lehmann at 37° and by the authors at 25° confirms not only the accuracy of the potentials obtained but also the value of the temperature coefficient. From this temperature coefficient by means of the Gibbs-Helmholtz equation Lehmann calculated ΔH to be −29,850 calories. The calculated value from purely thermal data given in Table V is −29,800 calories.

Two earlier potential measurements by Thunberg are quoted by Lehmann. The enzyme was prepared, presumably, from horse skeletal muscle, and contained fumarase. In order to prevent the hydrolysis of fumaric acid to *l*-malic acid 3 times its equivalent quantity of *l*-malic acid was added, the equilibrium ratio of *l*-malic acid to fumaric acid being 3. The first measurement was made colorimetrically at 30°, and at pH 6.7, and the result quoted is −0.015 volt. Since equivalent quantities of succinic and fumaric acids were used, the value of $\tilde{E}$ at 30° is −0.411 volt. A later presumably electrometric determination at pH 6.91 and 30° yielded a potential of −0.015 volt corresponding to $\tilde{E} = -0.433$ volt. At 25° these become −0.415 and −0.436 volt respectively. These values correspond in calories to free energy changes of −19,140 and −20,100 respectively.

Quastel and Whetham with resting *Bacillus coli* at 45° obtained for the reaction

Succinic acid + methylene blue ⇄ fumaric acid + leucomethylene blue

a value of 3.0 for the equilibrium constant. According to these authors the fumarase action under their conditions is very slow and their equilibrium value may be considered as unaffected by this secondary reaction. According to Clark, Cohen, and Gibbs (9) the potential of methylene blue at 30°, when the ratio of re-

ductant to oxidant is 1, is 0.004 volt. On the assumption that the temperature coefficient for methylene blue $\frac{dE_h}{dT} = -0.00135$, obtained by Clark, Cohen, and Gibbs at pH 8.62, obtains also at pH 7.2 and is linear up to 45°, the value for E'_0 at 45° at this pH would then be −0.016 volt.

Since at equilibrium the potential of the methylene blue system is the same as that of the succinate-fumarate system we may write $\frac{(\text{succinate})}{(\text{fumarate})} \times \frac{(\text{methylene blue})}{(\text{leucomethylene blue})} = 3$, when $\frac{(\text{methylene blue})}{(\text{leucomethylene blue})} = 1$, with a change in sign since here the reaction has been written reductant → oxidant + $2e$.

$0.016 = \tilde{E} + \frac{RT}{nF} \ln 3 + \frac{RT}{F} 7.2$, the correction term for ionization being negligible at pH 7.2. Therefore $\tilde{E}_{318°} = -0.424$.

With use of the same linear temperature coefficient $\tilde{E}_{298°} = -0.438$ and $-\Delta F_{298°} = -20{,}180$ calories.

Quastel and Whetham do not state the temperature at which their pH measurement was made, nor the concentration of the phosphate. We have assumed, therefore, in this calculation, that whatever error is incurred by taking the pH to have been 7.2 at 45° is neutralized by the error incurred by employing the potential for methylene blue at pH 7.2 instead of that which actually obtained at 45°.

The free energy change in the oxidation of succinic acid to fumaric acid was calculated from thermochemical data as follows:

Entropy of succinic acid, $S_{298°} = 42.0$ (10); carbon, $S = 1.3$ (11); O_2, $S = 49.03$ (12); H_2, $S = 31.23$ (13).

Therefore the entropy of formation of solid succinic acid calculated from the reaction, $4C + 3H_2 + 2O_2 \rightarrow C_4H_6O_4$, is

$$\Delta S(s) = 42.0 - 4(1.3) - 3(31.23) - 2(49.03) = -155.0$$

The value of 31.23 for the entropy of molecular hydrogen employed here is considerably different from the older value given by Lewis and Randall of 29.44. This revised value for the entropy of hydrogen, due to Giauque (13), has been accepted by Parks and his collaborators and is now employed by them in their calcu-

lations of the entropies of organic compounds by means of the third law of thermodynamics from specific heat data.[4]

The heat of combustion of succinic acid at constant pressure was found by Verkade, Hartman, and Coops (14) at 19° to be 357,100 calories. Reduced to 25° and to its weight in vacuum the value becomes 356,900. With the value 94,240 calories obtained by Roth (15) for the heat of combustion of carbon to carbon dioxide and 68,310 calories for the heat of formation of water recently obtained by the United States Bureau of Standards, the heat of formation of solid succinic acid calculated from the reaction $C_4H_6O_4 + 3\frac{1}{2}O_2 \rightarrow 4CO_2 + 3H_2O$, is

$$\Delta H(s)_{298^\circ} = 356{,}900 - 4(94{,}240) - 3(68{,}310) = -225{,}000 \text{ calories}$$

Since $\Delta S = \frac{\Delta H - \Delta F}{T}$, the free energy of solid succinic acid therefore is,

$$\Delta F\ (s)_{298^\circ} = -225{,}000 + 298 \times 155 = -178{,}800 \text{ calories}$$

In order to estimate the free energy of solution of succinic acid the information necessary is the solubility and activity coefficient of the undissociated form of succinic acid in its saturated solution. From the International Critical Tables the mol fraction x of succinic acid in its saturated solution is given by the expression

$$-\log x = \frac{1}{T} \cdot (0.5223)\ (32{,}380) - 3.778 = 1.895 \text{ at } 25^\circ$$

For the calculation of the activity coefficient of undissociated succinic acid we have employed the data in the International Critical Tables on the lowering of the vapor pressure and of the freezing point of aqueous solutions; and in the evaluation of these data

[4] Giauque states, "The value of the entropy of hydrogen which should be used in conjunction with data obtained from the third law of thermodynamics is 31.23 E.U. . . . It is obtained by subtracting the high temperature nuclear spin entropy $R \ln 4 = 2.75$ E.U. from the absolute entropy of hydrogen 33.98 − 2.75 = 31.23 E.U. This places hydrogen on the same basis as other molecules in most of which, and perhaps in all of which, the subtraction is taken care of by the fact that heat capacities are not usually measured below temperatures of a few degrees absolute."

the methods described by Lewis and Randall ((11) pp. 273, 286) involving the use of their h function with the vapor pressure lowering data, and their j function with the depression of the freezing point data. Table II contains the results of these computations showing the allowance made for the activity of the ions arising from the dissociation of succinic acid. No partial molal specific heat data for these solutions are available for computing the variation in the activities with temperature. We have, therefore, assumed that the activities of all the constituents of these

TABLE II

*Activities of Components of Succinic Acid in Aqueous Solutions from Vapor Pressure and Freezing Point Depression Data**

Molality	Total activity	Activity of					Data employed
		H^+	Succinate$^=$	Undissociated form			
				Molality	Activity a	Activity coefficient	
M	a_2	a_+	a_-	M			
0.01	0.0100			0.0100	0.0100	1.00	Freezing point
0.05	0.0509			0.05	0.051	1.02	" "
0.1	0.0988			0.1	0.099	0.99	" "
0.2	0.1976			0.2	0.198	0.99	" "
1.211	1.04	0.01	0.0092	1.19	1.02	0.86	Vapor pressure
2.817	2.36	0.0155	0.0139	2.78	2.33	0.84	" "
4.021	3.22	0.0185	0.0163	3.98	3.19	0.80	" "
4.722	3.59	0.0205	0.0176	4.68	3.55	0.76	" "
8.030	6.02	0.0267	0.0225	7.97	5.97	0.75	" "

* International critical tables of numerical data, physics, chemistry and technology, New York and London (1926).

solutions remain the same over the temperature range from approximately 0° to 100°, and have estimated the activity coefficient of the undissociated succinic acid in its saturated solution at 25° to be 0.87. It was estimated that in this solution 0.008 mol of succinic acid was dissociated. Since the molality of the saturated solution at 25° is 0.715, the activity of undissociated succinic acid is $0.87 \times 0.707 = 0.615$ molal. The free energy change, therefore, in the transfer from a saturated solution to one in which undissociated succinic acid is at 1 molal activity is

$$-\Delta F = RT \ln 0.615 = -288 \text{ calories}$$

The free energy of undissociated succinic acid in solution at 1 molal activity, therefore, is $-178{,}800 + 288 = -178{,}500$ calories.

For the reaction, H_2 succinic acid $\rightarrow$ succinate $^{=} + 2H^+$, in which all the participants are at activities of 1 molal, the free energy change $-\Delta F = RT \ln K_1 K_2$, where K_1 and K_2 are defined by the equations,

$$K_1 = \frac{(H^+)\ (H \cdot \text{succinate}^-)}{(H_2 \text{ succinate})}, \text{ and } K_2 = \frac{(H^+)\ (\text{succinate}^=)}{(H \cdot \text{succinate}^-)}$$

From the data of Sihvonen, the calculated values of pK_1 and pK_2 at 25° and at infinite dilution, since we are dealing with activities and therefore with true equilibrium constants, are 4.201 and 5.641 respectively. The free energy change in ionization therefore is —13,420 calories. Hence the free energy of the bivalent succinate ion is $-178{,}510 + 13{,}420 = -165{,}090$ calories.

The entropy of solid fumaric acid at this temperature is 39.7 (10). The entropy of formation therefore is

$$\Delta S(s)_{298^\circ} = 39.7 - 4(1.3) - 2(31.23) - 2(49.03) = -126.0$$

For the heat of combustion of fumaric acid we have used Roth's value of 319,700 calories at 19° and weighed in air, which on a vacuum basis and at 25° becomes 319,300 calories (15). Parks and Huffman (10) have employed Stohmann's value which is 600 calories greater. In a private communication Professor Parks wrote that in their present revision of their data they "have rather arbitrarily given Roth's result twice the weight of Stohmann's and thus have taken the value 319,900 calories for 19° weighed in air. This gives 319,500 calories for 25° on a vacuum basis." We have preferred to ignore the earlier value and to give full weight to the value obtained by Roth. The difference between this value and the mean value employed by Parks is only 200 calories. The heat of formation of solid fumaric acid is, therefore,

$$\Delta H(s)_{298^\circ} = 319{,}300 - 4(94{,}240) - 2(68{,}310) = -194{,}280 \text{ calories}$$

The free energy of formation of solid fumaric acid is

$$\Delta F(s)_{298^\circ} = -194{,}280 + 298(126.0) = -156{,}720 \text{ calories}$$

A saturated solution of fumaric acid in water at 25° contains 0.061 gm. per 100 gm. of water (16). In its saturated solution the ionization is approximately 12.5 per cent, from which the molality of the undissociated form in the saturated solution is 0.0469. In the absence of data by which we might have estimated the activity coefficient of undissociated fumaric acid at this concentration, we have assumed that it is the same as that of succinic acid at this concentration, 1.0. The free energy change, therefore, in the transfer of 1 mol of undissociated fumaric acid from its saturated solution at 25° to one in which its activity is 1 molal is RT ln 0.053 = −1822 calories.

The free energy, therefore, of undissociated fumaric acid in solution at 1 molal activity is

$$\Delta F(s)_{298^\circ} = -156{,}720 + 1820 = -154{,}900 \text{ calories}$$

The free energy change in ionization, H_2 fumaric → fumarate$^=$ + $2H^+$ is

$$RT \ln K_1K_2 = -10{,}270 \text{ calories}$$

where K_1 and K_2 are the first and second hydrogen dissociation constants of fumaric acid.

The free energy of the bivalent fumarate ion at 1 molal activity therefore is

$$\Delta F \text{ (1 M)} = -154{,}980 + 10{,}270 = -144{,}630 \text{ calories}$$

Therefore in the reaction, succinate$^=$ (1 M) → fumarate (1 M) + $2H^+$ (1 M) + $2e$.

$$-\Delta F_{298^\circ} = -165{,}090 + 144{,}630 = -20{,}460 \text{ calories}$$

This computation is summarized in Table III.

In Table IV are set out the free energy values for this reaction obtained from potential and equilibrium measurements and these are compared with the above calculated value. Excepting the first determination by Thunberg the correspondence is remarkable. The difference between the mean of the electrometric and equilibrium values and the thermal value is greater than the probable error in the estimation of activity coefficients, but is well within the experimental error of the direct heat measurements. For instance

an error in the entropy difference of 1 unit (Parks and Huffman state that an error of 2 units is possible) would amount to 300 calories, and would practically account for the whole difference between calculated and observed values. A considerably larger

TABLE III

Summary of the Calculation from Thermochemical Data of Free Energy Change in the Conversion of Succinic Acid to Fumaric Acid and Hydrogen Ions

	Succinic acid	Fumaric acid
	calories	*calories*
Free energy of formation of solid	−178,800	−156,720
" " " solution	+288	+1,820
" " " ionization	+13,420	+10,270
Standard free energy of bivalent ion	−165,090	−144,630
" " " change	−20,460	

TABLE IV

Comparison of Observed with Calculated Free Energy Changes in the Enzymatic Oxidation of Succinic Acid to Fumaric Acid

Investigator	Source of enzyme	Temperature of measurement	$-\Delta F$	Difference from calculated value of $-\Delta F$, −20,460 calories $(-\Delta F_{obs.}) - (-\Delta F_{cal.})$
		°C.	*calories*	*calories*
Thunberg, 1925	Horse skeletal muscle	30	−19,140	1320
" 1928	" " "	30	−20,100	360
Lehmann	" " "	37		
		18	−20,180	280
Quastel and Whetham	Resting *Bacillus coli*	45	−20,180	280
Authors	Beef heart muscle	25	−20,140	320
"	" diaphragm	25	−20,140	320

error is also possible in the heat of combustion values as the discussion above of the heat of combustion of fumaric acid indicates. The employment of a fixed thermal value for the free energy change for purposes of comparison with the electrometric data shows the variations in the electrometric values. Had the mean electro-

metric value been employed as the fixed value for purposes of comparison, the variations in the calculated thermal values would have been greater than those shown in Table IV among the electrometric values.

It seems permissible, therefore, from the correspondence shown in Table IV, to conclude, as a first approximation, that the enzyme or enzymes which effect either *in vitro* or *in vivo* the oxidation of succinic acid to fumaric acid may be classed as "perfect catalysts."

The correspondence between the calculated and observed values for the change in heat content, ΔH, is also remarkably close. This is shown in Table V.

The close correspondence of the values for ΔH calculated from electrometric and from thermal data is additional confirmation

TABLE V

Heats of Formation of Succinate$^=$ and Fumarate$^=$

	Succinic acid	Fumaric acid
Heat of formation of undissociated solid	−225,000	−194,280
" " solution	+6,400	+5,900
" " ionization	−320	−740
" " formation of bivalent ion in solution	−218,920	−189,120
Succinate$^= \xrightarrow{-\Delta H}$ fumarate$^=$	−29,800	
$-\Delta H$ calculated from $\frac{dE}{dT}$	−29,850	

of the conclusion based upon the agreement between calculated and observed values for ΔF, that in this reaction the enzyme, regardless of the source of its preparation, or of its site of action, operates as a "perfect" catalyst, *i.e.* the reaction proceeds in a perfectly reversible manner, the heat and free energy changes being unaffected by the intervention of the catalyst.

The ratio, ΔF:ΔH, in this reaction is 20,200 : 29,850 = 0.68. It was pointed out in a previous communication (17) that the ratio ΔF:ΔH was nearly unity for the combustion of both tripalmitin and of glucose. Since the maximum amount of work derivable is practically equal to the value of $-\Delta F$, any difference in efficiency of fat and carbohydrate as fuels for muscular work must be ascribed to differences in intermediary metabolism. The

conversion of succinic acid to fumaric acid is an example in which the difference between ΔF and ΔH is quite large, and therefore the theoretical maximum work derivable from this reaction alone is much less than the total heat change. Since the formation of a double bond is a typical first stage in the oxidation of fatty acids it is probable that this considerable difference between ΔF and ΔH in the case of the oxidation of succinic acid to fumaric acid is typical of the oxidation of fatty acids in general. Of course the conversion of succinic acid to fumaric acid is only a "half reaction." Eventually an exothermic reduction of oxygen must occur, in which the ratio of ΔF:ΔH is always nearer 1. Nevertheless even in the complete reaction involving oxygen, the chief responsibility for differences between ΔF and ΔH would rest with the oxidation of the organic metabolite. It must be added, of course, that ΔF will vary, also, with the actual concentrations of the metabolites and products of reaction, whereas ΔH will not be significantly changed.

SUMMARY

1. A modification of the Thunberg vacuum technique for the micro electrometric determination of oxidation-reduction potentials is described.

2. A general derivation of oxidation-reduction potential equations is presented possessing some advantages for purpose of thermodynamic calculations over the conventional derivation.

3. The molal electrode potential, $\tilde{E}$, for the succinate-enzyme-fumarate equilibrium was measured with enzymes prepared from beef heart, and from beef diaphragm, over a range of hydrogen ion activities from pH 6.15 to 7.9. It was found that the potential is independent (*a*) of the metabolite ratio employed, confirming the earlier observations of Lehmann, (*b*) of the hydrogen ion activity, and (*c*) of the ionic strength of the solution.

4. It is shown further that the value of -0.437 volt for the molal electrode potential, obtained with beef heart and with beef diaphragm, within the narrow limits of experimental error, is the same as that obtained with such different catalysts as resting *Bacillus coli*, or enzymes prepared from horse skeletal muscle.

5. The molal electrode potential of -0.437 volt corresponding to a standard free energy change at 25° for the reaction

$$\text{Succinate}^{=} \rightarrow \text{fumarate}^{=} + 2H^{+} + 2e$$

of —20,140 calories, agrees very closely with the standard free energy change of —20,460 calories calculated from the entropies and other physicochemical properties of succinic acid and fumaric acid.

6. Similarly the heat of reaction, $-\Delta H$, —29,850 calories, calculated from the temperature coefficient of the potential is shown to be independent of the enzyme employed and agrees very closely with the value of —29,800 calories calculated from the thermal data.

7. This close correspondence between calculated and observed values of $-\Delta F$ and $-\Delta H$ is taken as proof that the enzyme promoting this reaction is a perfect catalyst; and that the enzyme probably operates in this manner *in vivo* as well as *in vitro*.

8. The correspondence between these calculated and observed values is additional confirmation of the third law of thermodynamics.

9. The difference between $-\Delta F$ and $-\Delta H$ in this reaction is discussed in relation to energy changes in intermediary metabolism.

10. The free energy of formation of the bivalent succinate ion at 1 molal activity at 25° was estimated at —165,090 calories, and the corresponding standard free energy of the bivalent fumarate ion at —144,630 calories.

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