REVERSIBILITY IN BIOLOGICAL OXIDATIONS

Thesis by

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S U M M A R Y

I. The molal electrode potential for the succinate-enzyme-fumarate equilibrium was determined for a series of variations of all the constituents, at 25°.

The free energy and the heat of the reaction, determined electrometrically, are compared to the values calculated from known physico-chemical properties of succinic and fumaric acids. The values agreed within the limits of experimental error. This, together with the lack of dependence of the free energy on the source of the enzyme, is taken to indicate that the enzyme behaves as a perfect catalyst.

II. From the equilibrium constant for the reaction of fumarate and water to form l-malate in the presence of the enzyme fumarase, the free energy of the reaction was calculated. From this value and the known physico-chemical properties of fumaric acid, the free energy of the formation of l-malic acid was estimated.

III. It was demonstrated that in the presence of toluene-treated B. coli, lactate is oxidized to pyruvate, and that furthermore, pyruvate may also be reduced to lactate.

IV. It was shown that in the presence of toluene-treated B. coli electron or hydrogen transfer from one metabolite to another occurs only thru the mediation of a reversible oxidizing agent.

V. The implications of these findings for the theory of biological oxidations are discussed.

INTRODUCTION

The question of the mechanism of biological oxidations has been the subject of many investigations in the last twenty years.

Batelli and Stern (3) in a series of investigations begun in 1909 discovered that the oxygen consumption of tissues was increased by the addition of certain oxidizable substances, and that such supplements could restore the respiration after it had been nearly abolished by washing. They showed that the respiration could be divided into two or more sections according to its resistance to physical and chemical treatments. They found that the oxidation of various substances seemed to be brought about by separate enzymes differing in their labilities.

Warburg, in 1914, (48) found that respiration was laregly dependent upon an iron-containing enzyme which was readily and reversibly poisoned by HCN, CO and H_2S. This "respiration ferment", as he showed later, is a hemin compound that reacts with molecular oxygen which may then oxidize organic substances.

However, Wieland (50) in 1913 brought forth quite convincing evidence that the reactions were principally the result of hydrogen activation in the metabolized substances. This was confirmed by the work of Thunberg (45).

Fleisch (16) in 1924, and later in the same year, Szent Gyorgyi (44) reported experiments indicating that both a metabolite activator and an oxygen activator are necessary in biological oxidations. Keilin (24) has confirmed this view and has discovered the presence of cytochrome, a compound hemochromogen nearly universal in occurrence, which is reduced or oxidized, respectively, as either the oxygen activator or the metabolite activator is inhibited. This substance, it was suggested, might act as a mediator for the two enzyme systems.

> The term mediator, used by Barron and Hastings (2) to designate a reversibly oxidizable and reducible substance which may react with a heterogeneously activated metabolite, effectively transferring the reduction potential to an inert electrode, is hereby extended to include a substance which may react with each of the two enzyme systems, and thus transfer electrons (or hydrogen or oxygen) from the one to the other.

In 1924 Quastel and Whetham (36) demonstrated that in the presence of certain organisms, not only oxygen and reversible oxidizing agents like methylene blue, but also nitrates, chlorates, and fumarates were reduced.

Quastel has postulated the presence of active centers (32) at, or near, the surface of the cells, at which the molecules of the metabolite or of the oxidizing agent could be absorbed and activated. Each center would be specific for one or more substances. That some such heterogeneous form of catalysis exists is indicated by the failure to obtain any such enzymes in true solution, and by the failure of the enzyme metabolite systems to affect an inert electrode in the solution.

The following pages present some evidence as to the nature of this activation.

Note on electrode conventions:

Lewis and Randall (29) define the standard electrode potential in such a way that the standard potential of sodium against sodium ion is positive, while that of chlorine against chloride ion is negative. Abegg, Auerbach, and Luther (1) use the opposite terminology. Clark (9) seems to have followed the latter authors and uses negative potentials to indicate strong reducing intensity. Hence it is now customary for biochemists to use a terminology different from that used by most physical chemists. At the risk of confusing some readers who may be accustomed to the work of Clark and his followers, we shall use the conventions of Lewis and Randall thruout this work as we feel that this is the standard prevailing usuage.

I

The Role of the Enzyme in the Succinate-Enzyme-Fumarate Equilibrium

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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

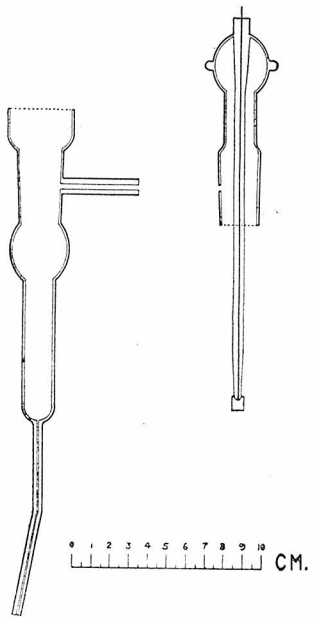

FIG. 1. Modified Thunberg tube for electrode potential measurements with a vacuum technique.

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 ($\frac{1}{2}$ 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°

$\frac{\text{Succinate}}{\text{Fumarate}} = \frac{9}{1}$			$\frac{\text{Succinate}}{\text{Fumarate}} = \frac{5}{5}$			$\frac{\text{Succinate}}{\text{Fumarate}} = \frac{1}{9}$			Enzyme
pH	Phosphate concentration	$-\tilde{E}$	pH	Phosphate concentration	$-\tilde{E}$	pH	Phosphate concentration	$-\tilde{E}$	
	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 $\frac{1}{2}$ 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

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

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) $\rightarrow$ 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

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

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 $\Delta F:\Delta H$ 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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II

The Free Energy, Heat, and Entropy of Formation of l-Malic Acid

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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 ± 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	$-(E'_h - E''_h)$	K for $\frac{(\text{malic})}{(\text{fumaric})} = K$
		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	−0.0428	−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

$$\text{Fumaric acid} + H_2O \rightleftarrows \textit{l}\text{-malic acid}$$

$$\frac{(\text{Malic})}{(\text{Fumaric})} = K$$

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

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

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

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

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

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

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

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

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

$$\Delta F_{(\textit{l}\text{-malic, 1 M})} = -201{,}860 - 11{,}720 = -213{,}580 \text{ calories}$$

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 $RT \ln \frac{0.0177}{0.317 \times 2} = -2120$ 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 $\Delta S = \frac{\Delta H - \Delta F}{T}$, the entropy of formation of solid *l*-malic acid at 25°,

$$\Delta S = \frac{-261{,}890 + 210{,}460}{298} = -169.2 \text{ E.U.}$$

The entropy of solid *l*-malic acid since

$$S_{\text{malic}} - S_{(4\,C)} - S_{(3H_2)} - S_{(2\frac{1}{2}O_2)} = -172.5 \text{ E.U.}$$

is

$$S(\text{solid})_{298^\circ} = -172.5 + 4(1.3) + 3(31.23) + 2.5(49.03)$$
$$= 52.2 \text{ E.U.}$$

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

$$\text{Fumarate}^{=} + H_2O \rightleftarrows \textit{l}\text{-malate}^{=}; \frac{(\textit{l}\text{-malate}^{=})}{(\text{fumarate}^{=})} = K$$

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 ± 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 52 E.U.

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III

THE ENZYMATIC REDUCTION OF PYRUVATE

That lactate may under certain circumstances be obtained from pyruvate has long been suspected. Since the enzyme which activates lactate in B.coli is not affected by treatment with toluene, (*37*) while all enzymes acting on pyruvate are destroyed by this process (*13*), toluene-treated B.coli seemed a particularly promising source of enzyme material.

Preparation of the Toluene-Treated B. Coli.

1) A simple medium consisting of 1% to 1.5% ammonium lactate and .5% to .7% sodium phosphates, (pH 6.8 to 7.8) with a trace of magnesium and ferrous sulphates was used.

2) A two liter flask containing 1500 ml of medium was inoculated with a culture of B.coli. It was then placed in the thermostat at 37° and throroly aerated with a stream of oxygen for a week or two.

3) The bacteria were centrifuged out and washed five or six times by suspending them in Ringer's solution and recentrifuging each time.

4) The organisms were suspended in Ringer's solution and oxygen was bubbled thru the thick suspension for several hours.

5) The material was stored in the cold room. About half an hour before using the bacteria were shaken with a little toluene.

Oxidation of Lactate to Pyruvate.

That lactate is oxidized to pyruvate in the presence of the toluene-treated organisms was demonstrated by aerating a buffered solution of sodium lactate and methylene blue, containing a suspension of this material at 37°. The procedure follows:

1) Large test tubes were fitted with two hole stoppers carrying glass inlet and outlet tubes.

2) Each tube contained

	10.0	ml of toluene treated B.coli suspension
	3.0	ml of M/2 phosphate buffer at pH 7.5
	0.5	ml of M/1000 methylene blue
and	2.0	ml of M/10 potassium d,l-lactate
or	2.0	ml of M/10 sodium pyruvate
or	2.0	ml water.

3) The solution was aerated for 20 hours at 37°.

4) It was then made up to 25 ml and centrifuged, aliquots of the supernatant solution being used for analysis.

5) Lactate was estimated as acetaldehyde-bisulfite according to the method of Friedmann and Kendall (17). One ml aliquots were used. The final titration was carried out with N/500 iodine solution.

6) Pyruvate was estimated as the 2,4-dinitrophenylhydrazone in 9 or 10 ml aliquots by the method of Simon and Neuberg (43).

The results are shown in Table I. The entries are in micromols (10^{-6} mols).

Table I.

The Oxidation of Lactates thru Toluene-Treated B.Coli

Lactate added	Pyruvate added	Lactate found	Lactate used	Pyruvate found
200	0	21.0 20.0 20.2	180	146 154
200	0	71.0 70.0	130	108 105
0	200	0.4 1.2	---	176 194
0	0	3.5 0.5	---	0 0

No acetaldehyde or methylglyoxal could be detected. The low yield of pyruvic acid may have been due in part to polimerization, although a certain loss of the hydrazone due to its appreciable solubility even at 0°. could not be avoided.

Reduction of Pyruvate to Lactate

A number of dyes were tested in an attempt to find a reversible oxidizing agent which would react readily with the enzyme system and at the same time have a sufficiently low rH. Chemical constitution was found to be an important rate determining factor, independent of the reduction potential.

Table II shows the reduction times of equal molal quantities of various dyes in the presence of toluene-treated B.coli and sodium formate at pH 7.5.

Table II.

Reduction of Various rH Indicators.

Name of Dye	E' at pH 7.0	Reduction Time
Thionine blue	ca -.01	2 min.
Methylene blue	-.011	2 min.
Methylene green	ca -.01	2 min.
Gallocyanine	-.021	30 min.
Nile blue A	+.142	25 min.
Brilliant alizarin blue	+.173	∞
Neutral red	+.340	∞
Safranine	ca +.3	∞
Methylene violet	ca +.27	2 hr.
Rosindulin 2 G	+.281	12 hr.

Quastel and Wheatley (35) show methylene violet to be relatively non-toxic to the oxidizing enzymes of B.coli and at the same time to be readily absorbed. The reduction potential of the dye seems sufficiently great for our purpose.

The Oxidation of Leuco Methylene Violet by Pyruvate.

That reduced methylene violet is oxidized by pyruvate in the pressence of toluene-treate B.coli was shown as follows:

1) A Thunberg tube containing toluene-treated B.coli, buffer, lithium pyruvate, and methylene violet was evacuated and filled with hydrogen.

2) After shaking several hours at 37° the dye became nearly all reduced. The hydrogen was then pumped off.

3) The color of the solution returned within half an hour.

In order to demonstrate that this oxidation of the dye was actually due to the reduction of pyruvate, experiments were carried out in which the lactate and pyruvate were estimated after the reaction of pyruvate and reduced methylene violet.

Thunberg tubes containing a small reservoir in the stopper were used for the experiment.

1) Into the dry stopper there was introduced 12 mg of sodium hydrosulfite.

2) The following solutions were introduced into the body of the vessel:

2.25 ml of M/2 phosphate buffer
3.0 ml of toluene-treated B.coli suspension
1.5 ml of M/1000 methylene violet
3.0 ml of a lithium pyruvate solution

3) The suspensions were incubated at 37° from 20 to 40 hours after evacuation and addition of hydrosulfite.

4) They were then made up to approximately 15 ml and centrifuged for one half hour in calibrated centrifuge tubes.

5) The volumes were adjusted to exactly 15 ml and aliquots were pipetted out for analysis.

6) Lactate was determined as before by the method of Friedmann and Kendall.

7) Pyruvate was first reduced to lactate with zinc and hydrochloric acid as recommended by Wendell (49) (On account of the presence of bisulfite resulting from the oxidation of hydrosulfite, the reduction of zinc had to be completed on the steam bath.) The resulting lactate was determined as usual.

Results are shown in Table III. Entries are in micromols.

Table III.

Reduction of Pyruvate by Toluene-Treated B. Coli

#	pH	Hours at 37°	Pyruvate originally present	Lactate found	Pyruvate found	Remarks
1.	7.6	20	30	9.19	19.29	
2.	7.6	20	0	0.48	2.55	
3.	7.6	20	45	0.16	29.80	No enzyme
4.	7.6	20	0	0.00	1.16	No enzyme
5.	5.8	20	30	14.10	19.50	
6.	5.8	20	0	1.63	2.53	
7.	7.6	40	10	3.10	7.42	
8.	7.6	40	30	7.45	23.20	
9.	7.6	40	100	9.30	87.15	
10.	7.6	40	300	14.80	-- --	

Thermodynamic Reversibility of the Lactate Pyruvate Activation

Two notes have recently appeared with regard to the oxidation-reduction potential of the lactate-pyruvate enzyme system.

Wurmser and Mayer-Reich (54) working in the neighborhood of pH 7.3 with a soluble enzyme preparation from B.coli and using cresyl violet as a mediator obtained a molal reduction potential for the lactate-pyruvate system of -.252 volts* at 37°. This gives for the reaction:

$$CH_3.CHOH.COO^- = CH_3.CO.COO^- + 2\,H^+ + 2\,E^-$$

$$\Delta F^\circ_{310} = 11{,}600 \text{ calories.}$$

Barron and Hastings (2) activated their metabolities with a preparation from gonococci. They also used cresyl violet. They worked over a pH range from 5.53 to 7.80. Their molal potential at 35° is

$$\tilde{E} = -.246 \text{ volts}^*$$

This gives

$$\Delta F^\circ_{308} = 11{,}300 \text{ calories for the above reaction.}$$

Since $\Delta H = 21{,}000$ calories, approximately, from the heat s of combustion, the change in ΔF° for a temperature change of 2° chould be only 65 calories. Hence the difference must be ascribed to a consistent error of 5 millivolts on the part of one of the above investigators, to a difference in the enzymes themselves, acting as imperfect catalysts, to some constant side reaction, or to an impurity in the materials used. A variation of 10 millivolts in the potential might be caused by using d,l-lactate when the enzyme activates only one of the optical isomers. Our own preparation of toluene-treated B.coli, for example, activated the levo-rotary isomer much more strongly than it did the dextro-rotary (sarco). Neither of the investigators indicates which form of the lactic acid was used.

*See note on electrode conventions on Page 2.

IV

SIMULTANEOUS OXIDATIONS and REDUCTIONS

Reaction of Pyruvate and Formate

Since, for the reaction:

$HCOO^- + H_2O + CH_3.CO.COO^- = HCO_3^- + CH_3.CHOH.COO^-$

$\Delta F^\circ = -11{,}400$ calories,

and since both formate and pyruvate are activated by toluene-treated B.coli we might expect a reaction to occur when these two metabolites are incubated with a suspension of this enzyme. Tables IV and V show the results of such experiments.

Table IV

Reaction of Pyruvate and Formate in the Presence of Toluene Treated B. Coli

Expt. No.	Pyruvate added	Formate added	Methylene violet	Lactate found	
1	20	24	1	4.3	
2	20	24	1	6.0	
3	20	24	1	0.3	Boiled enzyme
4	20	24	0	0.0	
5	20	0	0	0.6	
6	20	0	1	0.9	
7	0	24	1	0.8	

1) Each tube contained:

3 ml phosphate buffer at pH 7.5
4 ml toluene-treated B.coli
the substances listed above.
The total volume 11 ml.

2) The tubes were evacuated and set away at 37° for 24 hours.

3) The bacteria were removed by centrifuging.

4) Aliquot portions of the supernatant liquid were analyzed for lactate by the method of Friedmann and Kendall. Entries are in micromols.

Table V

Reaction of Pyruvate and Formate in the Presence of Toluene Treated B. Coli.

Expt.No.	Hours at 37	Pyruvate added	Formate added	Methyl. violet	Lactate found	Pyruvate found
1	20	200	25	1.1	5.96	185.
2	20	200	25	1.1	7.58	178.
3	20	0	25	1.1	.42	2.1
4	40	200	25	0	.65	187.
5	40	200	0	1.1	.35	---

1) Each vessel contained:

1.5 ml M/2 phosphate buffer at pH 6.3
2. ml of the toluene-treated B.coli suspension
Substances listed above.
Total volume 6 ml.

2) Solutions were evacuated and incubated at 37° for the time indicated.

3) They were then centrifuged and the lactate determined as before.

4) The pyruvate was determined by the method of Wendell (49).

The results of this investigation lead to the very surprising conclusion that formate and pyruvate will not react except in the presence of a mediator, and that there is no suitable mediator in the toluene-treated organisms. Friedmann and Kendall (26) have shown, however, that lactate is formed together with other products from pyruvate in the presence of resting B. coli. In order to determine whether our strain of bacteria also had this property, an experiment similar to the above was carried out using the living organism.

Anaerobic Decomposition of Pyruvate.

1) Into each of 5 small flasks there was introduced:

1 ml M/2 phosphate buffer at pH 6.7
2 ml of an untreated B.coli suspension
Sodium formate, lithium pyruvate, and HCN as indicated in Table VI.
Total volume 5 ml.

2) These were evacuated and incubated at 37° for 24 hours.

3) The solutions were centrifuged, and made up to 10 ml.

4) Aliquots of the supernatant liquid were analyzed for lactate by the method of Friedmann and Kendall.

5) Pyruvate was determined by means of its bisulfite binding capacity, as very small blanks were obtained by this method. It was found that quantitative results could be obtained by the following procedure:

a) A one or 2 ml aliquot was pipetted into a 50 ml beaker.

b) Two or 3 ml of 1% sodium bisulfite solution were added.

c) The solution was then placed on the steam bath for one minute. It was left at room temperature for 4 more minutes.

d) It was then placed in chopped ice for 5 minutes, and titrated while still cold with N/10 and then N/500 iodine to a faint color with starch.

e) The final titration was made with n/500 after liberation of the bisulfite with sodium bicarbonate.

The results of the experiment are shown in Table VI. The entries are in micromols.

Table VI

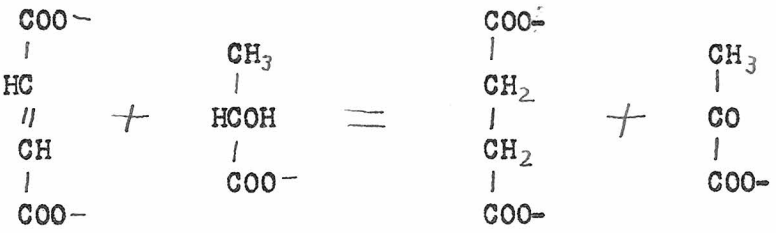
Annerobic Decomposition of Pyruvate by Resting B. Coli.

Expt.No.	Pyruvate added	Formate added	Cyanide added	Lactate found	Pyruvate found
1	200	200	0	19.3	24.1
2	200	0	0	19.0	13.5
3	200	200	20	17.0	30.8
4	200	0	20	18.6	24.9
5	0	200	0	0.4	0.3

It is clear that part of the Pyruvate that disappears reappears as lactate. However, if the mechanism involved reduction of the pyruvate by the formate produced by its decomposition, we might have expected to obtain larger yields of lactate when formate was present initially. Perhaps lactate is produced in some less direct manner, thru a carbohydrate stage, for example,- The presence of a simple mediator in vivo does not seem to be indicated.

Reaction of Fumarate and Lactate in the Presence of Toluene-Treated B.Coli.

Again we may write the reaction:



$\Delta F_{310} = -8{,}400$ calories.

Here also both metabolites, fumarate and lactate, are activated by toluene-treated B.coli. Hence we should expect to obtain a re-action.

An experiment was accordingly prepared in which sodium l-lactate and sodium fumarate were incubated in vacuo with toluene-treated B. coli, with and without the addition of methylene blue. Pyruvate was determined in the resultant mixture thru its bisulfite binding capacity as described above. Succinate was determined by means of the potential developed in the presence of succinic dehydrogenase solution and methylene blue. Sodium l-lactate was used in these experiments since it was oxidized about ten times as rapidly as the d-lactate (sarco lactate).

1) Into each of 5 small flasks was introduced:

1 ml M/2 phosphate buffer at pH6.7
2 ml of toluene-treated B.coli, l-lactate, fumarate, and methylene blue as indicated.
The total volume was 6 ml.

2) The vessels were evacuated and incubated for 24 hours at 45°. Solutions were then made up to 10 ml and centrifuged.

3) Aliquots of the supernatant solution were then analyzed for pyruvate and succinate.

4) Results are shown in Table VII. Entries are in micromols.

Table VII

Reaction of Lactate and Fumarate in the Presence of Toluene-Treated B. Coli.

Expt.No.	Lactate added	Fumarate added	Meth. Blue	Succin. found	Pyruvate found	Hours at 45
1	100	100	1	21.	20.0	15
2	100	100	1	41.2	40.0	24
3	100	100	0	0.0	2.4	24
4	100	0	1	----	5.9	24
5	0	100	1	0.0	0.8	24

The succanate concentration was determined as follows:

1) 2 ml aliquots were pipetted into electrode vessels, such as were described in Part I, together with
2 ml of succinic dehydrogenase, and
0.1 ml of M/1000 methylene blue, and
1.0 ml M/1000 sodium succinate or water was also added.

2) The vessels were evacuated and shaken at 37° until a steady E.M.F. value was reached.

3) The pH of the solutions were then measured and the corresponding values of $\tilde{E}$ calc= $\tilde{E}$ -.031 log Suc/Fum were calculated. The value of $\tilde{E}$ was taken to be -.409 volts, which was calculated from the results of experiment 5, in which no lactate is used. Altho this differs by 21 millivolts from the true value, it seems justified by the results. The method calculation is shown in Table VIII.

Table VIII

Potentiametric Determination of Succinate

	Succin. added	E calc	Diff. from 409	Log ratio	Ratio	Succ.	Fum.	Malate
1.	---	408	.001	.032	1.08	4.2	3.9	11.8
2.	---	384	.013	.423	2.65	8.0	3.0	9.0
	10	396	.025	.813	6.50	18.5	2.9	8.6
3.	unaccountably rapid reduction							
	10	400	.009	.292	1.96	10.0	5.0	15.0
5.	---	468	-.059	.92	.012	.06	4.98	14.96
	10	400	.009	(.301)	(2.00)	(10.0	(5.0)	(15.0)

Here again it seems that the reaction between the two metabolites does not take place in the presence of the toluene-treated B.coli except when a suitable mediator is added. In this connection we may note that when B.coli grow anaerobically on fumarate and lactate, succinate is formed, and lactate seems to be oxidized, according to the experiments of Quastel, Stephenson, and Whetham (34).

V

Discussion and Conclusions

Some Free Energy Values of Biological Interest:

Table IX.

Electrode Reaction	ΔF° calories	$\tilde{E}$ * volts	E_o' pH 7	Source & temp.
$H_2 = 2H^+ + 2E^-$ (1)	0	0.000	+.414	P.Ch.25°
hydrogen	0	0.000	+.430	P.Ch.37°
$CHO_2^- + H_2O = HCO_3^- + 2H^+ + 2E^-$	0	0.000	+.414	P.Ch.25°
formate bicarbonate (2)				
$C_3H_5O_3^- = C_3H_3O_3^- + 2H^+ + 2E^-$	11300	-0.246	+.183	Bio.35°
lactate pyruvate (3)	11600	-0.252	+.179	Bio.37°
$C_4H_4O_4^= = C_4H_2O_4^= + 2H^+ + 2E^-$	20140	-0.437	+.023	P.Ch.37°
succinate fumarate (4)	19820	-0.430	±.000	Bio. 37°
$NO_2^- + H_2O = NO_3^- + 2H^+ + 2E^-$	38560	-0.836	−.422	P.Ch.25°
nitrite nitrate (5)				
H_2O_2(aq.) $= O_2 + 2H^+ + 2E^-$	31470	-0.683	-.269	P.Ch.25°
hydrogen peroxide oxygen (6)				
$H_2O = \frac{1}{2}O_2 + 2H^+ + 2E^-$ (7)	56090	-1.218	-.788	P.Ch.37°
water oxygen				

Some of the above values, marked "Bio." were obtained with the aid of activating enzymes. The other values marked "P.Ch." were calculated from existing physical chemical data (22,23,29).

* See note on Electrode Conventions on Page 2.

In the presence of toluene-treated B.coli, reactions 1 and 2 proceed from left to right as written; 5, 6 and 7 proceed only from right to left; and 3 and 4 are reversible. None of these occur at ordinary temperatures in the absence of a catalyst.

It should be borne in mind that these are actually only half cells, requiring another oxidizable or reducible substance to complete the reaction. This substance may be a reversible-oxidation-reduction indicator with a suitable reduction potential. It has never been clearly demonstrated that two ordinarily inert substances may react after activation without the intervention of a mediator.

The electrode potentials above recorded cannot be obtained directly upon an inert electrode, but the systems react with a mediator as though such a potential were established at an active center. The electrode potentials might be formulated somewhat as follows for the lactate-pyruvate system, for example:

$$\text{Enzyme},\ C_3H_5O_3^- \ ,\ C_3H_3O_3^- \ ,\ H^+;\ \tilde{E} = -.246$$

In Table X. are tabulated some reversibly oxidizable and reducible systems with their reduction potentials, at neutrality. In addition to these there are naturally occurring substances such as glutathione and oxygen which may under some circumstances reduce or oxidize irreversibly, without requiring activation.

In Table XI. are the reduction potentials, at neutrality, of oxidation-reduction indicators which have been, or might be used as mediators, in studying biological oxidations.

Table X.

Naturally Occurring Oxidation-Reduction Systems *

Reductant	Oxidant	n	$\frac{dE}{d(pH)}$	$\tilde{E}$	E_o' pH 7.	Temp.	Rf.
Catechol	Orthoquinone	2	.06	-.794	-.382	24°	(4)
Epinenerine		2	.06	-.784	-.372	24°	(4)
	Cytochrome	1	0		-.260	20°	(15)
Hemoglobin	Methemoglobin	1			-.152	24°	(12)
	Chromodoris zebra pigment	1			-.102	30°	(31)
Piocyanin		2	.06		+.034		(18)
Hermidine	Cyanohermidine	1		-.366	+.093	30°	(6)
	Echinochrome	2	.06	-.200	+.215	30°	(7)
Hemin		1	0		+.230		(11)

*See note on electrode conventions on Page 2.

A few comments on the practical interpretations of such potentials might here be in order. To obtain the oxidation-reduction potential of a metabolite system activated at an enzyme surface, we must use an oxidation-reduction indicator, or a mediator, with a useful potential range, which includes the potential to be measured. The limit of usefulness of a mediator is about 60 millivolts either side of the E_o' value at the pH used, (the E' value being defined as the potential of the solution when the oxidized and reduced forms of the indicator are present in equal amounts. Methylene blue (E' = -011) is thus suitable for measuring the potentials of the enzyme-succinate-fumarate-hydrogen ion "electrode" (E_o' = 000), while cresyl violet (E_o' = +.167) is useless for this purpose, altho well suited to the lactate-pyruvate (E_o' = +.179) system.

In order to obtain a reaction between a reducible metabolite and an oxidizable one it is necessary to use a mediator with a useful potential range of which at least a part is more negative than that of the oxidizable metabolite and at least part is more positive than that of the reducible metabolite. In order for the reaction to proceed to completion the oxidizable metabolite should have a potential at least 100 millivolts greater than that of the reducible one. Thus, methylene violet (E_o' = +.27) is a suitable mediator for the formate-pyruvate reaction, and we should expect the reaction to go to completion.

Table XI

Some Oxidation-Reduction Indicators*

Oxidant	E° pH 7
Tungsticyanide	-.435
Ferricyanide	-.320
p-Quinone	-.275
Phenolindophenol	-.234
O-Cresol indophenol	-.188
1-Naphthol-2sulfonate-indophenol	-.123
Cresyl blue	-.032
Gallocyanine	-.021
Methylene blue	-.011
Indigotetrasulfonate	+.046
Indigo carmine	+.125
Gallophenine	+.142
Nile blue	+.142
Indigo monosulfonate	+.157
Cresyl violet	+.167
Brilliant alizarine blue	+.173
Anthraquinone sulfonate	+.230
Janus green	+.258
Methylene violet	ca +.27
Rosindulin 2G	+.281
Neutral red	+.340

*See note on electrode conventions on Page 2.

If we could find a mediator for the hydrogen-bicarbonate reaction we would expect a measurable equilibrium to be reached. It should be noted, however, that these activating enzymes exhibit a certain degree of specificity towards reversibly oxidizable dyes. So that a dye, in order to serve as a mediator, must in addition to the thermodynamic requirements listed above, be a substance which will react rapidly with the enzyme system or systems in question.

The Existence of Mediators in Vivo.

Although we have presented evidence that no mediators with a reduction potential at neutrality greater than -.06 is present in toluene-treated B.coli, there are the following indications that these organisms do contain mediators in vivo :

1) The existence of a mediator between reactions 2 and 3 is suggested by the production of lactate from pyruvate by resting B. coli. As we have already suggested, this might occur thru some indirect process, rather than by a simple mediator.

2) Growth may be obtained on a lactate-fumarate medium anaerobically, but not on either lactate or fumarate alone, as sole source of carbon. Succinate can be isolated from such a medium and the accompanying pH changes indicate that growth takes place principally at the expense of the lactate. That this phenomenon does not, however, prove the presence of a reversible mediator is brought out on Page 55 .

3) That in bacteria that have been grown on a medium rich in formate, a mediator exists which will link reactions 1 and 2 is indicated by the ability of these organisms to decompose formate into bicarbonate and hydrogen. Stephenson and Stickland (40) feel that this is counter-indicated by the presence of the formic splitting property in Bact. lactus aerogenes, with the absence of a hydrogen-activating enzyme. The enzyme, or mediator, is very labile to all sorts of poisons.

4) Oxidation of metabolites by the reduction of nitrate or of oxygen is a well estabilised phenomenon. The fact that as much nitrite is formed aerobically as anaerobically with growing B. coli indicates either that there is no mediator between reactions 5 and 7 or that the oxygen reaction is represented by 6, with appreciable amounts of peroxide always present. Recent studies by Cook, Haldane and Mapson (14) indicate that each of the activators concerned in reactions 2, 3,and 4, has associated with it a different oxygen-activator. This suggests that the active centers for oxygen and each metabolite exist in close proximity, reacting either directly, or thru special mediators.

There is also evidence that mediators are present in other biological systems. The common occurrence of such substances as are listed in Table XI clearly demonstrates that chemicals which may suitably act as mediators are found in all sorts of animal and plant tissues. The occurrence of cytochrome and other hematin derivatives seems to be practically universal in tissues using oxygen.

Some information concerning the mediators in brain tissue may be obtained from recent experiments by Quastel and Wheatley (35). They found that, when lactate and succinate were both being oxidized, as much lactate was burned as when lactate was being oxidized alone, while the oxidation of succinate was considerably decreased. Lactate, however, did not reduce fumarate in anaerobic experiments; also, lactate did not spare the oxidation of succinate when the lactate activating center was poisoned. These authors conclude that the effect may be due to competition for a carrier or mediator:

the sparing action might be due to a difference in accessibility of the mediator at the two centers; or, to an effect of the greater reducing intensity of the lactate system on the dynamic equilibrium controlling the state of reduction of the mediator. We may therefore draw the following conclusions:

1) That there is in brain a mediator (possibly cytochrome) with a potential well on the negative side of the succinate-fumarate system.

2) That there is no mediator with a potential range between that of lactate and that of fumarate.

According to Wieland (51), when alcohol is being oxidized to acetate by bacteria, the addition of quinone stops the ordinary respiration and the oxygen consumption becomes limited to the reoxidation of hydroquinone. This phenomenon suggests that the mediator between the oxygen and alcohol has a potential positive to that of the quinone, so that it is completely oxidized by the latter, and hence can no longer take up oxygen. If these experiments were done in an acid medium, cytochrome would behave in this manner; also, hemoglobin would answer the specifications.

The Mechanism for Deriving Energy from Oxidation.

The work of Quastel and Wooldridge (38) indicates that the following conditions must be fulfilled by a medium in order that anaerobic growth of B. coli may take place:

1) The medium must be thermodynamically unstable; that is: reaction must be possible involving a liberation of energy.

2) The compounds present must be activated by the bacteria.

3) Substances must be present, or producible by exothermic reactions, which are capable of participating in the synthetic reactions of growth.

Condition 3) seems to be satisfied if pyruvate is present or produced. Condition 2) is satisfied by any of the substances in Table X, for example; many others are known.

Of the media tried by Quastel and Stephenson (33) the lactate-fumarate seems to be the least unstable of those which supported growth. The free energy decrease involved in the reaction:

$$\text{lactate}^- + \text{fumarate}^= = \text{pyruvate}^- + \text{succinate}^= ,$$

being only 8200 calories, at 37°. That this is just about the minimum energy necessary for growth is indicated by the fact that an addition of two equivalents of succinate to a medium containing one equivalent each of lactate and fumarate completely inhibits growth, although this decreases the free energy of the reaction by only 400 calories; the inhibition might of course have other causes.

If the reaction between lactate and fumarate takes place in vivo, like it does in vitro, in the presence of methylene blue, it is difficult to see how any of the free energy could be utilized as chemical work, since the enzymes apparently are perfect catalysts and do not enter into the reaction and the methylene blue is practically unchanged. However, if the lactate and the fumarate were in separate half cells, each together with some of the enzyme preparation and some methylene blue, and the two solutions were connected by a salt bridge, electrodes in the two solutions would show a difference of potential of about .3 of a volt at the start. A conductor connecting the two electrodes would carry a current which could be used to do mechanical or chemical work, such as running a motor, or electrolyzing another cell. The net result of the latter process would be that instead of simply an oxidation of lactate and a reduction of fumarate there would also be an oxidation and reduction of two other substances which could not have exchanged electrons spontaneously.

Thus, we may say that in the process of growth on a lactate-fumarate medium some substances are oxidised by fumarate and different substances are reduced by lactate, and, that although the ~~two~~ new substances so produced could react spontaneously with each other, they do not do so. Thus the possibility of a mediator, or a chain of mediators, facilitating the oxidation of lactate at the expense of the reduction of fumarate is inconsistent with the possibility of energy-consuming syntheses occurring, and hence, presumably, with growth. Thus, the separation of the centers of activation for lactate and fumarate may serve much the same purpose as the separation

of the two halves of a voltaic cell, namely, to permit the utilization of some of the energy produced as work. In the case of aerobic oxidation we certainly can obtain complete oxidation of a metabolite at the expense of oxygen without the production of any work. Under what circumstances some of this energy is utilized for synthetic reactions is not all clear.

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