though all of the above evidence certainly seemed to support the conclusions of Bertram,[2] it was felt that additional proof of the configuration of vaccenic acid could be obtained by infrared absorption measurements in comparison with oleic and elaidic acids. The infrared absorption curves for oleic, elaidic and vaccenic acids, respectively, are given in Figs. 1, 2, and 3. On the assumption that oleic acid is *cis* and elaidic acid *trans*, it may be seen from the general similarity of the infrared patterns of elaidic and vaccenic acids that the latter is probably of *trans* configuration. Comparison of the curves for the three fatty acids in the region of wave length 3 to 7.5 μ shows a striking similarity. The minimum at 10.25 μ in the pattern for elaidic acid is also present in the vaccenic acid pattern, but absent for oleic acid. However, the doublets in the region of 8.8 to 9.2 μ present in the oleic acid curves are absent in the infrared pattern for vaccenic acid. Perhaps this may be attributed to the difference in the position of the double bond. Because of light scattering, no particular significance is attributed to small differences in the region 13 to 16 μ.

Although the vaccenic acid is probably of greater purity than the acid isolated by Bertram, the magnitude of the melting point in comparison with that reported by Bertram is somewhat surprising.

X-Ray diffraction analysis of the vaccenic acid is in progress and the results of this study will be reported in a separate communication.

In view of the pronounced similarity in infrared absorption of elaidic and vaccenic acids, additional evidence is therefore presented to confirm the *trans* configuration of vaccenic acid.

Infrared absorption measurements of highly purified vaccenic acid are reported in comparison with values for oleic and elaidic acids.

Acknowledgment.—The technical assistance of Miss Leatrice Klein in the preparation of the vaccenic acid is gratefully acknowledged.

Summary

Comparison of the infrared patterns of the three fatty acids seems to confirm the *trans* configuration of vaccenic acid.

PITTSBURGH, PENNSYLVANIA
RECEIVED NOVEMBER 6, 1947

[CONTRIBUTION NO. 637 FROM THE DEPARTMENT OF CHEMISTRY, UNIVERSITY OF PITTSBURGH]

Kinetics of the Fumarase System[1]

BY E. M. SCOTT AND RUTH POWELL

Dakin[2] in 1922 showed that an enzyme, fumarase, catalyzed the equilibrium reaction

$$\text{fumarate} + H_2O \rightleftarrows \text{l-malate}$$

Clutterbuck[2a] found that phosphate affected both the solubility of the enzyme and the rate of the reaction. Borsook and Schott[3] studied the equilibrium and calculated the heat of formation of *l*-malic acid. It was reported by Ionescu, *et al.*,[4] that the *p*H of optimum activity of the enzyme with *l*-malate as substrate was more alkaline than that with fumarate. Jacobsohn[5] found that while the reaction with both substrates appeared to follow the first-order course, the well-known relation of the first-order reaction constants to the equilibrium constant

$$K_1/K_2 = K_{eq.} \qquad (1)$$

did not apply.

The present investigation was designed to test Jacobsohn's observations and to determine why equation (1) did not hold. In contrast to the experiments cited above, highly purified enzyme prepared according to the crystallization procedure of Laki and Laki[6] was used in our investigations.

A titrimetric determination of fumarate similar to the method of Straub[6a] was found to be much more convenient and somewhat more accurate than the polarimetric methods used in earlier kinetic studies.[2a,4,5]

Experimental

Eastman Kodak Co. fumaric acid was recrystallized from water, dissolved and neutralized as one substrate; Eastman *l*-malic acid was dissolved and neutralized to provide the other. Purity of the substrates for present purposes was established by the following evidence: (1) Both acids gave correct neutral equivalents; (2) *l*-malic acid gave no reaction with permanganate under the conditions stated below; (3) both substrates gave the same equilibrium concentration of fumarate; and (4) no evidence of inhibition by either substrate was found (*vide infra*, "Effect of Substrates on Stability").

The enzyme used was an amorphous fraction, obtained after crystallization of the protein described by Laki and Laki. This preparation had about three times as much activity per unit protein N as did the crystals. The initial rate of hydration of fumarate at *p*H 7.29 and 30° by this enzyme was 0.015 mole/sec. g. of protein N.

Unless otherwise indicated, the enzyme tests were run in 10 ml. of a solution containing phosphate buffer (*p*H 7.29, ionic strength 0.2), 0.1 *M* sodium fumarate or *l*-malate, and enzyme to give a protein N concentration of 4.2 p. p. m. One ml. samples were removed, added to 10 ml. of water containing 0.5 ml. of concentrated

(1) Aided by grants of the National Institute of Health and the Buhl Foundation. Presented at the 111th Meeting of the American Chemical Society, April 14–18, 1947.

(2) Dakin, *J. Biol. Chem.*, **52**, 183 (1922).

(2a) Clutterbuck, *Biochem. J.*, **22**, 1193 (1928).

(3) Borsook and Schott, *J. Biol. Chem.*, **92**, 559 (1931).

(4) Ionescu, Stanciu and Radalescu, *Ber.*, **72B**, 1949 (1939).

(5) Jacobsohn, *Biochem. Z.*, **254**, 112 (1932); **274**, 167 (1934).

(6) Laki and Laki, *Enzymologia*, **9**, 139 (1941).

(6a) Straub, *Z. physiol. Chem.*, **236**, 43 (1935).

hydrochloric acid, and titrated with 0.02 *M* potassium permanganate. The end-point of the titration was taken when one drop (0.03 ml.) of potassium permanganate solution gave a pink color which persisted for sixty seconds. Analyses by this method of mixtures of known amounts of fumarate and *l*-malate without enzyme were accurate within 0.5% total substrate content. Acetic acid–acetate buffers were used below *p*H 5.8, phosphate buffers between 5.8 and 8.0, and ammonia–ammonium chloride buffers above *p*H 8.0. All buffers had an ionic strength of 0.2. Temperatures were maintained by immersion in a water-bath, controlled to within 0.05°.

Since the reaction did not follow a path predictable from an equation for a reaction of integral order, rates were expressed in terms of the initial value as determined graphically. This appeared to be justified by the data in Fig. 1, from which it was concluded that initial rate was directly proportional to enzyme concentration.

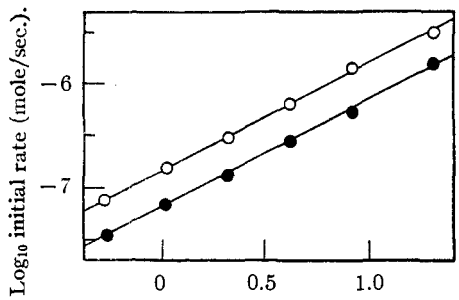

Fig. 1.—Effect of enzyme concentration on initial rate of reaction: -O-O-, fumarate as substrate; -●-●-, *l*-malate as substrate.

Results

Equilibrium Constant.—The equilibrium constant was found to be independent of substrate or enzyme concentration. It was independent of *p*H, except that below *p*H 6.0, *l*-malate was favored. Since *l*-malic is a somewhat weaker acid than fumaric, this was expected. The variation of equilibrium constant with temperature is shown in Fig. 2. From this figure, the heat change of hydration of fumarate ion was calculated as −3560 cal./mole and the free energy change at 25° was −826 cal./mole. These values agree satisfactorily with those of previous investigators.[3,5]

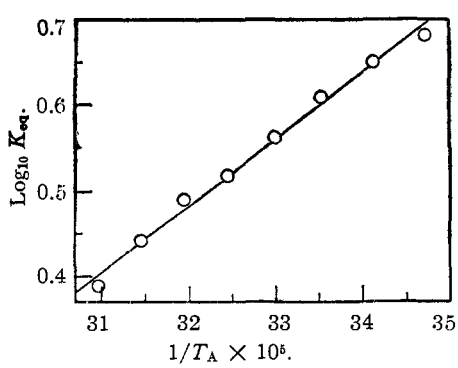

Fig. 2.—Effect of temperature on the equilibrium.

Course of Reaction.—Typical curves of the course of the reaction are shown in Fig. 3.

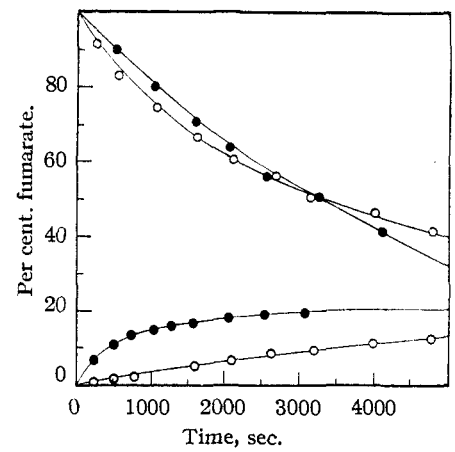

Fig. 3.—Path of the reaction: -O-O-, *p*H 8.46; -●-●-, *p*H 5.97.

When these were plotted according to the first-order equilibrium reaction equation

$$K_F + K_M = 1/t \ln\left(\frac{x_0 - x_e}{x - x_e}\right) \qquad (2)$$

(where x_0 is original concentration x_e the equilibrium concentration, x the concentration of fumarate at time t, and K_F and K_M the first-order reaction constants with fumarate and *l*-malate, respectively), the curves shown in Fig. 4 were obtained. As is apparent, the slopes of the curves do not agree when the reaction starting with 100% *l*-malate is compared with that starting with 100% fumarate. This confirms the work of Jacobsohn.

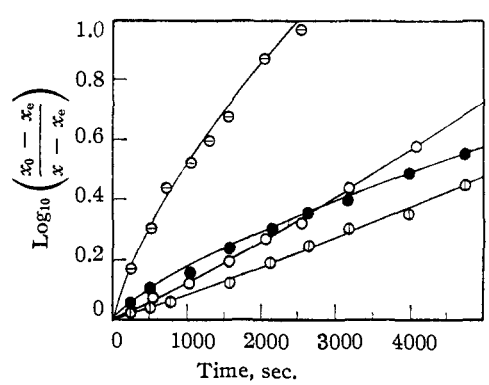

Fig. 4.—The reaction as fitted to the first order equation: -●-●-, fumarate, *p*H 5.97; -◑-◑-, *l*-malate, *p*H 5.97; -O-O-, fumarate, *p*H 8.46; -⊖-⊖-, *l*-malate, *p*H 8.46.

Effect of *p*H.—The relative rates of the reaction starting with either *l*-malate or fumarate varied greatly with *p*H as shown in Fig. 5. Thus, at *p*H 6.0 equilibrium was reached with fumarate in about one-half the time required for the reaction with *l*-malate, but at *p*H 9.0, over six times as long was required with fumarate as with *l*-malate.

Effect of Temperature.—Variation of initial rate of reaction with temperature at *p*H 7.29 is shown in Fig. 6. The apparent activation

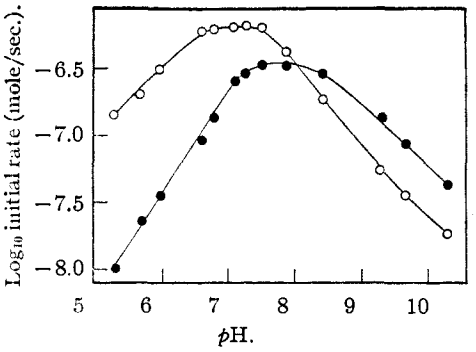

Fig. 5.—Effect of *p*H on reaction rate: –O–O–, fumarate; –●–●–, *l*-malate.

energies starting with the two substrates were: fumarate, 8,100 cal./mole; *l*-malate, 12,200 cal./mole.

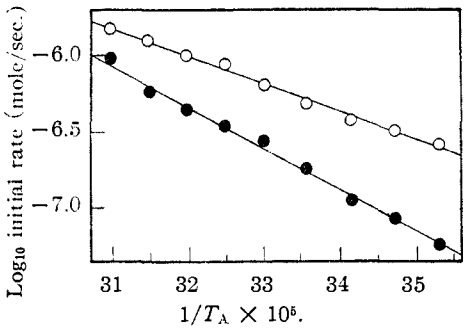

Fig. 6.—Effect of temperature on reaction rate: –O–O–, fumarate: –●–●–, *l*-malate.

Effect of Substrates on Stability.—In one experiment *l*-malate was added, the system brought to equilibrium, and then fumarate was added. The rate of hydration of fumarate was found to be the same as when fumarate was added, the system brought to equilibrium, and more fumarate added. This indicated that no inhibitors were being added with either substrate, and that the substrates had no differential effect on stability of the enzyme. Independent tests showed that under the usual conditions of our experiments, enzyme destruction was very slow or negligible.

Effect of Substrate Concentration.—Over the range shown in Table I, the initial rate of reaction at *p*H 7.29 appeared to be independent of substrate concentration. This could have been true only if the rate-controlling step of the reaction was not of the first order but was, at least at the start, of the zero order.

TABLE I

EFFECT OF SUBSTRATE CONCENTRATION ON INITIAL RATE

Substrate concentration, *M*	Initial rate, mole/sec. × 10⁷ Fumarate	*l*-Malate
0.4	5.0	2.8
.2	6.0	3.2
.1	5.9	3.2
.05	5.6	4.0
.025	5.4	2.5
.0125	6.0	3.0

Discussion

It is evident from the results that the reason equation (1) does not apply to the case of fumarase is that the reaction is not of the first order. Superficially it appears to follow the course predicted by the first-order equation because, in an equilibrium system, the reverse reaction is so important in determining rate that different orders of reaction are not clearly distinguishable. Equation (1) will apply ordinarily only to those reactions in which there is a single step conversion of one reactant to the other. Accordingly, these results can be interpreted as indicating that the fumarate–*l*-malate interconversion proceeds through a series of steps, any one of which may limit the rate. Consequently, the rate with one substrate may bear no obvious relationship to the rate with the other.

The only steps in enzyme-catalyzed reactions of which we have some conception at present are those of activation and complex formation. By complex formation is meant the fact that the substrate must combine with, or be adsorbed on, or at least come in contact with, the enzyme for the reaction to proceed. The reaction might then be pictured as

$$\underset{\text{(solution)}}{\text{F}} \rightleftharpoons \boxed{\text{F} \rightleftharpoons \underset{\text{Enzyme}}{\text{F = M}} \rightleftharpoons \text{M}} \rightleftharpoons \underset{\text{(solution)}}{\text{M}}$$

Complex formation — Activation or conversion — Complex formation

in which water is assumed to be present in great excess. The processes within the rectangle take place on the surface of the enzyme.

A priori, one cannot decide whether the process of complex formation involves a change in free energy, nor can one predict whether complex formation has an appreciable activation energy of its own. Three possibilities exist: (1) one can assume that the energy of activation of complex formation is small (*i. e.*, complex formation is much more rapid than conversion; this was the assumption of Michaelis and Menten[7]); (2) it can be assumed that the energy of activation of complex formation is appreciably greater than the energy of activation of the conversion process (*i. e.*, complex formation limits the rate); or (3) one can assume that the rates of complex formation and conversion are approximately the same, as did Briggs and Haldane.[8] In all three cases, one arrives at the following equation for the kinetic process

$$\alpha(x_0 - x) + \beta \ln\left(\frac{x_0 - x_e}{x - x_e}\right) = t \qquad (3)$$

α and β are constants which differ in meaning depending on the assumptions made.

(7) Michaelis and Menten. *Biochem. Z.*, **49**, 1333 (1913).
(8) Briggs and Haldane. *Biochem. J.*, **19**, 338 (1925).

Equation (3) fits much of the data in a quite satisfactory manner. Its essential applicability was indicated by the fact that, unlike several other possible equations, the same values of the constants were found whether starting with fumarate or with *l*-malate as substrate. In Fig. 3, the lines were drawn from this equation with values of α and β as follows: *p*H 5.97, $\alpha = -27{,}800$, $\beta = 2040$; *p*H 8.46, $\alpha = 35{,}700$, $\beta = 570$. Considering the great uncertainties in the processes involved in the fumarase reaction, however, the exact meaning of α and β is questionable, and equation (3) can be regarded as little more than an empirical expression that fits observed data. Thus, there is no indication in these data as to which of the three assumptions above may be correct, nor whether the real mechanism may not be in fact much more complicated than any of those here visualized.

Summary

The initial rate of the forward reaction in the system

$$\text{fumarate} + H_2O \underset{}{\overset{\text{fumarase}}{\rightleftharpoons}} \text{l-malate}$$

bears no obvious relation to the initial rate of the reverse reaction. This was shown to be due to the reaction being composed of a series of steps of which the rate-controlling step was of the zero order. The effect on the system of temperature, *p*H, and enzyme and substrate concentrations was studied, and an equation derived which described the data obtained.

PITTSBURGH, PENNSYLVANIA RECEIVED JUNE 11, 1947

[CONTRIBUTION FROM INSTITUTE OF POLYMER RESEARCH POLYTECHNIC INSTITUTE OF BROOKLYN]

Rate of Exchange of Cellulose with Heavy Water

BY V. J. FRILETTE,[1a] J. HANLE AND H. MARK

Introduction

The interchain structure of cellulose has aroused considerable interest in recent years. As with other polymers, there is reason to believe that the physical and chemical properties of cellulose are strongly influenced by the supermolecular texture that exists in a given sample. A generally accepted hypothesis concerning the texture of cellulose is[1b] that individual chain molecules having average extended lengths of several thousand Ångströms pass successively through regions of lower and higher lateral order. These ordered and disordered domains are not sharply distinguished and separated from each other but may be connected by areas of intermediate order. They are usually as a matter of convenience referred to as amorphous and crystalline constituents. One could expect different portions of a chain in such a structure to have different degrees of reactivity with small reagent molecules, for the behavior of an individual chain segment would depend upon how tightly it is bound laterally to its nearest neighbors. The reacting groups in the most disordered regions of a sample would be expected to be most easily accessible, while they would be inaccessible in the areas of high lateral order. Consequently, one would expect hydroxyl groups or glucoside bonds in amorphous regions to be most susceptible to reactions so that if the rate or extent of a particular reaction is followed quantitatively, it may be interpreted in terms of structural accessibility.

Until now, three chemical reactions have been used to characterize the reactivity of cellulose samples. Goldfinger, Siggia and Mark[2] measured the rate of periodate oxidation of various cellulose samples and established the existence of a rapid and a slow reaction. Assaf, Haas and Purves[3] treated cellulose with thallous ethylate in different solvents and found that only a fraction of the existing hydroxyl groups were converted to the thallium salt. Finally, Nickerson[4] and Conrad and Scroggie[5] studied the oxidative hydrolysis of cellulose to carbon dioxide and water. The rate of carbon dioxide evolution, which at first is rapid, gradually decreases to an almost constant value. It is claimed that by an analysis of the rate curves an estimate can be made of the amount of easily accessible (amorphous) cellulose originally present in the sample.

In this paper we shall describe a fourth reaction suitable for characterizing cellulose. It was first observed by Bonhoeffer[6] that the hydroxyl groups of cellulose react with heavy water. Champetier and Viallard,[7] who made a more systematic study of this effect, reported that a moderately slow reaction occurred and that practically all of the hydroxyl groups exchanged. The curves reported by these investigators showed little promise of yielding any information on accessibility, but a careful reading of the paper revealed that the cell in which the reaction was conducted had been

(1a) The material for this paper was abstracted in part from the thesis of V. J. Frilette, submitted to the Faculty of the Graduate School of the Polytechnic Institute in partial fulfillment of the requirements for the degree of Doctor of Philosophy, June, 1947.

(1b) O. Kratky, *Z. angew. Chem.*, **53**, 153 (1940).

(2) Goldfinger, Siggia and Mark, *Ind. Eng. Chem.*, **35**, 1083 (1943).

(3) Assaf, Haas and Purves, THIS JOURNAL, **66**, 59 (1934).

(4) Nickerson, *Ind. Eng. Chem.*, **34**, 85 (1942).

(5) Conrad and Scroggie, *ibid.*, **37**, 592 (1945).

(6) Bonhoeffer, *Z. Elektrochem.*, **40**, 469 (1934).

(7) Champetier and Viallard, *Bull. soc. chim.*, [5] **33**, 1042 (1938).