the hemiacetal exhibits general acid and base catalysis.[11] It is of interest to note that step 5 is in this case faster than step 6, which is considered in general to be the slowest one in acetal formation, as described above. This fact may be accounted for by considering step 6 as involving an intramolecular methylene bridge formation between neighboring hydroxyl groups, affording a stable six-membered ring.[12]

(11) G. W. Meadows and B. B. Darwent, *Trans. Faraday Soc.*, **48**, 1015 (1952).

(12) The formation of the acetal ring, step 6, is not conceivable as rate-determining, since then rate equation 7' ($dx/dt = K_4K_5k_6$[OH in polyvinyl alc.]^2[CH_2O][H^+]/[H_2O]) is derived and it is not attainable that the rate is proportional to the square of the concentration of the hydroxyl group. Perhaps this mechanism is pertinent to the formation of the non-cyclic acetal. In the case of polyvinyl alcohol, the evidence for the presence of intermolecular crosslinking has scarcely been obtained.

Other explanations for the accelerative influence of sulfuric acid, for instance the contribution of the simultaneous reaction of polyvinyl sulfate with formaldehyde or the influence of ionic strength, were eliminated, as shown in the Complementary Experiment and the salt effect in Table Ia.

Acknowledgments.—The authors wish to express their gratitude to Professors R. Oda and I. Sakurada for their aid in performing these experiments.

KYOTO, JAPAN

[CONTRIBUTION FROM GIBBS CHEMICAL LABORATORY, HARVARD UNIVERSITY]

The Kinetics of Ester Hydrolysis by Liver Esterase

BY G. B. KISTIAKOWSKY AND PAUL C. MANGELSDORF, JR.[1]

RECEIVED DECEMBER 9, 1955

The kinetics of hydrolysis of ethyl *n*-butyrate and methyl *n*-butyrate esters by purified horse liver esterase were studied in unbuffered solutions by a conductometric method over a range of *p*H at 25°. The enzyme was found to be reversibly inactivated at measurable rates in solutions of *p*H higher than about 10, a slower irreversible denaturation being also observed. After correction for this inactivation was made, it was found that the enzymatic activity is independent of *p*H from about 9.2 to higher than 10.6. The dependence of the rate on substrate concentration was studied in detail at *p*H 10.18. The data show significant deviations from the Michaelis–Menten mechanism and two more parameters suffice to describe the data satisfactorily, except for the fall-off of the rate at the highest substrate concentrations. The additional parameters may be interpreted as indicating the existence of two sites on an enzyme molecule (or of two distinct enzymes); the relative numerical magnitude of the kinetic parameters observed is such as to suggest this hypothesis. The relation of the present data, in particular of a lower value of the average K_m observed, to previous work is discussed.

Previous work from this Laboratory on urease[2] and the studies of fumarase by Alberty[3] and coworkers have demonstrated that the Michaelis–Menten mechanism is but a first approximation, accurate rate measurements revealing finer details of the kinetic behavior of enzymes. The present paper deals with some aspects of the kinetics of liver esterase, chosen because of its broad activity for the ester linkage, in contrast to the complete specificity of urease for the urea molecule. The particular enzyme used was horse liver esterase prepared according to the method of Connors, Pihl, Dounce and Stotz,[4] which method yielded them a liver esterase of the highest purity ever achieved. The kinetics of ester hydrolysis by horse liver esterase, which had previously been studied by Bamann and Schmeller[5] over a wide range of methyl butyrate concentrations, were reinvestigated by Connors, *et al.*, using their purified enzyme, but over a narrower range of ester concentrations and only in buffered solutions. It has been demonstrated that buffers and, in fact, any neutral salts may exert profound influence on enzyme kinetics.[6] The present experiments, therefore, were carried out in unbuffered solutions of low ionic strength, using essentially a conductometric titration method to follow the liberation of acid from the ester by the enzyme.

(1) Junior Fellow, Society of Fellows, Harvard University.

(2) G. B. Kistiakowsky and A. J. Rosenberg, THIS JOURNAL, **74**, 5020 (1952).

(3) R. A. Alberty, V. Massey, C. Frieden and A. R. Fuhlbrigge, *ibid.*, **76**, 2485 (1954).

(4) W. M. Connors, A. Pihl, A. L. Dounce and E. Stotz, *J. Biol. Chem.*, **184**, 29 (1950).

(5) E. Bamann and M. Schmeller, *Z. physiol. Chem.*, **194**, 1 (1931).

(6) G. B. Kistiakowsky, P. C. Mangeldorf, Jr., A. J. Rosenberg and W. H. R. Shaw, THIS JOURNAL, **74**, 5015 (1952); G. B. Kistiakowsky and W. H. R. Shaw, *ibid.*, **75**, 2751 (1953).

Experimental Details

In order to avoid complicating effects of carbonic acid, all the experimental equipment and procedures were designed to exclude CO_2 from the solutions used.

Glass distilled water was used for all solutions and was freed from CO_2 by prolonged bubbling of nitrogen at reduced pressure. All operations preparatory to the rate measurements were carried out in a CO_2-free "dry box" made from a fume hood which had been made air-tight and was provided with snug-fitting flexible armholes, hatches and a fan with suitable ducting for removing CO_2 by continuous circulation of air over moist sodium hydroxide sticks. All stock solutions were made and stored in this box in sealed vessels.

The reactions were carried out in a flanged Pyrex vessel 4 inches deep and 2 inches in diameter with rounded bottom. A lid made of $^1/_4$ inch aluminum was clamped tightly to the lip of the vessel by means of a rubber gasket. It is interesting to note that when originally a transparent polyester plastic sheet was used for the lid, the enzymatic activity was reduced by a factor of 20, presumably due to inhibition by the volatile plasticizer, since the lid came into no contact with the liquid.

The electrode system was supported by two narrow glass tubes which were sealed through the lid and carried electrical leads. The electrodes were two parallel multiperforate bright platinum sheets, 2 × 2 cm., held 1 to 2 mm. apart by a surrounding horizontal open-ended Pyrex cylinder to which the edges of the electrodes were sealed by gently melting the glass along the lines of contact. With the lid in place the electrodes hung well below the level of the liquid, which was stirred by a small magnet sealed in a polyethylene tube. The whole was submerged in a water thermostat, the temperature being held constant to 0.01° at 25°.

The resistance of solutions was measured on a Leeds and

Northrup Jones conductivity bridge in a conventional circuit, with a one-kilocycle oscillator for the input and an amplifier for the output. The unbalance signal was observed with an oscilloscope but a hundred-fold finer discrimination of resistance changes was possible with a rectifying circuit and a d.c. galvanometer. The former consisted essentially of a 6SN7 double triode, the d.c. voltage for the galvanometer arising between the two cathodes, when the bridge output was applied between the plates and the oscillator signal was used as the common grid excitation. Since this detector was sensitive to both the phase and the amplitude of the bridge output, it was used only after the approximate capacitance and resistance balances had been obtained with the aid of the oscilloscope.

The lid on the reaction vessel was provided with an inlet for nitrogen, a slight overpressure being maintained inside. A stoppered hole permitted the pipetting of solutions into the vessel, meanwhile letting nitrogen flow gently outward. The pipetting was done mechanically, with a valve system providing suction and overpressure.

The method of Connors, *et al.*,[4] was followed very closely in preparing the enzyme concentrate, the only deviations being the use of a Blendor instead of a Burr mill for grinding and of a cotton string sleeve instead of muslin sleeve for pressure filtration. The total activity at each stage changed just about as they reported. From the final solution which was kept at 1°, a 0.085-cc. aliquot was withdrawn to make up each 50-cc. of standard enzyme solution, of which 1 or 2 cc. was used for each run.

An approximately 3×10^{-3} sodium hydroxide solution was kept in a paraffin-lined tincture bottle.

The substrates, ethyl *n*-butyrate and methyl *n*-butyrate, Eastman White Label Reagents, were redistilled. Boiling range of each was less than 1°, bracketing the tabulated b.p. Refractive index of each was about 0.0004 unit less than the tabulated values.

Fifteen to twenty drops of an ester from a dropper gravimetrically calibrated for the ester, was made up with water in a volumetric flask. By using repeated dilutions in volumetric flasks, a 500-fold range of ester concentrations was conveniently available.

The reaction vessel was filled with water and the substrate solution to the desired concentration in the dry box, using bulb operated volumetric pipets. After its transfer to the thermostat a few minutes were allowed for thermal equilibration, while stirring and passing nitrogen. The NaOH solution was then added from a pipet and resistance readings were taken at one-minute intervals until a steady rate of thermal hydrolysis was determined. The addition of the enzyme solution followed, and the resistance readings at one-minute intervals were continued until the run was completed. Upon emptying, the vessel and the electrodes were rinsed several times with glass distilled water and were soaked in it for 15 minutes with stirring before being returned to the dry box.

Results and Discussion

The conductometric method employed in these experiments is well suited for studying of the *p*H dependence of enzymatic activity by measuring conductivity continuously in experiments with fairly concentrated substrate solutions. Given the conductivity of the solution at the theoretical neutral point (which could be taken as the measured conductivity at the minimum), one can derive the instantaneous concentration of the hydrogen or hydroxyl ions directly as the surplus conductivity, divided by the appropriate ionic conductance factors. Moreover, the instantaneous reaction rate is proportional to the rate of conductivity change. In acid solutions a correction is necessary to allow for the incomplete dissociation of the acid. But this is readily applied when the *pK* of the acid is known, as is the case for *n*-butyric acid.

The rates of hydrolysis observed upon the addition of the enzyme were corrected for the thermal hydrolysis rates. The latter were found to be proportional to the hydroxyl ion concentration in alkaline solutions. Hence a simple measurement of the hydrolysis rate, before the enzyme was added, sufficed to apply the necessary corrections to the rates measured subsequently over a range of *p*H values, providing that the concentration of the substrate remained sensibly constant. In dilute substrate solutions the change in the hydroxyl and in the substrate concentrations were commensurate and the above procedure, therefore, did not apply. In such solutions only the initial rates following the addition of the enzyme were determined and the true enzyme-catalyzed rate was obtained by extrapolating the rates before and after the addition of the enzyme to the instant of the addition. This procedure corrected also for slow conductivity drifts from other causes, which became significant in very dilute substrate solutions because of the low rates of enzymatic hydrolysis.

Previous work[4] indicated that liver esterase has a sharp activity maximum at about *p*H 8 but this could not be confirmed, the rate appearing virtually constant over the range *p*H 9.3 to 10.3 and beyond, that is, between 15 and 200 μmoles of NaOH per liter. On closer study, this independence of *p*H proved to be true only of the rates observed immediately after the enzyme had been added to alkaline solutions. If the titration was repeated, by adding more hydroxide when the neutrality point was reached, or if the initial solution was as much as 400 μmolar in alkali, a slow falling off of the rate with the exposure of the enzyme to alkaline *p*H was observed. This is shown in Fig. 1.

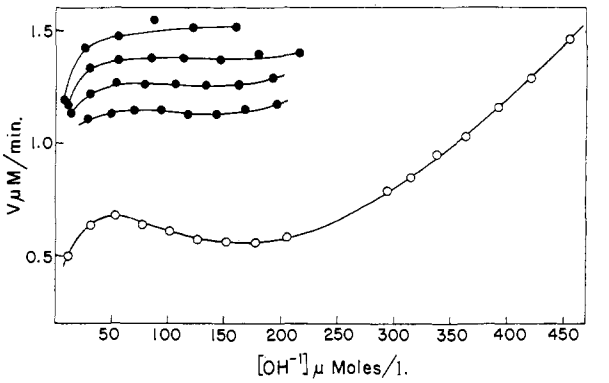

Fig. 1.—The rate of hydrolysis as a function of hydroxyl ion concentration. Solid circles show four successive runs with the same enzyme. Open circles show a run with the same enzyme concentration but twice the initial concentration of hydroxide.

The four consecutive runs, in each of which the solution was titrated to 200 μmolar hydroxide concentration, show not only a progressive decrease of the rate but also a progressively more pronounced minimum. Both the decreasing rate and the minimum were still more pronounced when the initial amount of hydroxide was doubled, suggesting a distinct tendency for the enzyme to recover its activity as it approached neutrality, after the exposure to strongly alkaline solutions.

The extent of the inactivation of the enzyme was studied by adding the enzyme to alkaline solutions at varying intervals before the substrate was added.

Separate controls were run to obtain the corrections for the thermal hydrolysis. The results of one such series of runs is shown in Fig. 2, but on the whole they were not as reproducible as Fig. 2 might suggest. The initial fall-off of activity was roughly exponential, with a time constant of five to ten minutes in a 200 μmolar hydroxide solution, tending to a finite asymptote. Superimposed upon this was a much slower linear decrease in activity. It is likely that the former represents a reversible and the latter an irreversible denaturation of the enzyme. If this is the correct explanation, the minima in the rate curves are due to the recovery of the enzyme at lower *p*H and, being indicative of the denaturation equilibrium states of the enzyme, could be used to determine the reversible *p*H–denaturation curve. But another explanation also fits the experimental data, which is that the exposure to alkaline solutions alters the *p*H–enzymatic activity curve of the enzyme, with a maximum developing near the neutral point. It was not possible to choose between the two mechanisms because of the complicating effects of irreversible denaturation.

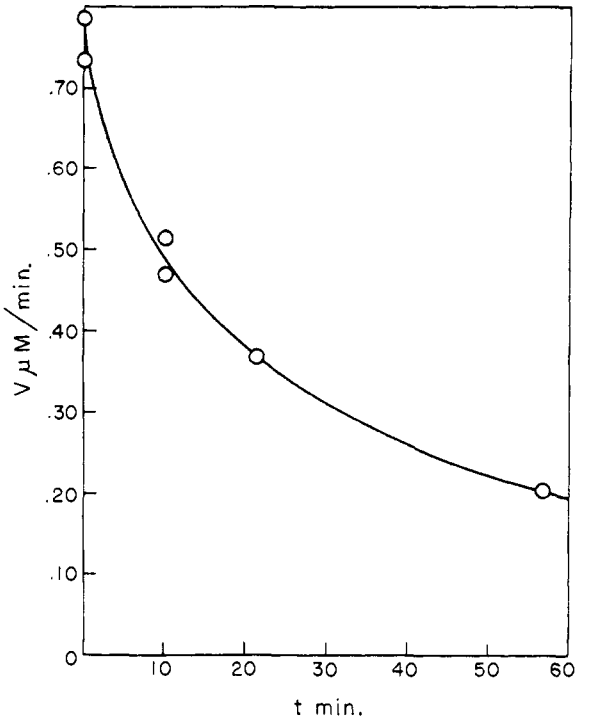

Fig. 2.—The effect on the initial rate of hydrolysis of the exposure of enzyme to a 200 μmolar hydroxide solution for varying lengths of time.

Several of the runs were followed to the acid side of the neutral point, establishing that at *p*H 5.9 the enzymatic activity was roughly 5% of its activity at *p*H 9.6. In Fig. 3 the activity–*p*H curve as observed in the present research is contrasted with that found by Connors, *et al.*[4] The present experiments indicate a rise of activity from *p*H 6 to *p*H 9, although the exact shape of the curve near the neutral point could not be readily determined with our method. This rise is followed by a region of constant activity, if the latter is measured immediately after adding the enzyme to alkaline solutions; the other branch of the curve in these alkaline solutions indicates the effect of reversible denaturation of the enzyme, as would be observed after exposing the enzyme to the indicated *p*H for some time.

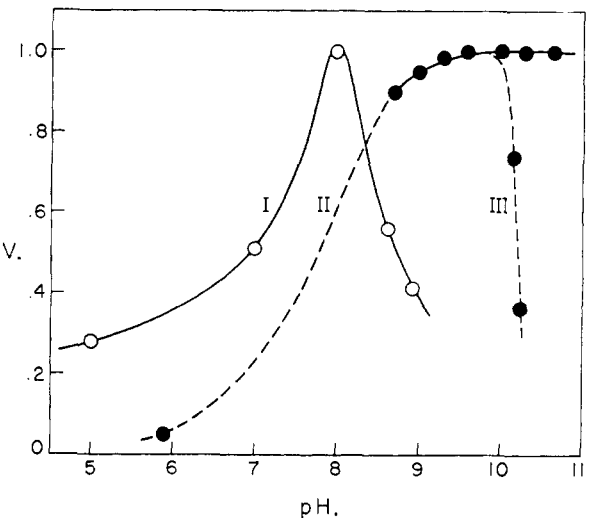

Fig. 3.—A comparison of the *p*H enzymatic activity curve observed with methyl *n*-butyrate in this research (curve II) with that reported by Connors, *et al.* (curve I). Line III shows the effect on enzymatic activity of protracted exposure to alkaline solutions.

The observation of Willstaetter, Haurowitz and Memmen[7] that the activity maximum shifts to higher *p*H as the purity of the enzyme is improved, suggests that the enzyme used in the present experiments was highly purified. But it is not likely to be purer than that used by Connors, *et al.*[4] and the discrepancy revealed by Fig. 3 must be due to other causes. These are, most likely, the use of the unbuffered solutions of low ionic strength and measurements of rates immediately upon the addition of the enzyme to alkaline solutions, employed in the present research.

Attempts were made to study the synthetic action of the enzyme on aqueous solutions of alcohol and acid, using for this purpose a sensitive recording *p*H meter developed in this Laboratory by Dr. Robert Davis for the study of the kinetics of carbonic anhydrase. No synthetic action was observed, apparently[7,8] because the purified esterase decreases so rapidly in activity with decreasing *p*H that it fails to produce measurable rates in the *p*H range where the equilibrium constant for ester formation is favorable to observe synthetic action. This finding is rather disappointing because the knowledge of the two maximum rates and the two Michaelis constants of a simple two-way Michaelis–Menten mechanism would suffice to evaluate all four rate constants.

The dependence of the enzymatic rate of hydrolysis on substrate concentration was studied in detail at *p*H 10.18. Table I shows the results of

(7) R. Willstaetter, F. Haurowitz and F. Memmen, *Z. physiol. Chem.*, **140**, 203 (1924).

(8) P. Rona, A. Ammon and H. Fischgold, *Biochem. Z.*, **241**, 460 (1951).

three series of measurements with ethyl *n*-butyrate, corrected for non-enzymatic rate. Since slightly different enzyme stock solutions were used for each series of measurements, the second and the third were multiplied each by a single conversion factor. These were obtained from the vertical displacements necessary to bring the log v–log S plots of the three series into the best agreement among themselves. In Table II are shown three series of measurements with methyl *n*-butyrate, also reduced to a standard concentration of active enzyme by means of correction factors. The rates shown correspond to the same enzyme concentration as used for Table I, the conversion factor having been obtained from experiments in which aliquots of the same enzyme solution were made to hydrolyze the ethyl *n*-butyrate and the methyl *n*-butyrate esters. The unit "cc. enzyme" refers to the standard solution and is roughly equivalent to 1.7 micro-liters of the concentrated stock solution.

TABLE I

THE RATE OF HYDROLYSIS OF ETHYL *n*-BUTYRATE AT *p*H 10.18

Substrate, M	Rates in μmole/mln., cc. enzyme 1st series	2nd series	3rd series
1.2×10^{-5}	.....	0.020, 0.022	.....
2.4	.....	.045	0.043, 0.0525
4.8	.....	.....	.083
6.1	.....	.106	.....
1.2×10^{-4}	.....	.150	.130
2.4	.....	.240	.233
4.8	.....	.....	.320
6.1	.....	.280	.....
1.2×10^{-3}	0.440, 0.415	.400	.445
2.4	.525, .520	.....	.485, .535
4.8	.585, .590	.....	.580
9.6	.580, .595	.....	.....
9.7	.....	.615	.....
1.2×10^{-2}	.....	.....	.615
1.9	.580, .555	.....	.....
3.4	.555	.....	.....
3.8	.555	.....	.....

TABLE II

THE RATE OF HYDROLYSIS OF METHYL *n*-BUTYRATE AT *p*H 10.18

Substrate, M	Rates In μmole/min., cc. enzyme 1st series	2nd series	3rd series
5.1×10^{-5}	.....	...	0.037
1.01×10^{-4}	.....	...	.068
2.3	.....	0.137	.....
2.5	.....	...	.137
5.1	.....	...	.227, 0.232
5.2	.....	.246	.....
1.01×10^{-3}	.....	...	.366
1.14	.....	.425	.....
1.2	0.448, 0.459	...	.....
2.4	.654, .654	.616	.....
2.5	.....	...	.550
4.9	.900, .900	...	.....
5.1	.....	...	.880, .905
1.0×10^{-2}	1.110, 1.090	...	.....
1.01	.....	...	1.120
2.0	1.205, 1.190	...	.....
2.5	.....	...	1.230
4.0	1.245	...	.....

With neither ester did the enzyme display the true Michaelis–Menten kinetics, even if the high substrate fall-off, characteristic of esterases, is ignored. The plots of $1/v$ *vs.* $1/S$ have a pronounced concavity downward, such as has been found by Rosenberg[2] with the urease–urea system. Hence more than the two parameters provided by the Michaelis–Menten mechanism are required to describe the data, but the form of this mechanism is such that the additional parameters must be introduced in pairs, since each new distinguishable enzyme–substrate compound must have both a specific rate and a characteristic substrate concentration parameter.

With the increasing number of adjustable parameters, doubts arise about their correct evaluation from necessarily imperfect experimental data. To overcome this universal difficulty, a series expansion of the rate expression appears to be indicated. If the denominator of a general enzymatic rate expression is factored and the expression is developed in partial fractions accordingly, the resulting expression will be of the form of a sum of Michaelis-type terms

$$v = \sum_i \frac{a_i S}{K_i + S} \tag{1}$$

in which, of course, none of the quantities K_i could have negative real values.

Expansion of this expression in powers of S or $1/S$ is unsatisfactory since the resultant expressions diverge in a region where S is surrounded by values of K_i. Introducing the variable

$$q = \frac{S - K}{S + K} \tag{2}$$

and the parameters

$$p_i = \frac{K_i - K}{K_i + K} \tag{3}$$

where K is an arbitrary quantity, the general rate expression transforms to

$$v = \sum_i \frac{a_i}{2} \frac{(1 + q)(1 - p_i)}{1 - p_i q} \tag{4}$$

The absolute magnitude of q and all p_i is always less than unity, so that this expression may be expanded in powers of q with convergence over the entire experimental range. Using the definitions

$$\hat{p}^N = \Sigma \frac{a_i p_i^N}{\Sigma a_i}; \; v_{max} = \Sigma a_i \tag{5}$$

the rate is obtained as

$$v = \frac{v_{max}}{2} [(1 - \hat{p}) + q(1 - \hat{p}^2) + q^2(\hat{p} - \hat{p}^3) + \ldots.] \tag{6}$$

If the arbitrary parameter K is taken as that substrate concentration where $v = v_{max}/2$, $\hat{p}$ is equal to zero, so that the "mean" K_m defined in this way is identical to that employed by enzymologists before the procedure of Lineweaver and Burk became established.

The direct experimental data must be transformed suitably to compare with expression (6). The value of q corresponding to each experimental substrate concentration must be calculated and a series expression for v in powers of q must be fitted

to the data. Lacking a suitable computer to do this by a least squares method, we instead set out by fitting a smooth curve to the experimental data by eye. Interpolated values of the rate were then taken from this curve at regular intervals of q and analyzed by the calculus of finite differences.

After applying a correction for the fall-off of the rate at high substrate concentrations, the "true" value of v_{max} was determined as 660 μmoles of ethyl n-butyrate per minute per cc. of enzyme. The concentration of this substrate at which the rate fell to 1/2 v_{max} was 0.521 mmole/l., by definition equal to K. The second and third "moments" of the power series (6) calculated using these quantities were

$$\hat{p}_2 = 0.191$$
$$\hat{p}_3 = -0.10$$

This information suffices to calculate the four parameters of the two-site[1] (or two-enzyme) mechanism fitting best the available experimental data: for ethyl n-butyrate

	Site I	Site II	
v_{max}	0.163	0.497	μmole/min., cc. enzyme
K_m	.082	.882	mmole/l.

With these parameters the curve of Fig. 4 was obtained, which is seen to fit the experimental data

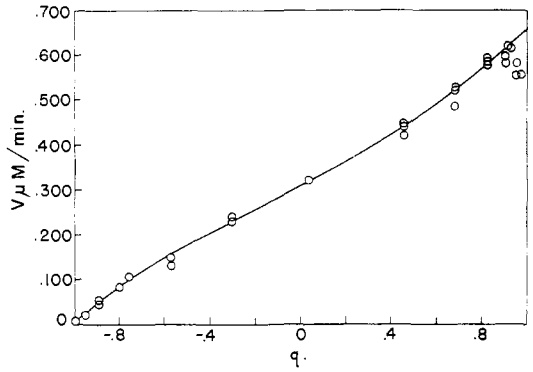

Fig. 4.—Dependence of the rate of hydrolysis of ethyl n-butyrate on substrate concentration. The rate is plotted against the parameter q defined in eq. 2. Solid line is the calculated rate from a four parameter equation.

extremely well, except in the region of the highest substrate concentrations where the fall-off, not allowed for in the expansion, is seen to exist.

This method of analysis proved to be unnecessary with methyl n-butyrate because the Lineweaver–Burk plot could be made to straighten out by a simple subtraction of a constant amount from all measured rates, as shown in Fig. 5. This constant amount is too large to be attributable to experimental errors, but can be interpreted by the two-site mechanism as the v_{max} of a site with a K_m much lower than the lowest ester concentration used in the experiments. Thus the rate parameters for methyl n-butyrate are derived to be

	Site I	Site II	
v_{max}	0.015	1.335	μmoles/min., cc. enzyme
K_m	$\leq$.01	2.48	mmoles/l.

It is interesting to note that on Site II the ratio of v_{max} to K_m is the same for both esters. The same

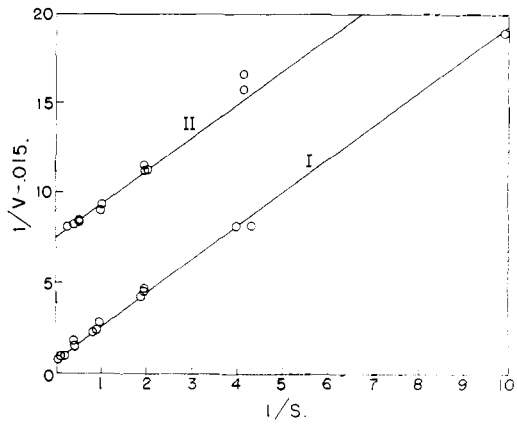

Fig. 5.—Dependence of the rate of hydrolysis of methyl n-butyrate on substrate concentration. Curve II is plotted on scales expanded tenfold to better show the region of high substrate concentrations.

relation holding for Site I would also fit the experimental data. This finding supports the hypothesis that two independent sites (or enzymes) do exist, the kinetic mechanism of each being

$$E + S \xrightarrow{k_1} ES \xrightarrow{k_3} E + P$$

where k_1 is ester independent, while k_3 varies with the ester and the site. The values of k_1 on this hypothesis are: Site I, k_1 = 2 ml./min., cc. enzyme; Site II, k_1 = 0.5 ml./min., cc. enzyme.

The larger of the two values of K_m reported here for methyl n-butyrate (2.5 mM) is considerably smaller than any reported heretofore for horse liver esterase. Connors, *et al.*,[4] found 22 mM in heavily buffered solutions. Bamann and Schmeller's data fit a K_m of about 10 mM in an unbuffered solution.[9] The present measurements were made at a higher pH than the above, but a few experiments with dilute substrate solutions indicated that the enzymatic rates with these were as independent of pH as those with more concentrated solutions. Thus K_m appears to be fairly independent of pH and its smallness in the present experi-

(9) Only after we had submitted this manuscript for publication did the paper of G. W. Schwert and A. J. Glaid, *J. Biol. Chem.*, **199**, 613 (1952), come to our attention. These authors, using enzyme prepared by the method of Connors, *et al.*, and working with unbuffered solutions, obtained values of K_m for both ethyl and methyl n-butyrate with which our results are in good accord. Because they restricted their studies to relatively narrow ranges of relatively high substrate concentrations the values of K_m and V_{max} which they report describe only the limiting behavior of the enzyme at the high substrate limit. With a two-site mechanism the quantities corresponding to the limiting values of K_m and V_{max} are, respectively, $(V^1_{max}K^1_m + V^2_{max}K^2_m)/(V^1_{max} + V^2_{max})$ and $(V^1_{max} + V^2_{max})$. Using these relations:

	We find at pH 10.18, 25°	Schwert and Glaid find at pH 8, 25°
For limiting K_m methyl n-butyrate	2.46 mM	3.14 mM
For limting K_m ethyl n-butyrate	0.685 mM	0.683 mM
For limiting ratio V_{max} MB/V_{max} EB	2.04	1.93

Taking the two unit pH difference into account the agreement is certainly satisfactory. Unfortunately, however, this agreement carries with it the implication that the inhibition constants and activation energies measured by Schwert and Glaid might have been substantially different if their measurements had been extended to lower ranges of substrate concentrations.

ments must be due to the absence of inhibitors in our reaction solutions, since competitive inhibitors, at least, cause an increase in the apparent K_m.

The observed deviations from the Michaelis–Menten mechanism may have an explanation entirely different from the two-site hypothesis discussed above. Falconer and Taylor[10] noted certain anomalies in the solubility of pig liver esterase, which they attributed to the presence of two separate enzymes. But their observations, as well as the results presented here, may find an explanation in terms of a more or less continuous range of enzyme variation. Given the pronounced alteration in the enzyme's kinetic properties as it is purified,[7,8,11,12] it is not unreasonable to suppose that liver impurities clinging to the enzyme could result in several molecular species having different enzymatic parameters, whose assembly would then show deviations from the Michaelis–Menten kinetics. The existence of such rather firmly bound complex species is also suggested by the failure to crystallize any of the liver esterases, notwithstanding all attempts to purify them.

Thanks are due to the Rockefeller Foundation for financial aid which made this research possible.

(10) J. S. Falconer and D. B. Taylor, *Biochem. J.*, **40**, 831, 835 (1946).

(11) H. Sobotka and D. Glick, *J. Biol. Chem.*, **105**, 221 (1934).

(12) K. Gyotoku, *Biochem. Z.*, **193**, 18, 27 (1928).

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, UNIVERSITY OF WISCONSIN]

Diffusion Currents at Spherical Electrodes

BY ROBERT P. FRANKENTHAL[1] AND IRVING SHAIN

RECEIVED JANUARY 9, 1956

The theory of diffusion currents at microelectrodes has been extended to the case of spherical electrodes under conditions where the curvature of the electrode surface must be considered and where the applied voltage varies linearly with time. Theoretical current–voltage curves were constructed and compared with experimental results for the reduction of lead and thallous ions. The stationary spherical mercury electrode used in this work shows great promise as an analytical tool.

Diffusion currents at microelectrodes under conditions where the equations for linear diffusion apply have been studied by Randles,[2] Sevcik,[3] and Berzins and Delahay.[4] They showed that their equations apply to electrodes of any shape provided that the rate of voltage change is high and/or the electrode surface is large. However, when these conditions are not met and spherical or cylindrical microelectrodes are used, the curvature of the electrode surface must be considered in a theoretical treatment of the current–voltage relationships observed. Nicholson[5] studied the current–voltage curves obtained with cylindrical platinum microelectrodes. The development of stationary spherical mercury microelectrodes by Gerischer[6] and Berzins and Delahay[7] has made it possible to extend the theory to spherical electrodes.

Theory

Consider the reduction of a substance O to a substance R which is soluble in the solution or in the electrode. Further conditions are that the only way substance O can reach the electrode surface is through diffusion, that the electrode reaction is reversible, that the voltage varies linearly with time, and that the electrode is a sphere. The current flowing at the electrode will be a function of the flux of substance O at the electrode surface, which, under these conditions, will be determined by the general equation for spherical diffusion[8]

$$\frac{\partial C_o(r,t)}{\partial t} = D_o\left[\frac{\partial^2 C_o(r,t)}{\partial r^2} + \frac{2}{r}\frac{\partial C_o(r,t)}{\partial r}\right] \quad (1)$$

C_o is the concentration of substance O, r is the distance from the center of the electrode, t is the time, and D_o is the diffusion coefficient of substance O. This equation must be solved for certain initial and boundary conditions.

The initial conditions are that the concentration of substance O is uniform throughout the solution at zero time, and that the concentration of substance R is zero in the solution (or in the electrode, if an amalgam is formed) at zero time.

The first boundary condition is derived from the Nernst equation. If the voltage varies linearly with time

$$E = E_i - vt \quad (2)$$

where E is the potential of the electrode, E_i is the potential at zero time, and v is the rate of voltage change. The negative sign indicates that the potential of the working electrode varies toward more cathodic potentials. Combining equation 2 and the Nernst equation[9]

$$\frac{C_o(r_0,t)}{C_R(r_0,t)} = \theta \exp(-\sigma t) \quad (3)$$

where

$$\theta = \frac{f_R}{f_o}\exp\left[\frac{nF}{RT}(E_i - E^0)\right] \quad (4)$$

and

$$\sigma = \frac{nF}{RT}v \quad (5)$$

(1) Based on the Ph.D. thesis of R. P. Frankenthal, 1956. Procter and Gamble Fellow 1954–1955.

(2) J. E. B. Randles, *Trans. Faraday Soc.*, **44**, 327 (1948).

(3) A. Sevcik, *Collection Czechoslov. Chem. Communs.*, **13**, 349 (1948).

(4) T. Berzins and P. Delahay, THIS JOURNAL, **75**, 555 (1953).

(5) M. M. Nicholson, *ibid.*, **76**, 2539 (1954).

(6) H. Gerischer, *Z. physik. Chem.*, **202**, 302 (1953).

(7) T. Berzins and P. Delahay, THIS JOURNAL, **77**, 6448 (1955).

(8) P. Delahay, "New Instrumental Methods in Electrochemistry," Interscience Publishers, New York, N. Y., 1954, p. 60.

(9) Ref. 8, pp. 116–117.