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TEMPERATURE AND pH EFFECTS ON IMMOBILIZED LACTATE DEHYDROGENASE KINETICS

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Summary

Rabbit muscle lactate dehydrogenase (EC 1.1.1.27) was attached covalently to the inner surface of nylon tubing, and kinetic measurements made. The results were interpreted on the basis of the Kobayashi-Laidler treatment of immobilized enzymes in flow systems, various tests being applied to determine the degree of diffusion control. It was established in various ways that the degree of diffusion control increases with (a) decrease in flow rate, (b) decrease in substrate concentration, and (c) decrease in temperature. A number of quantitative relationships, predicted by the theory, were obeyed by the results, for example: (a) K_m(app) varies linearly with $v_f^{-1/3}$, where v_f is the flow rate, (b) the logarithm of the product concentration at the exit varies linearly with the logarithm of the flow rate, and (c) absolute calculations of product concentrations are in reasonable agreement with experiment. A value of 5 kcal · mol^{-1} is estimated for the activation energy of the diffusion processes, and of 1 kcal · mol^{-1} for the chemical processes. When the pH is varied the rates pass through a flat maximum, the pH dependence being less than with the free enzyme.

Introduction

Several recent reviews [1—4] have dealt with theoretical and practical aspects of the kinetics of immobilized enzymes. Two systems have been studied in considerable detail: enzyme trapped in a gel, slices of which are immersed in substrate solution [5—7], and enzyme attached to the inner surface of tubing

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through which the substrate solution flows [7—10]. Most of the previous work has been done with single-substrate systems, but in a recent paper [11] we have reported results with lactate dehydrogenase, which catalyzes the reaction between pyruvate and reduced nicotinamide adenine dinucleotide (NADH). The enzyme was covalently attached to the inner surface of nylon tubing and the kinetics were studied over a range of concentrations of both substrates, and at different flow rates. Some preliminary results were reported at different temperatures. Under the conditions of the experiments the kinetics were largely diffusion-controlled, but extrapolation procedures led to information about the diffusion-free process.

The present paper describes more detailed kinetic studies of the same system, carried out over a range of temperatures and pH values.

Materials and Methods

Chemicals. Pyruvic acid, reduced nicotinamide adenine dinucleotide and crystalline rabbit muscle lactate dehydrogenase (EC 1.1.1.27, type I, lot 55C-9500, suspended in 2.1 M ammonium sulfate at pH 6.0) were purchased from the Sigma Chemical Company, St. Louis, MO. The nylon tubing of 0.152 cm internal diameter was obtained from Canus Plastics Ltd., Ottawa.

Attachment procedure. The method used to attach the enzyme to the inner surface of the nylon tubing involves three steps: (1) Partial hydrolysis of the surface, with cleavage of some of the secondary amide linkages, (2) Coupling of the released carboxyl groups to benzidine, by the use of dicyclohexylcarbodiimide, and reaction of the amino groups with glutaraldehyde, (3) Reaction of the activated surface with the enzyme. Details are given in our previous paper [11]. As discussed in that paper, other methods leave a positive or negative charge on the surface, and such charges appear to be unfavorable to dehydrogenase. The present method leads to a neutral surface, and the tube showed little loss in activity over a period of months. When not in use the tubes were filled with buffer at pH 7.5 and stored at 4°C.

Rate measurements. All measurements were made with a tube 50 cm in length with an internal radius of 0.076 cm. The reaction was followed by the decrease in absorbance of NADH at 340 nm, use being made of an SP 1800 Pye Unicam spectrophotometer. Substrate solutions were pumped through the tube by means of an LKB Varioperpex II peristaltic pump, model 2120. One end of the nylon tube was connected to the feed solution by means of tygon tubing, while the other was connected to a flow cell in the spectrophotometer. Our value of the absorption coefficient of NADH at 340 nm was $6.22 \cdot 10^3$ $dm^3 \cdot mol^{-1} \cdot cm^{-1}$, which is consistent with previously obtained values [12,13].

Theoretical

Kobayashi and Laidler [14] have given a theoretical treatment of the flow kinetics of an enzyme attached to the inner surface of a tube, for the case of single-substrate systems. This treatment has been found to be successful in leading to an interpretation of the kinetic results with several enzymes [7—10]. The theory for single-substrate systems is quite complicated, but some useful

generalizations have been obtained. The Michaelis-Menten equation is obeyed to a good approximation, even when there is considerable diffusion control. A diffusion layer is established at the inner surface of the tube, and the rate with which substrate diffuses through this tube can be expressed in terms of a mass transfer coefficient k_L given by

$$k_L = 1.28\left(\frac{D^2 v_f}{rL}\right)^{1/3} \tag{1}$$

where D is the diffusion coefficient ($cm^2 \cdot s^{-1}$), v_f is the flow rate ($cm \cdot s^{-1}$), r the internal radius of the tube (cm) and L its length (cm). At high flow rates there is a reduction in the effective thickness of the diffusion layer, and diffusion then exerts little control over the rate. The apparent Michaelis constant $K_m(app)$ at any flow rate is related to the inherent value K'_m (i.e. the value in the absence of diffusional effects) by

$$K_m(app) = K'_m + \frac{V'_m}{2k_L} \tag{2}$$

where V'_m is the limiting rate at high substrate concentrations. In view of Eqn. 1 it follows that

$$K_m(app) = K'_m + 0.39V'_m\left(\frac{RL}{D^2 v_f}\right)^{1/3} \tag{3}$$

so that $K_m(app)$ should be linear in $v_f^{-1/3}$ and at very high flow rates becomes K'_m. This has been confirmed for a number of systems [7—11].

At high flow rates the rate equation becomes

$$v = \frac{2\pi rLk'_c[E]_s[S]}{K'_m + [S]} \tag{4}$$

where k'_c is the inherent rate coefficient (influenced by conformational and environmental effects but not by diffusion), and $[E]_s$ is the enzyme concentration per unit surface area. The product concentration at the tube exit is this rate divided by the volume of solution that emerges in unit time, viz. $\pi r^2 v_f$, and is thus given by

$$[P]_0 = \frac{2Lk'_c[E]_s[S]}{rv_f(K'_m + [S])} \tag{5}$$

At low flow rates diffusional effects become dominant, and at very low substrate concentrations the theory leads to the result that the rate of product formation is

$$v_r = 8.06(v_f D^2 r^2 L^2)^{1/3}[S] \tag{6}$$

The product concentration at the tube exit is now

$$[P]_D = 2.56\left(\frac{DL}{r^2 v_f}\right)^{2/3}[S] \tag{7}$$

When there is full diffusional control the rate is independent of the kinetic parameters for the enzyme.

A theoretical treatment of the flow kinetics for a two-substrate system has

not yet been developed, and presents great difficulty. For the interpretation of experimental results it seems best to concentrate on the two limiting cases, with one substrate present in excess and the concentration of the other one varied. When, for example, NADH is present in excess, it will saturate the enzyme at the surface; the pyruvate will travel through the diffusion layer and react with the enzyme-NADH complex at the surface. Under these conditions the variation of rate with substrate concentration is expected to be of the Michaelis-Menten form:

$$v_r = \frac{V[S]}{K_m(\text{app}) + [S]} \tag{8}$$

With pyruvate in excess, the situation may be different. It is known from numerous studies with lactate dehydrogenase in free solution that the reaction occurs by an ordered ternary complex mechanism, the NADH being bound first and enzyme-NADH reacting with pyruvate [15—20]. If the same is true of the bound enzyme, the mechanism in the flow experiments with pyruvate in excess will be that the NADH will diffuse to the surface and form a complex, which will react rapidly with readily-available pyruvate. The rate Eqn. 8 will again be obeyed.

Results and Analysis

Kinetic parameters

Rates were measured at five temperatures, 10, 20, 30, 40 and 50°C and at flow rates ranging from 0.36 cm · s^{-1} to 1.50 cm · s^{-1}. In one series the concentration of pyruvic acid (A) was held at a high value (3.00 mM) and the concentration of NADH (B) was varied; in another the concentration of NADH was

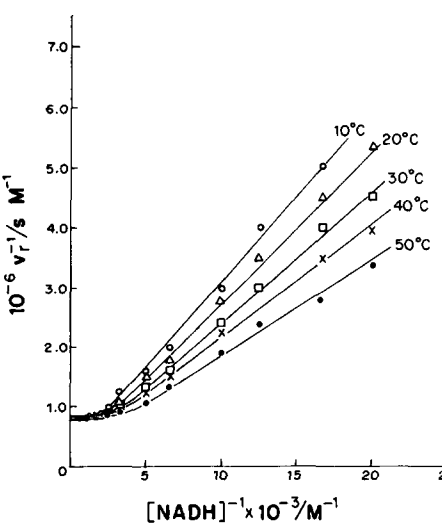

Fig. 1. Typical Lineweaver-Burk plots. These curves are for pyruvic acid in excess at 3.00 mM and with the NADH concentration varied from 0.05 mM to 0.70 mM. The nylon tube was 50 cm long with an internal radius of 0.076 cm; pH 7.5.

TABLE I

VALUES OF K_m(app) (mmol · dm^{-3}) OBTAINED FROM LINEWEAVER-BURK PLOTS

v_f/cm · s^{-1}	10°C	20°C	30°C	40°C	50°C
Variable [NADH], and [pyruvate] fixed at 3 mM					
0.36	0.55	0.40	0.32	0.25	0.18
0.72	0.46	0.34	0.26	0.21	0.15
1.08	0.42	0.30	0.24	0.19	0.13
1.50	0.40	0.28	0.23	0.17	0.12
Variable [pyruvate], [NADH] fixed at 2 mM					
0.36	0.60	0.44	0.38	0.31	0.25
0.72	0.51	0.38	0.31	0.24	0.19
1.08	0.45	0.34	0.28	0.20	0.16
1.50	0.42	0.30	0.25	0.18	0.14

held at 2.00 mM and the concentration of pyruvic acid was varied. To test the applicability of Eqn. 8, plots were made of $1/v$ against 1/[A] and 1/[B] for both series of experiments, at different temperatures and flow rates. Fig. 1 shows such a test, for pyruvic acid in excess at 3.00 mM and the concentration of NADH varying between 0.05 mM and 0.70 mM. There is seen to be good linearity at the lower substrate concentrations, when substantial diffusion control is expected, but there are deviations from linearity at NADH concentrations above 0.2 mM. This type of curvature of the Lineweaver-Burk plots is typical of systems of this kind where there is a change in the degree of diffusion control, and was also found in our measurements with NADH in excess. The K_m(app) values obtained from the linear portions of these plots are shown in Table I, use being made of the method of least squares.

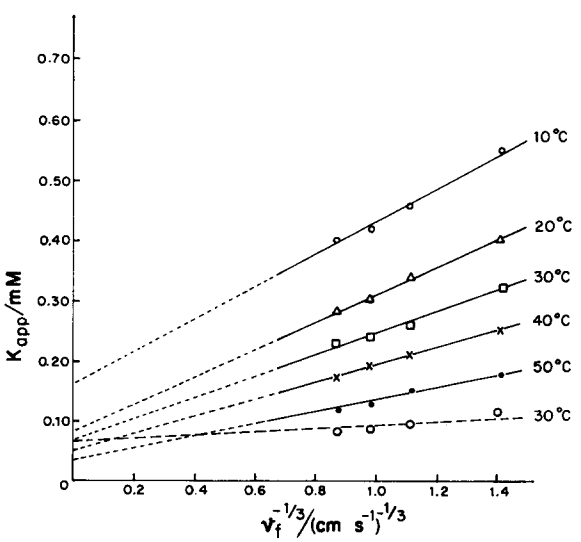

Fig. 2. Variation of the apparent Michaelis constant K_m(app) with flow rate. Pyruvic acid in excess at 3 mM; pH, 7.5.

TABLE II

VALUES OF K'_m OBTAINED FROM PLOTS OF K_m(app) AGAINST $v_f^{-1/3}$

T (°C)	K'_m (NADH) (mM)	K'_m (pyruvate) (mM)
10.0	0.160	0.075
20.0	0.080	0.045
30.0	0.070	0.026
40.0	0.049	0.015
50.0	0.035	0.005

Dependence of K_m(app) on flow rate

Eqn. 3 predicts the dependence of K_m(app) on flow rate, and Fig. 2 shows plots of K_m(app) against $v_f^{-1/3}$ for pyruvic acid in excess. Similar plots were obtained for NADH in excess. The values extrapolated to $v_f^{-1/3}$ (infinite flow rate) correspond to K'_m, values of which are listed in Table II. If conformational and environmental factors were unimportant the K'_m values would be close to the Michaelis constants, K^f_m, for the enzyme in free solution. Values for the latter at 25°C are [11]

K^f_m(NADH) = 0.009 mM

K^f_m(pyruvate) = 0.030 mM

The latter value is similar to the K'_m values, but the former is significantly smaller, indicating that attachment to the surface has brought about some change in the enzyme.

Temperature dependence

Since the above evidence suggests that there is considerable diffusional control under many of the conditions we employed, Eqn. 6 should provide a good basis for interpreting the temperature dependence, especially at low [S] and v_f. In this equation only the diffusion coefficient D is temperature dependent, its variation being given to a good approximation by an equation of the Arrhenius form. If there is considerable diffusion control, plots of log v_r against $1/T$ should be linear, and the slopes will be $2E_D/3 \cdot 2.303\,R$, where E_D is the activation energy for diffusion.

Fig. 3 shows Arrhenius plots for variable [NADH], with [pyruvate] held at 3 mM; similar plots were obtained at different flow rates, and with variable [pyruvate]. The calculated activation energies are listed in Table III. Within the experimental error, flow rate has no significant effect on the values. There is a tendency for the activation energies to decrease with increasing substrate concentration, presumably because there is a decreasing degree of diffusion control. A plot of the activation energies against substrate concentration leads to an extrapolated value, at [S] = 0, of about 5.0 kcal · mol^{-1}, which we take to be E_D, the activation energy for the diffusion process.

Plots of the activation energies against 1/[S] lead to an extrapolated value of about 1.0 kcal · mol^{-1} as [S] = ∞. This was the case at different flow rates, and with variable [NADH] and variable [pyruvate]. This limiting value of 1 kcal ·

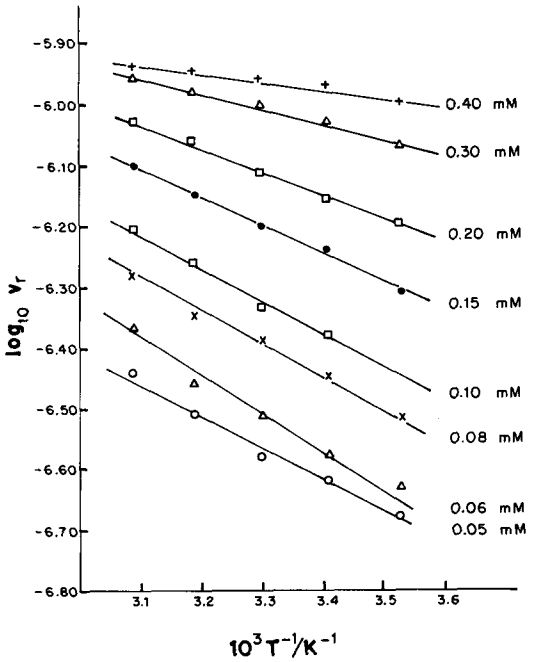

Fig. 3. Arrhenius plots of rate of product formation, with $v_f = 0.36$ cm · s^{-1} and [pyruvate] fixed at 3 mM; the concentrations of NADH are shown.

mol^{-1} is the activation energy corresponding to no diffusion control, and is therefore the value for the purely chemical process occurring at the surface.

Dependence of product concentration on flow rate

Eqns. 5 and 7 give the theoretical product concentrations for the extreme cases of no diffusion control and complete diffusion control. In the former

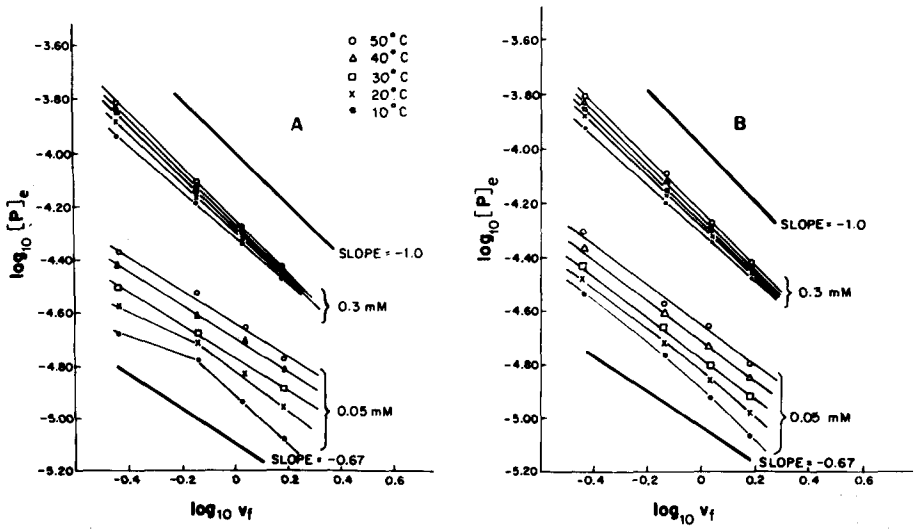

Fig. 4. Double-logarithmic plots of the product concentration at the tube exit, against flow rate. A, Pyruvate in excess at 3 mM; B, NADH in excess at 2 mM.

TABLE III

ACTIVATION ENERGIES (kcal · mol^{-1}) OBTAINED FROM PLOTS OF RATE OF PRODUCT FORMATION

v_f/cm · s^{-1}	[S]/mM	(A) Variable [NADH] fixed [pyruvate]	(B) Variable [pyruvate] fixed [NADH]
		E	*E*
0.36	0.06	4.10	5.08
	0.08	3.95	3.49
	0.10	3.54	2.85
	0.15	2.85	2.28
	0.20	2.85	2.05
	0.30	1.80	1.50
	0.40	0.92	1.00
0.72	0.06	3.43	4.48
	0.08	3.68	3.45
	0.10	3.10	3.42
	0.15	2.53	2.74
	0.20	2.28	1.95
	0.30	1.05	1.84
	0.40	0.68	1.70
1.08	0.06	3.27	4.39
	0.08	2.90	4.10
	0.10	2.85	3.66
	0.15	2.70	2.85
	0.20	1.92	2.26
	0.30	0.88	2.07
	0.40	0.90	1.47
1.50	0.06	3.78	4.75
	0.08	3.42	4.56
	0.10	3.02	3.60
	0.15	2.49	2.84
	0.20	1.95	2.24
	0.30	1.14	1.95
	0.40	0.94	1.00

case, plots of log $[P]_e$ against log v_f will have slopes of -1; in the latter case the slope is -0.67. Such plots are shown in Fig. 4, for a variety of temperatures and substrate concentrations; Fig. 4A is for pyruvic acid in excess, and Fig. 4B for NADH in excess at 2 mM. At the higher substrate concentrations (0.30 mM) the slopes are very close to -1, indicating little diffusion control, while at the lower substrate concentrations the rates are closer to -0.67, indicating considerable diffusion control. These plots again show that there is less diffusion control at the higher flow rates and at the higher temperatures.

Eqn. 7 gives the theoretical product concentration at the tube exit for full diffusion control, and it is of interest to compare the predictions of this equation with results obtained under conditions leading to substantial diffusion control; i.e. low substrate concentrations and flow rates. Table IV gives the results of calculations with D taken to be $4 \cdot 10^{-6}$ $cm^2 \cdot s^{-1}$ at 25°C and with an activation energy for diffusion of 5 kcal · mol^{-1}, as estimated above. Considering that this is an absolute calculation, and that the reactions are not fully diffusion-controlled, the agreement is quite satisfactory.

TABLE IV

VALUES OF $[P]_e$ AT THE TUBE EXIT, CALCULATED USING EQN. 7, COMPARED WITH EXPERIMENTAL VALUES

$r = 0.076$ cm; $L = 50$ cm; $D(25°C) = 4 \cdot 10^{-6}$ cm$^2 \cdot$ s^{-1}; $E_D = 5$ kcal $\cdot$ mol^{-1}.

v_f/cm $\cdot$ s^{-1}	[S] (mM)	T (°C)	[P]calc (μM)	[P]exp (μM)
0.36	0.05	10.0	20.0	29.0 *
0.36	0.05	20.0	24.5	33.4 *
0.36	0.05	30.0	29.6	36.1 *
0.36	0.05	40.0	35.2	43.1 *
0.36	0.05	50.0	41.5	50.0 *
0.72	0.08	10.0	20.1	22.6 *
0.72	0.05	50.0	26.2	26.4 *
0.36	0.05	10.0	20.0	20.9
0.36	0.05	30.0	29.6	31.3
0.72	0.08	10.0	20.1	23.6
0.72	0.08	50.0	41.8	41.6

* With [NADH] fixed at 2 mM and [pyruvate] varied. The unstarred experimental values are for [pyruvate] fixed at 3 mM and [NADH] varied.

Dimensionless parameters

Kobayashi and Laidler [14] showed that a good estimate of the amount of diffusion control is provided by plotting two dimensionless parameters

$$\phi = \frac{[P]_e}{[S]} \left(\frac{v_f r^2}{DL}\right)^{2/3} \tag{9}$$

and

$$\rho = \frac{K_m(app)}{[S]} \tag{10}$$

against each other. A quantity η is a measure of the extent to which the reaction is diffusion-free, and in previous papers [7—11] we have divided the ϕ—ρ plots into three regions; Region 1, in which $\eta > 0.95$, corresponding to essentially diffusion-free kinetics; Region 2, in which $0.95 > \eta > 0.6$, an intermediate region; and Region 3, with $\eta < 0.6$, where there is substantial diffusion control. For present purposes it was more expedient to divide the plots into a larger number of regions, the boundaries between them corresponding to $\eta = 0.10$, 0.40, 0.60, 0.70, 0.80, 0.90 and 0.95.

Figs. 5 and 6 show plots of ϕ against ρ. In calculating ϕ the diffusion coefficient D was taken to be $4 \cdot 10^{-6}$ cm$^2 \cdot$ s^{-1} at 25°C and to vary with temperature with an activation energy of 5.0 kcal $\cdot$ mol^{-1}, as obtained above. Fig. 5 is for [pyruvate] fixed at 3.00 mM and [NADH] varied, and Fig. 6 is for [NADH] fixed at 2.00 mM and [pyruvate] varied. To avoid confusing the diagram only a small selection of values have been plotted, for two temperatures (10°C and 50°C) and two flow rates (0.36 cm $\cdot$ s^{-1} and 1.50 cm $\cdot$ s^{-1}), but the other values show consistent results and lead to the same general conclusions, which may be summarized as follows:

(1) Both diagrams show that at low concentrations of one substrate there is

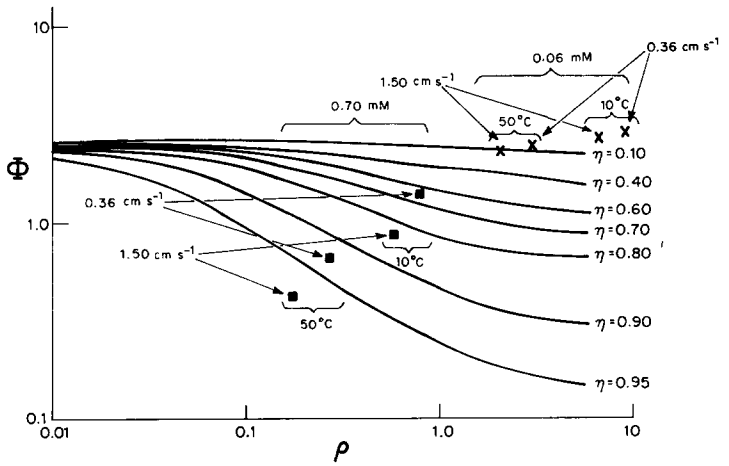

Fig. 5. Double-logarithmic plots of the kinetic parameters ϕ and ρ (Eqns. 9 and 10), for [pyruvate] fixed at 3.00 mM and at two NADH concentrations (0.06 mM (X) and 0.70 mM (■), two temperatures and two flow rates.

very considerable diffusion control, and that there is little effect of temperature and flow rate.

(2) The extent of diffusion control is substantially less at the higher substrate concentrations.

(3) At the higher substrate concentrations the extent of diffusion control is decreased by increasing the flow rate. This is predicted by the theory, and is the behavior found in all systems we have investigated [7—10].

(4) At the higher substrate concentrations, increasing the temperature decreases the extent of diffusion control.

Effect of pH

Rates were measured in a 50 cm nylon tube coated with enzyme, at a

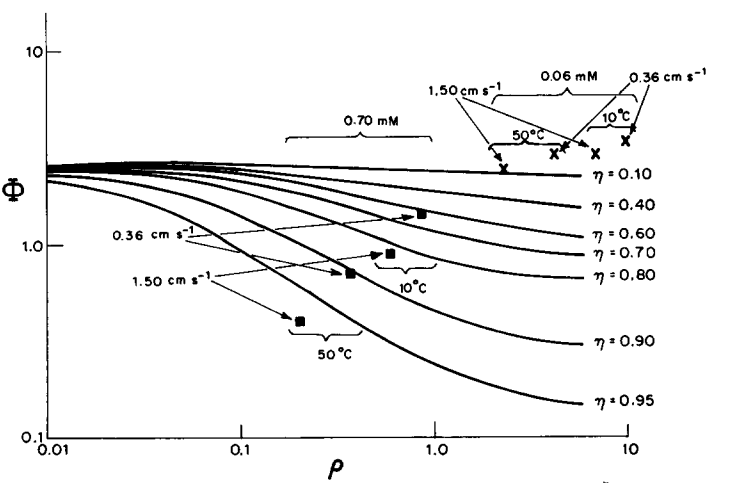

Fig. 6. Similar plots to those in Fig. 5, for [NADH] fixed at 2.0 mM and at two pyruvate concentrations (0.06 mM (X) and 0.70 mM (■)), two temperatures and two flow rates.

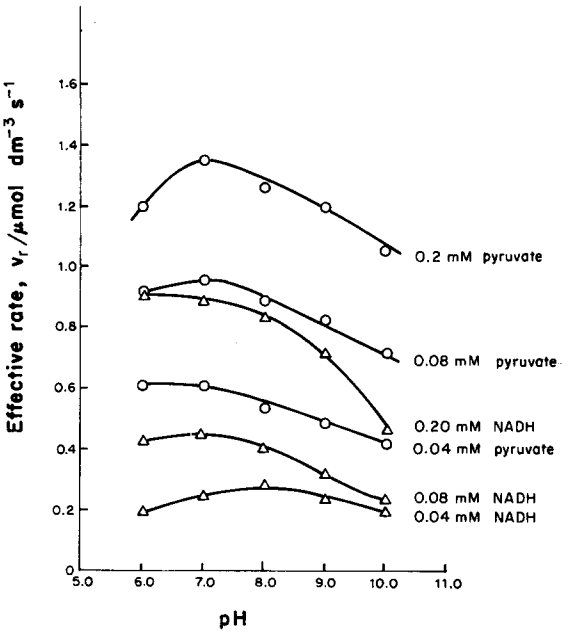

Fig. 7. Plots of rates against pH; T, 35° C.

temperature of 35.0°C and a flow rate of 1.0 cm · s^{-1}. The pH was varied from 6 to 10 by the use of various NaH_2PO_4-K_2HPO_4 and Na_2HPO_4-K_3PO_4 buffer mixtures containing 0.10 mM EDTA and 0.10 mM β-mercaptoethanol, the pH values being determined with a pH meter. The results at various substrate concentrations are shown in Fig. 7. There is a much smaller variation with pH than found by Schwert et al. [21] and Stinson and Holbrook [19] for the free enzyme.

Discussion

In the present work various lines of evidence point to the following general conclusions:

(1) The degree of diffusion control increases as the flow rate is decreased. This is shown by the variation of K_m(app) with flow rate (Fig. 2), which demonstrates the quantitative relationship between K_m(app) and v_f. It is also shown very clearly by the plots of the dimensionless parameters (Figs. 5 and 6).

(2) The degree of diffusion control increases as the substrate concentration is decreased. This is suggested by the curvature of the Lineweaver-Burk plots (Fig. 1), and is shown more clearly by the dependence of product concentration on flow rate (Figs. 4A and B). It is also shown by the plots of the dimensionless parameters (Figs. 5 and 6).

(3) The degree of diffusion control decreases as the temperature is raised. This is shown by the plots of log $[P]_e$ against log v_f (Fig. 4) and of the dimensionless parameters (Figs. 5 and 6). This behavior is expected in view of the fact that the activation energy for the chemical process (~1 kcal · mol^{-1}) is less than that (~5 kcal · mol^{-1}) for the diffusion processes. As the temperature is raised

the rates of diffusion increase to a greater extent than the chemical rates, and there is therefore less diffusion control.

(4) The activation energy for the diffusion processes is approx. 5 kcal · mol^{-1}. This value is obtained directly from the Arrhenius plots (Fig. 3), with extrapolation to zero substrate concentration for which there is full diffusion control. This value of 5 kcal · mol^{-1} also leads to a reasonable interpretation of the temperature-dependence of the product concentrations at the tube exit (Table IV). Also, the use of this value in calculating the dimensionless parameter ϕ leads to self-consistent results (Figs. 5 and 6).

(5) The enzyme has been substantially altered when attached to the surface, as indicated by the difference between the K'_m values (Table II) and those for the free enzyme.

(6) The pH dependence for the tube-supported enzyme (Fig. 7) is much smaller than that for the free enzyme. This may be partly due to the fact that attachment to the surface has modified the enzyme. However, a more significant factor is probably the substantial degree of diffusion control, the diffusion processes being essentially pH-independent.

The present system is one in which the chemical process occurring at the surface is relatively rapid and has a very low activation energy of about 1 kcal · mol^{-1}. It is therefore not difficult to find a range of conditions in which the diffusion processes are slow and rate limiting. Even at the highest substrate concentrations and flow rates there is still very much diffusion control. This system is in sharp contrast to alcohol dehydrogenase (Mazid, M.A. and Laidler, K.J., unpublished data), in which the activation energy for the chemical process is much higher (~10 kcal · mol^{-1}); there is therefore much less diffusion control.

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