Biochimica et Biophysica Acta, 377 (1975) 317—330

BBA 67404

IMMOBILIZED ELECTRIC EEL ACETYLCHOLINESTERASE

II. FLOW KINETICS OF ACETYLCHOLINESTERASE CHEMICALLY ATTACHED TO NYLON TUBING

T.T. NGO and K.J. LAIDLER

Department of Chemistry, University of Ottawa, Ottawa, K1N 6N5 (Canada)

(Received July 16th, 1974)

Summary

Acetylcholinesterase has been attached covalently to the inner surface of nylon tubing. An experimental study has been carried out on the flow kinetics; solutions of acetylthiocholine at various concentrations were passed through tubing at various flow rates, and measurements made of the rates of formation of product. The results were analyzed in the light of the theoretical treatment of Kobayashi and Laidler, four different methods of analysis being employed. It is found that at lower substrate concentrations and flow rates the reactions are largely diffusion controlled. The K_m (app) values are substantially higher than the K_m' value for diffusion-free conditions, but approach it as the flow rate is increased, when the diffusion layer becomes less important. The results are entirely consistent with the Kobayashi—Laidler theory, and provide guidelines for the design of open tubular heterogeneous enzyme reactors, both for industrial and analytic purposes.

Introduction

During recent years there has been particular interest in the attachment of enzymes to the inner surfaces of tubes; such systems are generally known as open tubular heterogeneous enzyme reactors. There are several important applications of such systems. One is in the automated analysis of biochemically-important metabolites, biological fluid being pumped into one end of the tube and an analysis made of the effluent; Hornby and co-workers [1—3] have designed several open tubular heterogeneous enzyme reactors for use in clinical analysis. This procedure is much superior to using the enzyme in free solution, when it cannot conveniently be used over and over again; the enzyme attached

to the tube remains intact and can be used for many analyses, with a large reduction in cost. Another application is in biochemical preparations: enzymes, being highly specific and efficient catalysts, are now commonly used in industrial systems, for example in the manufacture of drugs that are otherwise difficult to synthesize. Sofer et al. [4] have recently synthesized several N-oxide drugs using a hepatic microsomal mixed-function flavoprotein oxidase, covalently attached to the inner surface of nylon tubing; these N-oxides are difficult to prepare by direct chemical synthesis. In addition, enzymes immobilized by attachment to the inner surfaces of tubes are beginning to be used in extracorporeal shunts, to remove undesirable substances from the body. L-Asparaginase, which is used in the treatment of certain tumors, has been immobilized on the inner surface of nylon tubing [5—7] and used in physiological experiments [7]. Such systems have advantages over the direct administration of large amounts of enzyme by injection, because the asparaginase immobilized in a tube is protected against proteolytic degradation and there are no toxic and undesirable immunological effects associated with the injection of free asparaginase. Apart from the practical uses mentioned above, enzyme-tubes can also serve as models for enzyme systems in vivo; for example, many enzymes are attached to the interior walls of blood vessels [8]. It is therefore important to pursue investigations of the kinetics of action of enzymes when they are attached to tubes.

The present studies were done with electric eel acetylcholinesterase attached to the inner surface of nylon tubing. A solution of the substrate acetylthiocholine was caused to flow through the tube, and the kinetics investigated over a range of substrate concentrations and flow rates. The experiments were carried out with reference to a recent theoretical treatment of such flow systems by Kobayashi and Laidler [9]. The theory has also been tested experimentally by Bunting and Laidler [7] with asparaginase attached to nylon tube.

Materials

The enzyme, acetylcholinesterase from electric eel (Type III), was obtained from Sigma Chemical Co. The substrate acetylthiocholine (ASCh) and the chromogenic agent 5,5′-dithiobisnitrobenzoic acid (DTNB) were obtained from Sigma Chemical Co. Glutaraldehyde was obtained from Eastman Organic Chemicals. Type-6 nylon tubing to which the acetylcholinesterase was attached, was obtained from John Tullis, Tullibody, Alloa, Scotland. Dipotassium hydrogen phosphate was obtained from Fisher Scientific Co. Phthalic acid and boric acid were obtained from British Drug Houses, and sodium chloride from Canadian Laboratory Supplies Ltd.

Methods

All solutions used were made up in buffer which consists of 0.15 M NaCl, 2 mM phthalate, 2 mM phosphate and 2 mM borate, pH 7.0.

Attachment of acetylcholinesterase to the interior surface of a nylon tube

The method used for attachment of the acetylcholinesterase was a modifi-

cation of the techniques of Sundaram and Hornby [2] and of Bunting and Laidler [7]. A coil of nylon tubing (Type 6), 1 m long, was placed in a 35°C water bath and its inner surface was partially hydrolyzed by pumping 4.5 M HCl through the tubing for 10 min at a flow rate of 5 ml per min. Hydrolysis was arrested by pumping water for 15 min at a flow rate of 5 ml per min. The coil was then transferred to an ice bath and rinsed with 0.2 M sodium bicarbonate buffer, pH 9.0, for 2 min. The tubing was then perfused with a 5% (v/v) solution of glutaraldehyde in 0.2 M sodium bicarbonate buffer, pH 9.0, for 15 min at a flow rate of 2 ml per min. After the tubing was rinsed with 0.05 M potassium—phosphate buffer, at pH 8.0, it was perfused for 2.5 hours with 1.1 mg of enzyme in the same buffer. Unattached enzyme was removed by perfusion of the tubing at 4°C with 0.2 M, pH 9.0, sodium bicarbonate buffer for 10 min, 0.15 M NaCl for 10 min and finally with distilled water for 10 min each, at a flow rate of 5 ml per min. When not in use, the tube was stored at 4°C in distilled water. The acetylcholinesterase activity of the tubing was determined by measuring the thiocholine produced when solutions of acetylthiocholine were passed through the tubing at a selected flow rate.

Kinetic procedure

Nylon tubing was attached to each end of the enzyme-tube, one end being connected to a peristaltic pump, which in turn was connected by nylon tubing to a microvolume flow cell (1 cm pathlength) obtained from Hellma Co. The other end of the tygon tubing dipped into the thermostatted substrate solution. A Watson—Marlow MHRE 200 pump was used for kinetic runs at high flow rates, while an LKB Varioperplex pump was used for runs at low flow rates. The rate of the reaction was monitored spectrophotometrically by measuring the absorbance change at 412 nm on an Pye Unicam SP 1800 recording spectrophotometer. After each kinetic determination, the enzyme-tube was washed thoroughly with buffer to remove all traces of product, which might have accumulated in the tubing. The kinetic procedure has been described in more detail by Ngo et al. [11].

Analysis of experimental results

A detailed treatment of flow kinetics for an enzyme-tube, with full derivations, has been given by Kobayashi and Laidler [9]. The reaction rate may be limited by the rate of mass transfer of the substrate to the surface of the immobilized enzyme; it may also be limited by the rate of diffusion within the layer of immobilized enzyme. The width of the diffusion boundary layer established at the interior surface of a tube depends on the magnitude of the mass transfer coefficient (k_L), which is a measure of the rate at which substrate diffuses through this layer, in a direction perpendicular to that of substrate flow. The theory leads to the following expression for the mass transfer coefficient:

$$k_L = \text{constant} \times \left(\frac{D^2 v_F}{rL}\right)^{1/3} \quad (1)$$

where D is the diffusion constant for the solute through the solution, v_F is the

flow rate, r is the radius of the tube and L its length. It is to be seen that increasing flow-rate increases the rate of substrate diffusion to the surface; this is because there is a reduction in the effective thickness of the diffusion boundary layer. At sufficiently high flow rates there is therefore no diffusion control; at low flow rates, however, a substantial diffusion layer is set up, and the overall kinetic behavior of the immobilized enzyme is expected to deviate from that which is obtained with the enzyme in free solution.

The theoretical treatment of Sundaram et al. [12] shows that the rate of a reaction catalyzed by immobilized enzyme can be approximated by an equation of the Michaelis—Menten form

$$v = \frac{k_c'[\mathrm{E}]\ [\mathrm{S}]}{K_m(\mathrm{app}) + [\mathrm{S}]} \tag{2}$$

where [S] is the concentration of the substrate in the bulk solution, [E] the concentration of immobilized enzyme, k_c' is a catalytic constant modified by conformational and environmental effects (but not influenced by diffusion), and K_m (app) is an apparent Michaelis constant; it is influenced by conformational, environmental and diffusional effects.

Four methods can be used to analyze and relate the experimental results to the theory.

Method A. The theory [9] shows that at low substrate concentrations ([S] $\ll K_m$ (app)) and low flow rates, the reaction is diffusion controlled; the concentration of product formed at the exit of the tube, $P_{e(D)}$, is given by

$$P_{e(D)} = 2.56\left(\frac{DL}{r^2 v_F}\right)^{2/3} [\mathrm{S}] \tag{3}$$

and the rate is given by

$$v_{(D)} = 8.06(v_F D^2 r^2 L^2)^{1/3} [\mathrm{S}] \tag{4}$$

It is to be noted that Eqns 3 and 4 do not involve the concentration or activity of the enzyme; this is the case if the rates are entirely diffusion controlled. It is assumed that there is enough enzyme at the surface of the tube to convert substrate as fast as it comes into contact with the enzyme.

When the enzyme reaction is very slow (i.e. low [E]) or the mass transfer rate is very high (i.e. low L or high D), the concentration of product formed at the exit of the tube, $P_{e(O)}$, is given by

$$P_{e(O)} = \frac{2L}{r v_F} v_r \tag{5}$$

and the rate is given by

$$v_{(O)} = 2\pi r L v_r \tag{6}$$

where

$$v_r = \frac{k_c'[\mathrm{E}][\mathrm{S}]}{K_m' + [\mathrm{S}]} \tag{7}$$

K'_m is the intrinsic Michaelis constant of the immobilized enzyme which is diffusion-free. It is to be noted that, in contrast to Eqns 3 and 4, Eqns 5 and 6 do involve the concentration or activity of the attached enzyme; this is the case if the rates are diffusion-free. Thus the Lineweaver—Burk plots are straight lines, similar to those for the native enzyme in free solution.

Eqns 3 and 4 permit the product concentrations, and rates, to be calculated and compared with experiment. Also, product concentrations may be plotted against flow rate, as double-logarithmic plots. If the reaction rates are fully diffusion-controlled, Eqn 3 shows that the slope, in the double-logarithmic plot, should be −0.67. If the rates are not diffusion-controlled, Eqn 5 shows that the slope, in the double-logarithmic plot, should be −1.0. The same values of slopes hold for the double logarithmic plots of rates against flow rates. The slope of an actual experimental plot therefore, leads to a qualitative conclusion as to the extent of diffusion control.

Method B. Lineweaver—Burk plots of $1/v$ against $1/[S]$ can give an indication of the extent of diffusion control. At low substrate concentrations and low flow rates (a thick diffusion boundary layer) the Lineweaver—Burk plots are predicted by Eqn 4 to be straight lines passing through the origin. At high substrate concentrations, the reactions are diffusion-free and the Lineweaver—Burk plots are predicted by Eqn 6 to be straight lines similar to those for free enzyme. However, in practice the Lineweaver—Burk plots are obtained using substrate concentrations ranging from low to high values, and the lines obtained are therefore composite, covering diffusion-free and diffusion-controlled behavior. The lines thus tend to be convex to the $1/[S]$ axis, the degree of convexity depending on the extent of diffusion control; the more diffusion control, the more convex are the lines. Regardless of the extent of diffusion control, there is only one value of V', and it is most convenient to determine V' first. From this value K_m (app) can be determined; it is the substrate concentration at which the rate is $V'/2$. The K_m (app) values so determined will not necessarily relate to diffusion-free behavior.

Method C. The theory also predicts that the K_m (app) for enzyme attached to a tube will increase with increasing diffusional control according to the equation

$$K_m\,(\text{app}) = K'_m + \frac{V'}{2k_L} \tag{8}$$

K'_m is the diffusion-free value of the Michaelis constant and V'_m is the maximal rate for the immobilized enzyme. Combination of Eqns 1 and 8 leads to the equation

$$K_m(\text{app}) = K'_m + \text{constant} \times \frac{V'}{2}\left(\frac{rL}{D^2}\right)^{1/3} \times v_F^{-1/3} \tag{9}$$

The K_m (app) values obtained from a Lineweaver—Burk plot (Method B) can then be plotted against $v_F^{-1/3}$; such a plot will yield K'_m as the intercept on the K_m (app) axis; this is the diffusion-free value. The slope can yield the diffusion-free V'.

Method D. The extent of diffusion control on a reaction can also be determined using two dimensionless moduli, defined by Kobayashi and Laidler [9]:

$$\phi = \frac{P_{e(D)}}{[S]}\left(\frac{v_F r^2}{DL}\right)^{2/3} \tag{10}$$

$$\rho = \frac{K_m\,(app)}{[S]} \tag{11}$$

Fig. 1 shows the relationship between ϕ and ρ for simple Michaelis—Menten kinetics. All of the quantities in Eqns 10 and 11 are experimentally obtainable, so that a plot of the data on the diagram indicates whether the rates are diffusion-controlled or not. Region I represents essentially diffusion-free rates (better than 95% free from diffusion); Region 3 represents largely diffusion-controlled rates (60% or more diffusion controlled) and Region 2 represents intermediate behavior between Regions 1 and 2. This diagram may be used once the K_m (app) values have been determined.

Results

The time required for a 41.5 cm acetylcholinesterase tube to attain a steady-state rate of acetylthiocholine hydrolysis is shown in Fig. 2. At the substrate concentrations and the flow rates used, the steady-state rate of hydrolysis was achieved in all cases within 3 min. According to the theory of Sundaram et al. [12], for a membrane, the time (τ) required to come within 10% of the steady-state substrate concentration is $\tau \approx 0.257\ l^2/D$, where l is the membrane thickness (cm) and D is the diffusion coefficient. A similar relationship should apply to the thin film on the inner surface of a tube. If D is $4 \cdot 10^{-6}$ cm^2 $\cdot$ s^{-1} (such a value has previously been found experimentally by Bunting and Laidler [7]) the thickness of the immobilized enzyme layer is calculated to be about 3 μ.

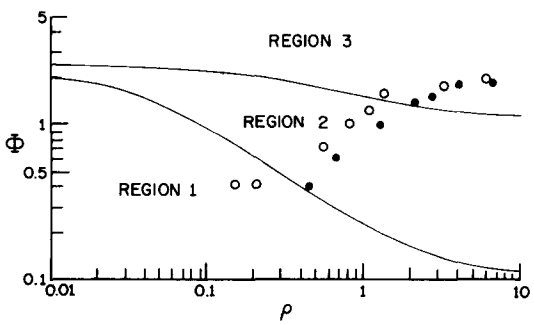

Fig. 1. Double-logarithmic plot of ϕ against ρ. The two lines divide the diagram into three regions corresponding to (1) small, (2) moderate, (3) large diffusional effects. The points are experimental values. Open circles are values obtained with 100 cm tube at a flow rate of 4.24 cm/s and the closed circles are those obtained at a flow rate of 0.45 cm/s.

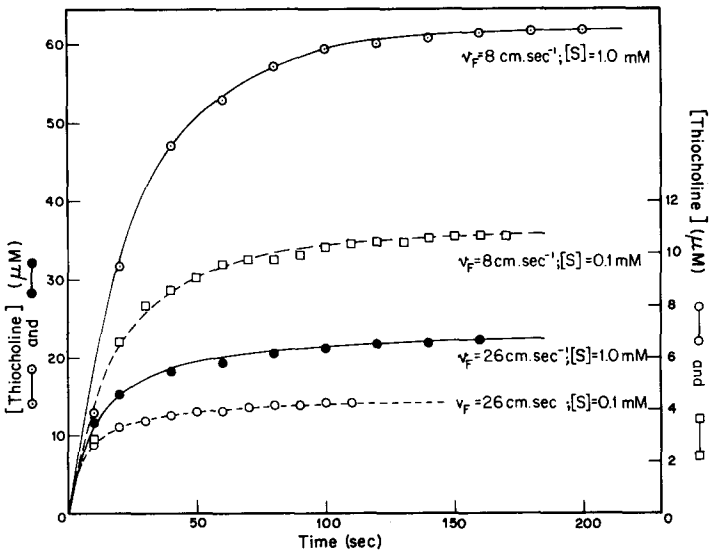

Fig. 2. The time required to attain a steady-state rate of acetylthiocholine hydrolysis by an 41.5 cm acetylcholinesterase-tube. T = 30° C. Flow rate (v_F) and substrate concentrations are indicated.

Table I shows results of experiments carried out with a tube of 10 cm length; it gives the concentration of thiocholine produced at the exit, for various flow rates and substrate concentrations. The numbers in parentheses are the concentrations calculated using Eqn 3; similar results were obtained for a 50 cm tube.

TABLE I

CONCENTRATIONS OF THIOCHOLINE (μM) PRODUCED AT THE EXIT OF 10 CM TUBE AT VARIOUS SUBSTRATE CONCENTRATIONS AND FLOW RATES

The values in parentheses were calculated using Eqn 3 as described in Method A with $D = 4 \cdot 10^{-6}$ cm^2/s, L = 10 cm and r = 0.05 cm.
The values in square brackets are

$$\frac{\text{Experimental value}}{\text{Value calculated using Eqn 4}} \times 100$$

Acetyl-thiocholine (mM)	Flow rate, v_F (cm/s)							
	4.24		1.70		0.70		0.45	
0.125	5.77 (7.76)	[74]	11.31 (14.24)	[79]	22.00 (25.63)	[86]	30.38 (34.56)	[88]
0.300	11.54 (18.62)	[62]	24.62 (34.23)	[72]	50.77 (61.82)	[82]	66.92 (83.10)	[81]
0.500	18.15 (31.04)	[58]	36.92 (57.05)	[65]	73.31 (103.04)	[71]	110.06 (138.49)	[79]
0.750	21.54 (46.56)	[46]	46.46 (85.57)	[54]	95.67 (154.56)	[62]	141.47 (207.74)	[68]
1.00	24.62 (62.08)	[40]	55.69 (114.10)	[49]	121.26 (206.08)	[59]	154.49 (276.99)	[56]
1.50	27.69 (93.12)	[30]	63.85 (171.15)	[37]	139.87 (309.12)	[45]	188.61 (415.48)	[45]
3.00	30.00 (186.24)	[16]	68.46 (342.30)	[20]	151.58 (618.24)	[25]	202.02 (830.96)	[24]
5.00	50.00 (310.40)	[16]	91.54 (570.50)	[16]	165.34 (1043.20)	[16]	211.07 (1384.96)	[15]

TABLE II

RATE OF ACETYLTHIOCHOLINE HYDROLYSIS (μmole/l/s) BY THE 10 CM TUBE AT VARIOUS SUBSTRATE CONCENTRATIONS AND FLOW RATES

The values in parentheses were calculated using Eqn 4 as described in Method A, with $D = 4 \cdot 10^{-6}$ cm^2/s, $L = 10$ cm and $r = 0.05$ cm.
The values in square brackets are

$$\frac{\text{Experimental value}}{\text{Value calculated using Eqn 4}} \times 100$$

Acetyl-thiocholine (mM)	Flow rate, v_F (cm/s)							
	4.24		1.70		0.70		0.45	
0.125	0.192 (0.259)	[74]	0.151 (0.191)	[79]	0.121 (0.142)	[85]	0.107 (0.123)	[87]
0.300	0.385 (0.621)	[62]	0.329 (0.329)	[72]	0.279 (0.341)	[82]	0.237 (0.294)	[81]
0.500	0.605 (1.035)	[58]	0.493 (0.763)	[65]	0.403 (0.568)	[71]	0.389 (0.490)	[79]
0.750	0.718 (1.553)	[46]	0.620 (1.145)	[54]	0.526 (0.852)	[62]	0.500 (0.735)	[68]
1.00	0.821 (2.070)	[40]	0.744 (1.526)	[49]	0.666 (1.136)	[59]	0.546 (0.980)	[56]
1.50	0.923 (3.105)	[30]	0.853 (2.290)	[37]	0.769 (1.704)	[45]	0.666 (1.470)	[45]
3.00	0.999 (6.210)	[16]	0.914 (4.579)	[20]	0.641 (3.407)	[19]	0.714 (2.941)	[24]
5.00	1.66 (10.351)	[16]	1.222 (7.632)	[16]	0.909 (5.679)	[16]	0.746 (4.901)	[15]

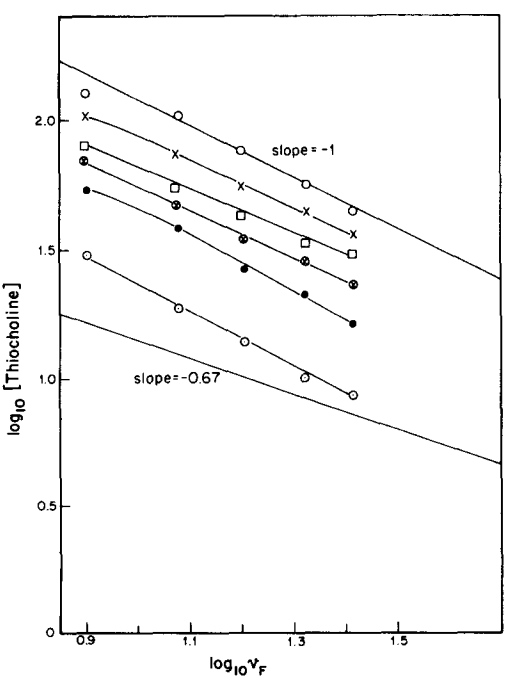

Fig. 3. Double-logarithmic plots of product concentrations against flow rates, for 50 cm tube and for various substrate concentrations. mM; ○, 3.0; X, 1.5; □, 1.0; ⊗, 0.75; ●, 0.5; ⊙, 0.25.

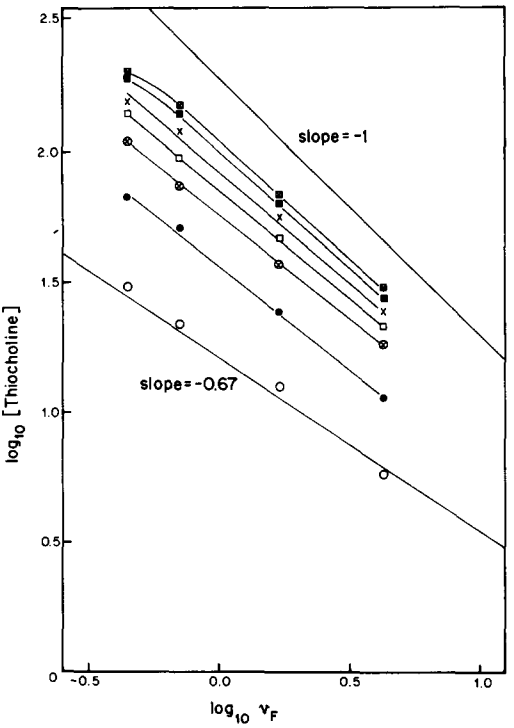

Fig. 4. Double-logarithmic plots of product concentrations against flow rates, for 10 cm tube and for various substrate concentrations mM; ⊠, 3.0; ■, 1.5; X, 1.0; □, 0.75; ⊗, 0.5; ●, 0.3; ○, 0.5.

The results can also be expressed in terms of reaction rates. Table II give the rates at various flow rates and substrate concentrations; the numbers in parentheses are the rates calculated according to Eqn 4.

The extent of diffusion control can readily be seen from double-logarithmic plots of the concentration of product at the exit of the tube, against flow rate. Such plots are shown in Fig. 3 for 50 cm tube and Fig. 4 for the 10 cm tube. As explained for Method A, the slope will be −0.67 for full diffusion control and −1.0 for no diffusion control.

The Lineweaver—Burk plots for the 10 cm tube at various flow rates are shown in Fig. 5. Similar results were obtained for the 50 cm-length tube. At high substrate concentrations all curves approach a common $1/V'$. Using the values of V' obtained from Fig. 5, the values of K_m (app) at various flow rates are calculated. The K_m (app) values were found to increase with decreasing flow rates, as predicted by Eqn 9 described in Method C. Fig. 6 shows plots of K_m (app) against $v_F^{-1/3}$, for the two tube lengths. The intercepts on the K_m (app) axis yield K_m' values of $1.7 \cdot 10^{-4}$ M for the 50 cm tube and $1.8 \cdot 10^{-4}$ M for the 10 cm tube. Both long and short tubes give essentially the same value of K_m'.

Tables III and IV give the calculated values of the parameters ϕ and ρ as defined by Eqns 10 and 11 of Method D. The numbers in parentheses indicate the region in which the system lies. Some of the results with the 10 cm tube are also plotted in Fig. 1 to indicate the extent of diffusion control.

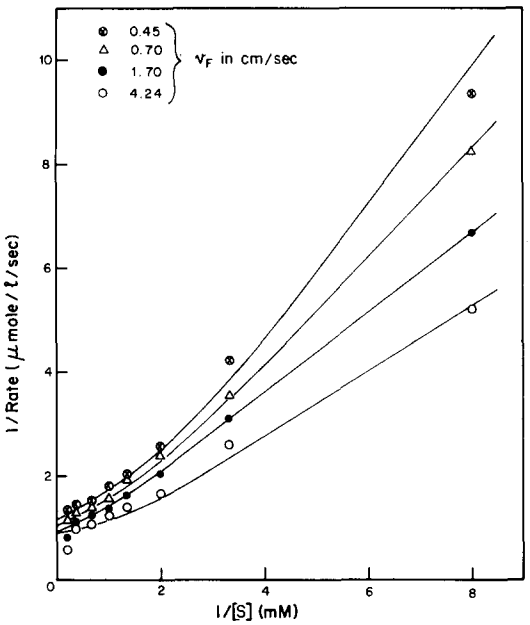

Fig. 5. Lineweaver—Burk plots for 10 cm tube, at various flow rates (cm/s) which are indicated in the graph.

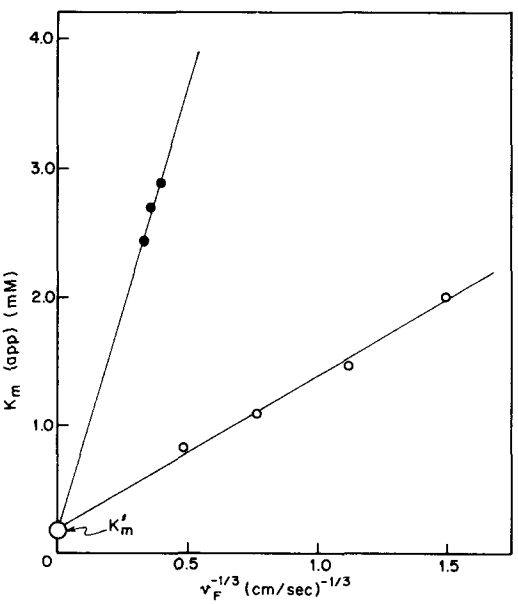

Fig. 6. Plots of the different Michaelis constants, K_m(app), against the flow rate v_F to the power of —1/3, for 10 cm (○) and 50 cm (●) tubes.

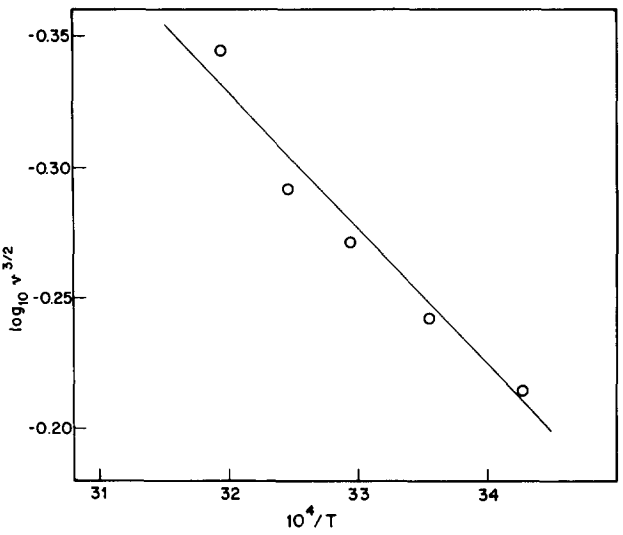

Fig. 7. Arrhenius plot of $\log_{10} v_l^{3/2}$ against $1/T$, to obtain the activation energy for diffusion, E_D. The rate v_l was obtained using 50 cm tube at a flow rate of 8 cm/s and a substrate concentration of 0.05 mM; pH 7.0.

Discussion

The concentrations of thiocholine formed at the exit of the 10 cm tube for various acetylthiocholine concentrations and flow rates of the substrate solution are shown in Table I. The values in parentheses are the concentrations of thiocholine calculated; using Eqn 3. The numbers in square brackets show the closeness of the agreement between experimental and theoretical values; a value

TABLE III

VALUES OF THE PARAMETERS ϕ AND ρ, FOR 10 CM TUBE, AT DIFFERENT SUBSTRATE CONCENTRATIONS AND FLOW RATES. THE NUMBERS IN PARENTHESES INDICATE THE REGION (CF. FIG 1)

Acetyl-thiocholine (mM)	Flow rate, v_F (cm/s)											
	4.24			1.70			0.70			0.45		
	ϕ		ρ	ϕ		ρ	ϕ		ρ	ϕ		ρ
0.125	1.88	(3)	6.25	2.03	(3)	8.80	2.19	(3)	11.84	2.25	(3)	16.00
0.30	1.59	(3)	2.72	1.84	(3)	3.67	2.10	(3)	4.93	2.06	(3)	6.67
0.50	1.50	(3)	1.63	1.66	(3)	2.2	1.82	(3)	2.96	2.04	(3)	4.00
0.75	1.19	(2)	1.09	1.39	(2)	1.47	1.58	(3)	1.97	1.74	(3)	2.67
1.00	1.02	(2)	0.81	1.25	(2)	1.10	1.51	(3)	1.48	1.43	(3)	2.00
1.50	0.76	(2)	0.54	0.96	(2)	0.73	1.16	(2)	0.99	1.16	(2)	1.33
3.00	0.41	(1)	0.22	0.51	(2)	0.37	0.63	(2)	0.49	0.62	(2)	0.67
5.00	0.41	(1)	0.16	0.41	(1)	0.22	0.41	(1)	0.30	0.39	(2)	0.40

TABLE IV

VALUES OF THE PARAMETERS ϕ AND ρ, FOR THE 50 CM TUBE, AT DIFFERENT FLOW RATES AND SUBSTRATE CONCENTRATIONS. THE NUMBERS IN PARENTHESES INDICATE THE REGION (CF. FIG 1).

Acetyl-thiocholine (mM)	Flow rate, v_F (cm/s)					
	26			16		
	ϕ		ρ	ϕ		ρ
0.25	3.17	(3)	9.8	3.24	(3)	11.6
1.50	2.11	(3)	1.63	2.23	(3)	1.93
3.00	1.46	(3)	0.82	1.57	(2)	0.97

of 100 represents complete agreement between experimental and theoretical results. It is clear from Table I that the agreement between experiment and theory is better at low substrate concentrations and low flow rates (the upper right-hand corner of Table I) than under other conditions; not only does the theory predict the correct trend, but it also provides reasonably good absolute values. As the substrate concentrations and flow rates increase, the agreement between experimental values and the theoretical values becomes worse (lower left-hand corner of Table I); this is because Eqn 3 is based on the assumption of full diffusion control of the rates, an assumption which is best satisfied at low flow rates and low substrate concentrations. At higher substrate concentrations the enzymic reaction will no longer be able to keep up with the diffusion processes, and the experimental rates will therefore be lower than the calculated ones. This is well confirmed by the values in square brackets, which are less than 100 in all cases. Eqn 5 should be used instead of Eqn 3 when comparing the theory with the results obtained with high substrate concentrations. However, Eqn 5 requires knowledge of the concentration of active enzyme; this is not available. For certain immobilized enzymes, their concentration can be determined by active-site directed titrant; for example the active concentration of chymotrypsin supported by polyelectrolytes has been determined by active-site titration with N-trans-cinnamoylimidazole [13].

The results can also be expressed as reaction rates and compared with Eqn 4, which is the theoretical equation for the rate which is fully diffusion controlled. Again the agreement between experiment and theory is very satisfactory at low substrate concentrations and low flow rates (the upper right-hand corner of Table II) and less satisfactory for the results at higher substrate concentrations and high flow rates which are substantially diffusion-free.

An alternative way of testing the results for the amount of diffusion control is to make double-logarithmic plots of product formed against flow rate. Such plots are shown in Fig. 3 for the 50 cm tube and in Fig. 4 for the 10 cm tube. Fig. 2 shows that, at the highest substrate concentrations used, the rate is substantially diffusion-free, the slope being approximately -1; Fig. 4 shows that at the lowest substrate concentration used, the rate is fully diffusion-controlled with a slope of -0.67. These plots confirm that there is full

diffusion control for the results at low flow rates and low substrate concentrations, and less diffusion control at higher flow rates and higher substrate concentrations.

The values of K_m (app) were obtained from the Lineweaver—Burk plots (Fig. 5). They were found to increase with decreasing flow rate, as predicted by Eqn 9 and as found in the work of Bunting and Laidler on asparaginase-tube [7]. In theory there is only one value of V' for one enzyme-tube; in practice, however, there is still a little diffusion control on the rate even at the highest substrate concentration used. This is probably why the lines in the Lineweaver —Burk plots do not meet exactly at one point on the 1/rate axis.

The K_m (app) values obtained from Fig. 5 are plotted against $v_F^{-1/3}$ in Fig. 6. The intercepts on the vertical axis yield K_m' values for the 10 cm and 50 cm tubes. It is significant that both tubes give essentially the same intercept ($1.7 - 1.8 \cdot 10^{-4}$ M), and that the values are consistent with the value of $1.6 \cdot 10^{-4}$ M found with acetylcholinesterase immobilized in polyacrylamide membrane (Part II of this series). The K_m value of acetylcholinesterase in free solution is $1.2 \cdot 10^{-4}$ M.

Table III gives the calculated values for the parameters ϕ and ρ, defined by Eqns 10 and 11 for the 10 cm tube. Some of the values from this table were plotted in Fig. 1 to show the region in which the system falls. A few calculated values of the same parameters for the 50 cm tube are shown in Table IV. In both Tables III and IV, the numbers in parentheses indicate the region as defined in Fig. 1. It is to be seen that the majority of the rates fall in region 3, which corresponds to diffusion-controlled rates. Only the results at high substrate concentration and high flow rates fall in region 1 (diffusion-free rates). This conclusion confirms that reached on the basis of the other two methods. The values used for the determination of V' were indeed virtually free from diffusional effects.

In Part I of this series [10] we have explained a method for obtaining the activation energy for the diffusion of the substrate within the membrane, the procedure being to combine the results of temperature studies with thin and thick membranes at low substrate concentration. The activation energy (E_D) for the diffusion of substrate through the diffusion boundary layer can be obtained by plotting $\log {}_{10}v_l^{3/2}$ against $1/T$; such a relationship is obtained from Eqn 4. v_l is the rate of acetylthiocholine hydrolysis at low flow rate and low substrate concentration. The Arrhenius plot of $\log_{10}v_l^{3/2}$ against $1/T$ is shown in Fig. 7, and the value of E_D is 2.4 kcal. This is entirely acceptable for the diffusion of a substrate molecule through the aqueous diffusion layer.

The results presented in Figs. 1—4 strongly support the theoretical treatments of Kobayashi and Laidler [9].

Open tubular heterogeneous enzyme reactors, such as described in this paper, are superior to packed bed enzyme reactors in many ways. They permit an unobstructed flow of substrate solution, and their low pressure drop and favorable hydrodynamic properties render themselves very useful in biomedical and analytical experiments; the problems of pressure drop associated with packed bed enzyme reactors have frequently been pointed out [14—15]. Two basic types of open tubular heterogeneous enzyme reactors can be distinguished; (1) diffusionally controlled and (2) kinetically controlled. For most

industrial preparative work, it is desirable to attach large quantities of enzyme to the tube wall so that the reaction becomes diffusion controlled. In addition to the relatively high concentration of product formed, such reactors have the advantage that their design does not require knowledge of the enzyme-kinetic parameters; this is clearly shown by Eqns 3 and 4, no enzyme term being involved in these equations. Thus Eqns 3 and 4 are very useful guidelines for the design of diffusion-controlled open tubular heterogeneous enzyme reactors.

For analytical work, however, the kinetically controlled type can also be used; with $[S] \ll K_m$ the rate be proportional to the concentration of the substance to be analyzed. Eqns 5 and 6 will be useful for the design of kinetically controlled enzyme reactors. Such reactors are usually prepared by attaching small amounts of enzyme to the tube, so that the rates of substrate diffusion will not be rate limiting. The true Michaelis constant (K_m) of such reactors can be determined using the relationship shown in Eqn 9. Once the values of K_m (app) have been obtained, Eqns 10 and 11 can be used to determine whether or not diffusion control exists under particular experimental conditions.

Acknowledgement

This work was supported by a grant from the National Research Council of Canada.

References

1 Hornby, W.E., Filippusson, H. and McDonald, A. (1970) FEBS (Fed. Eur. Biochem. Soc.) Lett. 9, 8—10
2 Sundaram, P.V. and Hornby, W.E. (1970) FEBS Lett. 10, 325—327
3 Inman, D.J. and Hornby, W.E. (1974) Biochem. J. 137, 25—32
4 Sofer, S.S., Ziegler, D.M. and Popovich, R.P. (1974) Biochem. Biophys. Res. Comm. 57, 183—189
5 Allison, J.P., Davidson, L., Gutierrez-Hartman, A. and Kitto, G.B. (1972) Biochem. Biophys. Res. Comm. 47, 66—73
6 Horvath, C., Sardi, A. and Woods, J.S. (1973), J. Appl. Physiol. 34, 181—187
7 Bunting, P.S. and Laidler, K.J. (1974) Biotechnol. Bioeng. XIV, 119—134
8 Kirk, J.E. Enzymes of the Arterial Wall, Academic Press, New York, 1969
9 Kobayashi, T. and Laidler, K.J. (1974) Biotechnol. Bioeng. XVI, 99—118
10 Ngo, T.T. and Laidler, K.J. (1975) Biochim. Biophys. Acta 377, 303—316
11 Ngo, T.T., Bunting, P.S. and Laidler, K.J. (1974) Can. J. Biochem. (in press)
12 Sundaram, P.V., Tweedale, A. and Laidler, K.J. (1970) Can. J. Chem. 48, 1498—1504
13 Goldstein, L. (1972) Biochem. 11, 4072—4084
14 Mari, T., Sato, T., Tosa, T. and Chibata, I. (1972) Enzymologia, 43, 213—226
15 Tosa, T., Sato, T., Mori, T., Matuo, Y., and Chibata, I. (1973) Biotechnol. Bioeng. XV, 69—84