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The Kinetics of Carboxymethylcellulose–Ficin in Packed Beds

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1. The kinetics of the hydrolysis of benzoylarginine ethyl ester in packed columns of CM-cellulose-70–ficin and CM-cellulose-90–ficin were studied. 2. The apparent Michaelis constant, K'_m, of these preparations was calculated and shown to be dependent on the flow rate at low rates of perfusion through the columns. 3. The values for k_3 of these preparations were calculated and shown to be nearly independent of flow rate. 4. A modified form of the integrated Michaelis rate equation was used to describe the action of these materials and its limitations are discussed. 5. The hydrolysis of solutions of casein by these columns was studied.

Although a variety of enzymes have been linked to chemical polymers to produce insoluble enzyme preparations, the kinetics of these materials when packed into columns have not been extensively studied.

Bar-Eli & Katchalski (1963) gave results for the hydrolysis of L-arginine methyl ester over a limited range of substrate concentrations and flow rates for columns of an insoluble derivative of polytyrosyl-trypsin. Columns of CM-cellulose–trypsin and CM-cellulose–ribonuclease have been used by Epstein & Anfinsen (1962) to study the reversible reduction of disulphide bonds in these enzymes. Some kinetic data have been reported by Nikolaev & Mardashev (1961) for columns of asparaginase adsorbed on CM-cellulose.

The present paper reports results of the kinetic studies on CM-cellulose–ficin preparations packed in columns and the derivation of a relationship fitting these results.

THEORETICAL

An expression relating the amount of substrate that has reacted in a given time in an enzyme-catalysed reaction obeying Michaelis–Menten kinetics can be obtained by integrating the Michaelis–Menten equation:

$$s_0 - s_t = k_3[\mathrm{E}]t + K_m \ln(s_t/s_0) \qquad (1)$$

where s_0 is the initial concentration of substrate, s_t is the substrate concentration after time t, and K_m, k_3 and [E] are the Michaelis constant, the rate constant for the decomposition of the enzyme–substrate complex and the concentration of total enzyme respectively.

Suppose that the reaction mixture may be caused to flow through a column in such a manner that all elements of fluid move through the column at equal velocities and that there is no overtaking. Then a horizontal cross-section of the liquid moves like an imaginary piston in the column and the flow may be referred to as 'piston flow'. Under these conditions, eqn. (1) applies to each infinitesimal cross-sectional piston volume and therefore expresses the total reaction taking place in each such volume during its passage through the column. Then t is the residence time of each fluid element in the column and s_0 and s_t are the concentrations of substrate entering and leaving the column.

If instead of moving with the liquid the enzyme is fixed relative to the column, the situation is unchanged provided that other conditions remain constant and that there is full access of substrate to the enzyme at every point.

The hydrolysis of BAEE* by preparations of ficin attached covalently to CM-cellulose has been shown to obey Michaelis–Menten kinetics in stirred suspensions (Hornby, Lilly & Crook, 1966). Eqn. (1) would therefore be expected to apply to a packed column of such a solid-supported enzyme if the appropriate values of k_3 and the apparent Michaelis constant, K'_m, be substituted. Then, assuming piston flow through the column, the residence time, t, is related to the void volume, V_t, and the flow rate through the column, Q, by:

$$t = V_t/Q \qquad (2)$$

The insoluble enzyme constituting the packed bed may be considered as a suspension of enzyme protein in a volume equal to the total volume of the cylindrical packed bed. Thus:

$$[\mathrm{E}] = \mathrm{E}/V_t \qquad (3)$$

* Abbreviation: BAEE, N^{α}-benzoylarginine ethyl ester hydrochloride.

where E is the total amount of enzyme (in moles) in the packed bed and V_t the total bed volume.

Therefore, from eqns. (1), (2) and (3):

$$(s_0-s_t)-K'_m\ln(s_t/s_0) = k_3(E/Q)(V_l/V_t) \quad (4)$$

Let P be the fraction of the substrate reacted in the column; then:

$$P = (s_0-s_t)/s_0$$

and if β, the voidage of the column, is defined as:

$$\beta = V_l/V_t$$

then substituting β and P in eqn. (4):

$$Ps_0-K'_m\ln(1-P) = k_3E\beta/Q = C/Q \quad (5)$$

where $C=k_3E\beta$ and is referred to as the reaction capacity of the column.

The curve of this equation passes through the point $P=1$ when $Q=0$ and asymptotes to $P=0$ as $Q\to\infty$, as would be expected since these give infinite and zero reaction times respectively. A somewhat less expected property is seen by differentiating P with respect to Q, whence it is found that the curve passes through the point $P=1$, $Q=0$ with zero slope, and that the slope increases rapidly at low values of Q, before decreasing again as the curve asymptotes. These properties are illustrated in the theoretical curves (Fig. 1) drawn for fixed values of C and K'_m and varying values of s_0.

Eqn. (5) may be rearranged:

$$Ps_0 = K'_m\ln(1-P)+C/Q \quad (6)$$

If values of P are measured when various initial concentrations of substrate are perfused through the same column at identical flow rates (i.e. Q is constant), then Ps_0 plotted against $\ln(1-P)$ will give a straight line if K'_m and C are constant at this flow rate. The slope of the line will be equal to K'_m and the intercept on the Ps_0 axis will be equal to C/Q. Thus K'_m and C may be determined for any flow rate of substrate through the column.

The validity of these relationships has been investigated and the results are described in this paper.

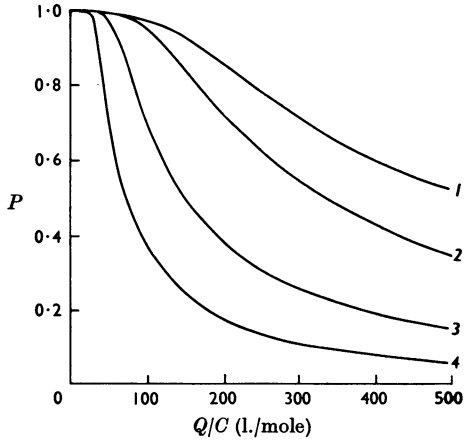

Fig. 1. Theoretical relationships between degree of reaction, P, column reaction capacity, C, and flow rate, Q, for K'_m 2·5 mM. Curves for $s_0=10^{-1}K'_m$ (*1*), $s_0=K'_m$ (*2*), $s_0=4K'_m$ (*3*) and $s_0=10K'_m$ (*4*) are shown.

MATERIALS AND METHODS

Solutions. Phosphate–NaCl solutions, pH 7·0 at 25°, had the following compositions: (1) KH_2PO_4, 12·0 g./l.; NaOH, 2·22 g./l.; NaCl, 11·9 g./l.; NaOH, 0·52 g./l.; NaCl, 2·97 g./l.; overall ionic strength I 0·4; (2) KH_2PO_4, 3·28 g./l.; overall ionic strength I 0·1. Both solutions also contained EDTA (1 mM) and cysteine (1 mM).

Preparation of CM-cellulose–ficin. This was prepared by the chemical attachment of ficin to CM-cellulose by the acid–azide method (Curtius reaction) and characterized as described by Hornby *et al.* (1966). In the present work two products were prepared by using CM-cellulose with ion-exchange capacities of 0·7 m-equiv./g. (Whatman Powder) and 0·9 m-equiv./g. (Mann Research Laboratories Inc., New York, N.Y., U.S.A.). These products were designated CM-cellulose-70–ficin and CM-cellulose-90–ficin. For purposes of calculation the ficin was assumed pure and of mol.wt. 20000.

Preparation of columns. Columns, 1·0 cm. in diameter and of various lengths, were packed by using a slurry of CM-cellulose–ficin suspended in phosphate–NaCl, I 0·4. The columns were surrounded by a jacket through which water at 25° was circulated. All solutions to be perfused through the column were held in a water bath at 25° and pumped into the sealed top of the column by using a peristaltic pump (type 4912A; LKB Produkter AB, Stockholm, Sweden). Phosphate–NaCl, I 0·4, was pumped through each new column until no more settling occurred. For use at lower ionic strengths, the column was then equilibrated with the appropriate buffer.

Flow rates were determined by collection of the column effluent and the dry weight of the bed was determined at the conclusion of each experiment by thoroughly washing the bed material with water, evaporating to dryness and weighing.

Determination of column flow characteristics. The pattern of fluid flow through a column can be observed in the following way. Under steady conditions the composition of the inlet stream is suddenly changed to produce an observable interface in the liquid and the composition of the effluent is continuously measured. If piston flow occurs, an abrupt change will appear in the effluent after a time equal to the residence time of the fluid in the column. If the change is spread over a period of time it will indicate relative fluid motion within the column and some degree of mixing. A plot of effluent composition against time (or effluent volume) is known as an F diagram (Danckwerts, 1953).

After equilibration of a column by perfusion with phosphate–NaCl, I 0·4, the perfusate was changed to one containing N^{α}-benzoylarginine and the appearance of this material in the column effluent was measured. Because the flow through the column approximated closely to piston flow (see below), changing the flow rate resulted in the establishment of a new steady state after the passage of 1 void volume of liquid through the column. Once the new steady state had been reached, the new degree of hydrolysis remained constant over long periods. The column

effluent was monitored continuously by passing the column effluent through a flow cell of 1 cm. light-path in the spectrophotometer.

Determination of enzymic activities. The esterase activity was determined by following the hydrolysis of BAEE (Koch–Light Laboratories Ltd., Colnbrook, Bucks.) in phosphate–NaCl, I 0·4. This was done spectrophotometrically by comparison of $E_{251\,m\mu}$ of the effluent with that of the perfusate in a Beckman model DB spectrophotometer (Schwert & Takenaka, 1955). On this instrument the maximum difference in extinction between 1 mM solutions of N^{α}-benzoylarginine (Koch–Light Laboratories Ltd.) and BAEE in phosphate–NaCl, I 0·4, was 1·05 at 251 mμ for a 1 cm. light-path.

The proteolytic activity was determined by the hydrolysis of solutions of casein by the method of Kunitz (1946).

RESULTS

Determination of column flow characteristics. The behaviour of solutions of BAEE and casein when passed through columns of CM-cellulose-90 was determined. It was found that, under the conditions used for the study of enzyme activities, BAEE and casein were not adsorbed and were completely recovered in the effluent.

The flow characteristics for a column of CM-cellulose-90–ficin (4·0 cm. × 1·0 cm. diam.) are shown in the form of an F diagram in Fig. 2. The void volume, V_l, was found to be 2·65 ml. and the voidage, β, was calculated as 0·75. From the F curve it is possible to determine the deviation from piston flow in terms of the holdback, H, which is the ratio of the area under the F curve between $V=0$ and $V=V_l$ to the total area between these limits and the zero and maximal values of $E_{251\,m\mu}$. The value for H was found to be 0·005 and it was calculated that 95% of the substrate in the perfusate has a residence time within 3% of the mean residence time. As the value of H for ideal piston flow is zero, the flow through the column approximates to piston flow. Similar results for voidage and holdback were obtained over a range of flow rates.

Effect of flow rate on the hydrolysis of BAEE. A column (4·0 cm. × 1·0 cm. diam.) was packed with 650 mg. of CM-cellulose-70–ficin containing 3·8% of protein having K'_m 2·5 mM and k_3 0·16 mole of BAEE hydrolysed/sec./mole of bound ficin when measured in a stirred suspension (Hornby *et al.* 1966). The effect of flow rate on the hydrolysis of solutions of BAEE by this column is shown in Fig. 3.

The corresponding results for a column (4·0 cm. × 1·0 cm. diam.) of 670 mg. of CM-cellulose-90–ficin containing 4·2% of protein (K'_m 2·5 mM and k_3 0·18 mole of BAEE hydrolysed/sec./mole of bound ficin) are shown in Fig. 4.

Determination of K'_m and k_3. These parameters were determined for the hydrolysis of BAEE by columns of CM-cellulose-70–ficin and CM-cellulose-90–ficin at various flow rates by the use of eqn. (6). The relationship between Ps_0 and $\log(1-P)$ for I 0·4 are shown in Figs. 5 and 6. Similar results were obtained with CM-cellulose-90–ficin at I 0·1. It is clear that good straight lines can be drawn through the points of constant flow rate. The slopes increase as the flow rate decreases. The

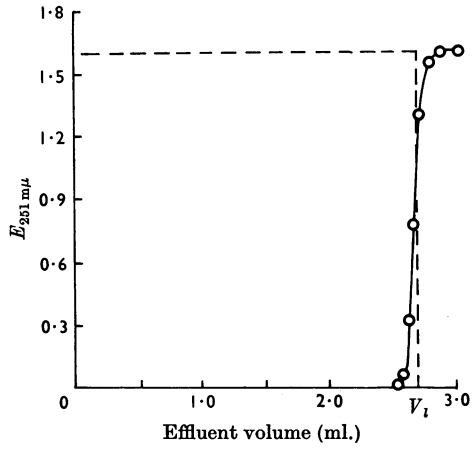

Fig. 2. F diagram showing flow characteristics of a column of CM-cellulose-90–ficin as determined by measurement of the appearance of N^{α}-benzoylarginine in the effluent after introduction of this compound into the column perfusate. Experimental details are given in the text.

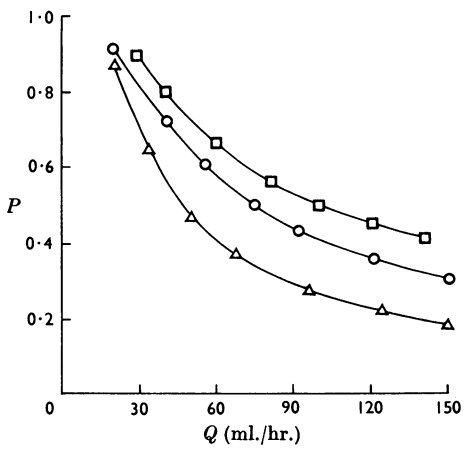

Fig. 3. Relation between degree of hydrolysis, P, of BAEE and flow rate, Q, for a column of CM-cellulose-70–ficin with initial substrate concentrations 0·5 mM (□), 2·5 mM (○) and 10 mM (△) in phosphate–NaCl, I 0·4. Experimental details are given in the text.

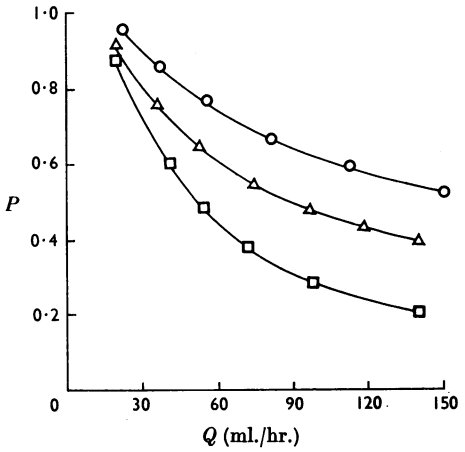

Fig. 4. Relation between degree of hydrolysis, P, of BAEE and flow rate, Q, for a column of CM-cellulose-90–ficin with initial substrate concentrations 0·5 mM (○), 5 mM (△) and 15 mM (□) in phosphate–NaCl, I 0·4. Experimental details are given in the text.

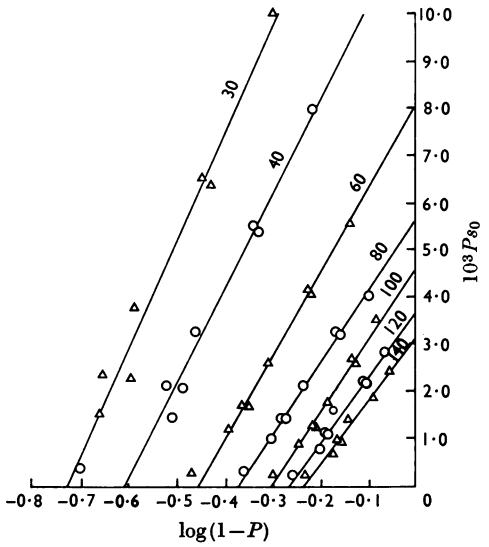

Fig. 5. Relationship between Ps_0 and $\log(1-P)$ for the hydrolysis of BAEE in phosphate–NaCl, I 0·4, by a column of CM-cellulose-70–ficin. Details are given in the text. The numbers on the curves indicate the flow rates, Q, in ml./hr.

values of K'_m at different flow rates obtained from these results are shown in Fig. 7. The values of the column reaction capacities obtained from Figs. 5 and 6 were found to be almost independent of flow rate and are given in Table 1.

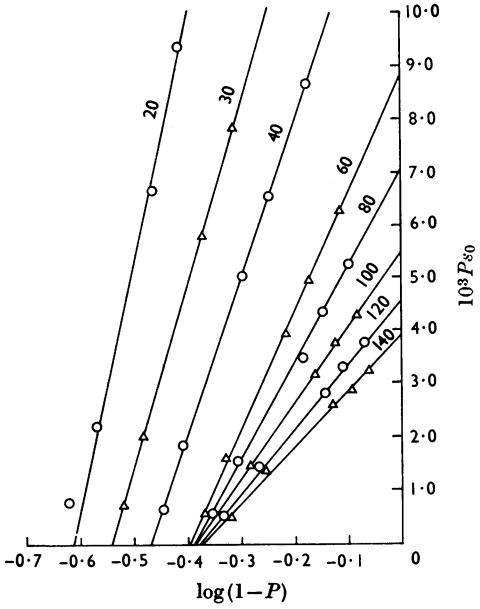

Fig. 6. Relationship between Ps_0 and $\log(1-P)$ for the hydrolysis of BAEE in phosphate–NaCl, I 0·4, by a column of CM-cellulose-90–ficin. Details are given in the text. The numbers on the curves indicate the flow rates, Q, in ml./hr.

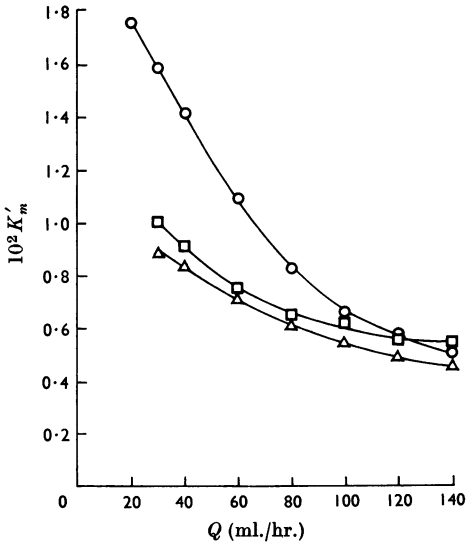

Fig. 7. Effect of flow rate, Q, on K'_m of a column of CM-cellulose-90–ficin for BAEE in phosphate–NaCl, I 0·4 (○), and phosphate–NaCl, I 0·1 (△), and of a column of CM-cellulose-70–ficin for BAEE in phosphate–NaCl, I 0·4 (□). Details are given in the text.

By using the previously defined values for the voidage and the amount of bound ficin in each column, values of k_3 were calculated and are shown in Table 2.

Effect of ionic strength on K'_m. The variation of K'_m with flow rate for the hydrolysis of BAEE by a column of CM-cellulose-90–ficin was investigated at a lower ionic strength. These results are also shown in Fig. 7 and Table 1.

Effect of flow rate on the hydrolysis of casein. The effect of flow rate on the hydrolysis of 0·5% and 2·0% (w/v) solutions of casein in mM-cysteine–mM-EDTA–0·1 M-sodium chloride–0·1 M-phosphate buffer, pH 7·0, by a column (4·0 cm. × 1·0 cm. diam.) packed with 650 mg. of CM-cellulose-90–ficin is shown in Fig. 8.

Table 1. *Effect of flow rate, Q, on column reaction capacity, C*

Experimental details are given in the text.

	10^7C (moles/sec.)		
	CM-cellulose–70-ficin column	CM-cellulose-90–ficin column	
Q (ml./hr.)	*I* 0·4	*I* 0·4	*I* 0·1
30	1·33	1·60	1·57
40	1·34	1·62	1·68
60	1·31	1·53	1·74
80	1·25	1·57	1·71
100	1·28	1·53	1·67
120	1·18	1·57	1·70
140	1·23	1·54	1·72
Average	1·28	1·57	1·68

DISCUSSION

By using the step-response technique it has been shown that the flow pattern through columns of CM-cellulose–ficin corresponds closely to piston flow over the range of flow rates used in the kinetic experiments. It has therefore been possible to test the validity of eqn. (5).

Figs. 3, 4 and 8 show that the experimental curves of P against flow rate are of the general shape expected from eqn. (5) (Fig. 1). Although

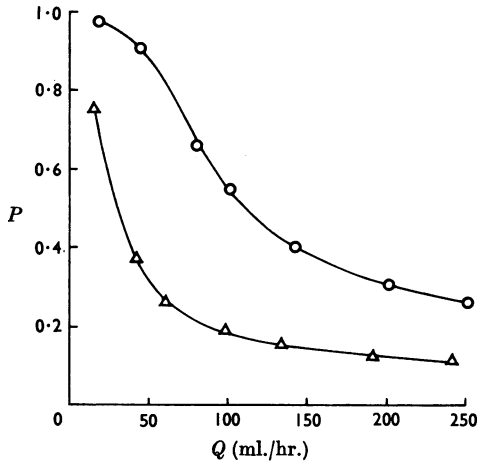

Fig. 8. Relation between degree of hydrolysis, P, of casein and flow rate, Q, for a column of CM-cellulose-90–ficin with initial casein concentrations 2% (w/v) (△) and 0·5% (w/v) (○) in mM-cysteine–mM-EDTA–0·1 M-NaCl–0·1 M-phosphate buffer, pH 7·0.

Table 2. *Values of the Michaelis constant and rate constant for the breakdown of the Michaelis complex for BAEE hydrolysed by ficin in free solution or attached to CM-cellulose*

	K'_m (mM)		k_3 (mole/sec./mole of ficin)	
Conditions	*I* 0·55	*I* 0·15	*I* 0·15	
Free solution	—	20·0	1·83	
Stirred suspension				
CM-cellulose-70–ficin	—	2·5	0·16	
CM-cellulose-90–ficin	7·0	2·5	0·18	
	I 0·4	*I* 0·1	*I* 0·4	*I* 0·1
Column				
CM-cellulose-70–ficin				
Flow: 30 ml./hr.	10·0	—		
Flow: 140 ml./hr.	5·4	—	0·14	—
Flow: very fast	5·2 (approx.)	—		
CM-cellulose-90–ficin				
Flow: 30 ml./hr.	15·9	9·0		
Flow: 140 ml./hr.	4·9	4·5	0·15	0·16
Flow: very fast	3·5 (approx.)	3·5		

the expected decrease in slope at low flow rates was not generally observed under the present experimental conditions it can be seen clearly in Fig. 8 because of the higher column reaction capacity for casein.

If eqn. (5) were to be applicable under all conditions, the parameters K'_m and C would have to be independent of flow rate, Q, and of initial substrate concentration, s_0. Although in these experiments there is no systematic effect of s_0 discernible, Figs. 5, 6 and 7 and Table 2 show that the flow rate can have a pronounced effect on K'_m. In all series this parameter increases at low flow rates by an amount depending on the ionic strength of the perfusate and on the degree of substitution of the CM-cellulose. In all experiments, K'_m showed signs of asymptoting towards a minimal value at high flow rates, but, in general, this value was higher than that observed under comparable conditions in stirred suspensions.

Also, Table 1 shows that, although values of C are nearly constant, there are some signs of a trend at high ionic strengths. Preliminary results suggest that at very low flow rates there is a tendency for C to rise, i.e. for k_3 to increase. Unfortunately, the present experimental system does not provide sufficiently accurate information to investigate this in detail.

The values of K'_m and k_3 obtained from the present experiments are collected in Table 2 and compared with those for the same preparations in stirred suspensions (Hornby *et al.* 1966). This shows that the apparent K'_m in columns was nearly always higher than in stirred suspensions. The only exception was for CM-cellulose-90–ficin at high ionic strengths. As the flow rate increased, K'_m decreased and tended to that value of K'_m obtained in stirred suspensions. This dependence of K'_m on flow rate at low flow rates may be explained by regarding each CM-cellulose–ficin particle as being surrounded by a diffusion layer (Nernst, 1904). The transfer of substrate through this diffusion layer will be inversely related to the thickness of the layer. Further, the effective thickness of the diffusion layer is inversely related to the flow of solution past the CM-cellulose–ficin particle (Glueckauf, 1955). This would mean that the rate of diffusion of substrate to the enzyme would be dependent on flow rate. At lower flow rates there will therefore be a significant difference between the concentration of substrate in the bulk of the solution and the concentration at the active site of the CM-cellulose–enzyme particle, which would be manifested in an elevation of the apparent K'_m.

The values of k_3 for CM-cellulose–ficin in a column are less than those previously reported for the same preparations in stirred suspensions, although CM-cellulose-90–ficin approaches that value at low ionic strengths. This apparent loss in activity may be due to the inaccessibility of some of the ficin molecules to substrate molecules because of the much closer proximity of the cellulose fibres inherent in the packing of the column. This could give rise to stagnant interfibrous regions that afford a barrier to the accessibility of the substrate to those enzyme molecules located within this zone, resulting in a decrease in the effective concentration of enzyme.

Values of K'_m for the hydrolysis of BAEE by columns of CM-cellulose-90–ficin were shown to increase with ionic strength. This dependence of K'_m on ionic strength has been described elsewhere (Goldstein, Levin & Katchalski, 1964; Hornby *et al.* 1966).

This work indicates that the apparent K_m, i.e. K'_m, for an insoluble enzyme preparation is dependent on factors affecting the rate of diffusion of substrate into the vicinity of the active site of the enzyme. Therefore, for such materials, any conclusions arrived at from measurement of K'_m can only be made when the system is not diffusion-limited.

Thus eqn. (5) describes satisfactorily the action of an insoluble enzyme preparation in a packed-bed assembly so far as the parameter C is concerned. The parameter K'_m varies with flow rate, but provided that the correct value is used the equation expresses the relationship between the initial substrate concentration and the degree of reaction in the column at a given flow rate.

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