Biochimica et Biophysica Acta, 485 (1977) 367—378

BBA 68299

EFFECTS OF CARRIER MORPHOLOGY AND BUFFER DIFFUSION ON THE EXPRESSION OF ENZYMATIC ACTIVITY

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(Received February 25th, 1977)
(Revised manuscript received July 11th, 1977)

Summary

A very stable esterase (EC 3.1.1.—), which hydrolyses ethyl acetate, cephalosporin C and other acetyl esters with a maximum turnover number of $3 \cdot 10^2$ s^{-1}, was isolated from *Bacillus subtilis* ATCC 6633 and immobilized on two supports: controlled-pore glass and powdered brick, a representative of carriers having a wide pore-size distribution. Carrier morphology determines diffusion rates and the expression of activity. Rate-limiting mass transfer of buffer leads to apparent losses of activity, gross distortions of molecular pH vs. activity profiles and to apparent deviations from Michaelis-Menten kinetics.

Introduction

A highly stable and non-specific esterase (EC 3.1.1.—) from *Bacillus subtilis* ATCC 6633 has been used in recent studies of enzyme carriers [1] and of the effects of buffer diffusion on the expression of catalytic activity [2]. Earlier, cells of the organism and crude preparations of the esterase were reported by Nüesch and coworkers [3] to deacetylate cephalosporin C.

The present work describes the isolation and characterization of the intracellular protein which has a number of properties in common with the intracellular esterase isolated by Higerd and coworkers [4,5] and the crude extracellular enzyme obtained by Abbot and Fukuda [6,7] from other strains of *B. subtilis*. There are however large differences between the pH vs. activity profiles and, in the case of the intracellular esterase, values of K_m and V/mg protein for ethyl acetate differ from ours by orders of magnitude.

In connection with work relating to the immobilized esterase, and with studies of its stabilization which are in progress, we report here the values of K_m and V for seven different substrates, as well as data relating to the stability of the enzyme and its activity in dilute buffers, solvent/water mixtures and in concentrated salt solutions. Some properties of the conjugates obtained by

coupling the protein to controlled-pore glass and brick powder are also described.

The two supports were chosen for practical and theoretical reasons. Both materials have high dimensional stability, which is essential in many preparative and analytical applications. Owing to the large, uniform pores of the glass and its large internal surface (82 m^2/g) enzyme conjugates with a very high activity per unit carrier volume can be prepared [1,8]. Such immobilized enzymes make it possible to outpace completely non-enzymatic side reactions, such as hydrolysis of the β-lactam ring of cephalosporins [9], without running into the problem of awkward or impossible catalyst volumes. Apart from these considerations, dimensionally stable particles permit the use of high flow rates in the measurement of activity in a differential recirculation reactor [10], with the advantage that variable effects of interfacial mass transfer on the results are largely eliminated.

Brick particles are representative of a wide class of carriers which, like pumice and many macroporous resins, have a relatively small internal surface and a wide pore-size distribution. It is a matter both of theoretical and practical interest to know how these differences in morphology effect binding capacity and the expression of activity.

Experimental

Materials

The sources and purities of the enzyme substrates were as follows: 2-methoxyethyl acetate (Aldrich, purity 100%), ethyl acetate (Fluka, puriss., 100%), Cephalothin ("Keflin", E. Lilly Co.), *p*-nitrophenyl acetate (Merck, "für biochemische Zwecke"); 7-aminocephalosporanic acid (93%), cephalosporin C (92%, with 2% deacetyl cephalosporin C and 4% water), Celospor (99%) and Boc-cephaloglycin (>95% presumably), all sodium salts, were obtained from the CIBA-Geigy research laboratories. They were assayed by high-pressure liquid chromatography [9]; cephalosporin C and Celospor, dissolved in 0.5 M KCl/7.5 mM Tris · HCl, pH 8, were also assayed by pH-stat titration utilizing the immobilized esterase. Polyethylene imine (mol. wt. 60 000) was obtained from the Dow Chemical Co., Glutaraldehyde (25%) from Fluka. All other materials were of analytical reagent grade.

Pure esterase (specific activity 120 units/mg protein) was used in all work except in coupling to brick powder, a preparation with a specific activity of 107 being employed in that case. The zirconia-clad alkylamino controlled-pore glass (550-Å pores, internal surface 82 m^2/g, packing density 0.46 g/ml, internal volume 1.2 ml/g, 0.2—0.8 mm particles, 0.14 mE of NH_2/g) was purchased from the Pierce Chemical Co. Red brick powder, stirred in 10% nitric acid until the evolution of carbon dioxide ceased, was washed, dried, sieved and then derivatized as described below. Its internal surface was 2 m^2/g, internal volume 0.6 ml/g, packing density (0.4—0.5 mm particles) 0.95 g/ml.

Activity measurements

Enzyme was assayed by the pH-stat method in 100 mM NaCl/10 mM sodium phosphate pH 8.0 at 25.0 ± 0.2°C under nitrogen, with 1% (85 mM)

2-methoxyethyl acetate as substrate. Units are expressed in μmol/min. Protein concen. (c) is based on the equation:

$$c \text{ (mg/ml)} = 1.55\, A_{280} - 0.76\, A_{260}$$

[11] where A_{280} and A_{260} are absorbances measured in a 1-cm cell.

Michaelis-Menten constants were determined in the manner described [12] the substrate compensation method being used to obtain linear titration curves at low concentrations of 2-methoxyethyl acetate. Hydrolysis of *p*-nitrophenyl acetate was followed spectrophotometrically at 405 nm, the reaction being started by adding 0.3 ml of substrate in acetonitrile to 2.7 ml of enzyme solution in pH 6.9 phosphate buffer.

All values are corrected for the spontaneous rates of hydrolysis in the alkaline range. Rates of base catalyzed hydrolysis of 2-methoxyethyl acetate as a function of pH have been reported earlier [2].

Activities of the immobilized preparations were determined in the differential recirculation reactor described by Ford and coworkers [10], also by pH-stat titration. The catalyst bed (0.2—1 g dry carrier) had a cross-section of 0.8 cm^2 and the flow rate used was 10 l/h in most of the work with controlled pore glass and 20 l/h with the brick particles. Reaction rates were insensitive to flow rates under the experimental conditions.

Molecular weights

The molecular weight of the esterase was determined by Dr. R. Fischer by gel chromatography on Sephadex G-200 with the following reference proteins: tetramer, trimer and dimer of γ-globulin, aldolase and the dimer and monomer of serum albumin. Molecular weight of the subunit was determined in the laboratory of Professor H. Zuber at the Eidgenössische Technische Hochschule, Zürich by SDS polyacrylamide gel electrophoresis [13] with albumin, horse liver alcohol dehydrogenase, lactate dehydrogenase mesophile and thermophile from B. stearothermophilus and carbonic anhydrase as reference materials.

Immobilization

Controlled-pore glass was derivatized with glutaraldehyde, washed well with distilled water and then used for coupling, all operations being carried out at room temperature, essentially as described by Weetall and Filbert [14]. The quantity of carrier in the coupling mixture was 35—50% v/v. After intermittent gentle agitation for 2—3 h, the mixture was allowed to stand at room temperature overnight. The catalyst was then washed with 1 M NaCl/10 mM phosphate pH 8 for several hours in the recirculation reactor, free activity in the washings being assayed. Brick powder was derivatized by the method of Royer [15]. 1 g of carrier was agitated gently with 32 ml of 0.5% polyethylene imine/HCl pH 8 for 1.5 h, washed four times with 100 ml water and then treated with 13 ml of 2.3% glutaraldehyde for 2 h, and finally washed with water. Coupling, in 25 mM phosphate pH 7, was carried out as described above. For satisfactory derivatization and coupling it is essential [14] to displace all air from the pores. Concentrations of enzyme in the carriers and activities refer to the dry weights of the supports.

Cell culture

The cells, supplied by Mr. J. Auden, were grown at 37°C in a 2500-l fermenter (3-blade turbine stirrer, diameter 595 mm, stirring rate 180 rev./min, head pressure 1 atm., aeration rate 1 l/min per l broth) on a medium containing 0.25% peptone C, 0.25% meat extract and 0.3% sodium acetate. The broth was inoculated with 30 l of culture grown under similar conditions but without sodium acetate. The cells were harvested after about 12 h when the esterase titer of the whole broth (100—125 units/l) did not increase further with time.

Isolation of enzyme

A lyophilisate of crude enzyme with a specific activity of 1 unit/mg protein was prepared as follows on a semitechnical scale. The cells were concentrated in an ALFA-Laval separator type BRPX-207 at 6—14°C. The concentrate (50 l) was extracted twice with 100 l acetone and then dried in vacuo at 30—35°C giving 6 kg solid containing 290 000 enzyme units. A suspension of the cells in 35 l 10 mM phosphate pH 8 was disintegrated by 2 passages through the Dyno mill type KD 5 and centrifuged. The diluted solution was then stirred with DEAE-cellulose (3 kg dry weight/10^5 units). The adsorbate was washed with 40 mM phosphate pH 8 and the enzyme desorbed by stirring with 400 mM phosphate pH 8. This was followed by diafiltration at pH 7 (DDS-Modul Type 20-0.72-LAB, membrane type 500) to reduce the buffer concentration and remove impurities with molecular weights of 20 000 or less. The solution was then lyophilized. The overall yield was 64%.

Esterase with a specific activity of 107 units/mg protein was obtained from this material in 75% yield by the following three steps, the first two carried out at room temperature, and the last at 4°C. Polyethylene imine (130 g of a 10% w/w solution adjusted to pH 7 with HCl) was added to 23 600 units of esterase in 1 l 10 mM phosphate, pH 7, and the solution was centrifuged. The enzyme precipitated quantitatively when 70 g of the polyethylene imine solution were subsequently added to the clear supernatant. The product was taken up in 1 l of 100 mM phosphate and subjected to fractional precipitation with solid ammonium sulfate at pH 8, the esterase precipitating in 80% yield when the salt concentration was raised from 36 to 42% w/w. After diafiltration on an Amicon filter XM-100 with 10 mM phosphate, pH 7.5, the enzyme was adsorbed at 4°C on a 40 ml column of DEAE cellulose. The column was washed with the same buffer to elute remaining traces of polyethylene imine, and a linear potassium phosphate gradient was then applied. The esterase, emerging as the main peak with 100 mM buffer, was lyophilized and stored at −20°C.

Pure protein, showing only one band in polyacrylamide gel electrophoresis (gel system N. 1a (pH 8.9, 7%) of Maurer [16]) had been obtained in earlier work from the crude lyophilisate by ammonium sulfate precipitation, chromatography on DEAE cellulose, chromatography on DEAE-Sephadex A-50 (elution with a linear gradient of NaCl in 60 mM Tris · HCl pH 8, activity emerging with 200 mM NaCl), and finally chromatography on Sephadex G-150. Specific activity of the pure product was 122 units/mg.

Physical properties and stability

The molecular weight of the enzyme, determined by gel chromatography

was about 150 000; SDS electrophoresis showed only one band with a molecular weight of 39 000. Thus the esterase is composed of four equal subunits. Its isoelectric pH was 4.7. The ultraviolet spectrum showed no unusual features, the absorbances at 280 nm (maximum), 260, 250 (minimum) and 240 nm being in the ratio 1.00 : 0.52 : 0.36 : 1.07.

The half life of the enzyme in 100 mM NaCl/10 mM phosphate pH 8 was about 2500 h at 50°C, and 70, 4 and 0.3 h at 60, 65 and 68°C respectively. In acetate buffer pH 6.0, 5.5, and 5.0 the losses of activity in 48 h at 25°C were 0, 0.2 and 30%, respectively. Only small losses were observed in a period of 1 month at room temperature in phosphate buffer pH 8 containing 40% v/v glycerol, dimethyl formamide or ethanol. The first two solvents (30% v/v) reduced activity by 25%, ethanol by 50% in relation to the value in water.

The Arrhenius plot of data for the hydrolysis of 85 mM 2-methoxyethyl acetate in 10 mM phosphate pH 8 was linear in the 15—35°C temperature range, the activation energy of the reaction being 7.6 kcal.

The immobilized preparations, which remained fully active for many weeks when stored under 100 mM phosphate pH 7 at 4°C, displayed satisfactory operational stability at 25°C even under conditions of drastic mechanical wear. The controlled pore glass conjugate containing 250 units of enzyme/g carrier, for example, retained 72% of its initial activity after 90 successive runs involving quantitative deacetylation of Celospor in a recirculation reactor under the following conditions: catalyst 6.7 g, substrate 5 g in 100 ml 20 mM phosphate, pH 8, duration of each run 0.5—1 h, linear flow rate 110 cm/min, space velocity 31 bed volumes/min. The experiments extended over a period of 2 weeks, and a substantial part of the activity loss probably occurred during the extensive washing of the catalyst prior to storage at 20°C. The brick conjugates seem to be at least as stable.

Catalysis by the free enzyme

Reactions of all substrates obeyed Michaelis-Menten kinetics. Hydrolysis of 85 mM 2-methoxyethyl acetate in 1 M acetate/10 mM phosphate was found to be 10% slower than in phosphate alone, but this decrease in rate probably reflects nonspecific salt effects, rather than product inhibition. With 10 mM phosphate pH 8 the relative rates in the presence of 0.1, 0.5 and 1.0 M NaCl were in the ratio of 1.00 : 0.91 : 0.80. In 0.1 M NaCl rates were independent of phosphate and Tris concentrations and were practically the same in both buffers [2]. All the following measurements described in this report were carried out in solutions containing 0.1 M NaCl. The buffer was 10 mM sodium phosphate, pH 8, temperature 25°C and the substrate 85 mM 2-methoxyethyl acetate unless other values and materials are specified.

As shown in Fig. 1 pH vs. activity profiles for the hydrolysis of the four substrates examined do not differ appreciably, the rate maximum being in the region of pH 8.3. Measurements of the Michaelis-Menten constants are shown graphically in Fig. 2 and the values of K_m and V/unit of enzyme (as defined in the experimental part) are summarized in Table I. Apparently the rate-determining step is the hydrolysis of the acetyl-enzyme intermediate since the values of V are practically the same.

Ethyl propionate hydrolyzed about 0.2 times as fast as 2-methoxyethyl ace-

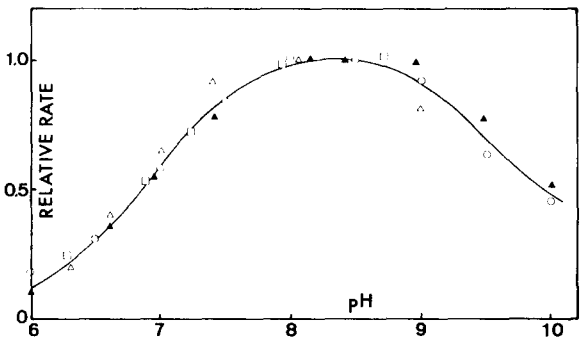

Fig. 1. Enzyme-catalyzed reaction rates at 25°C of 85 mM 2-methoxyethyl acetate (○), 1.4 mM *p*-nitrophenyl acetate (□), 80 mM Celospor (△) and of 1% Cephalotin (▲) as a function of pH. Rates expressed relative to the values at pH 8.

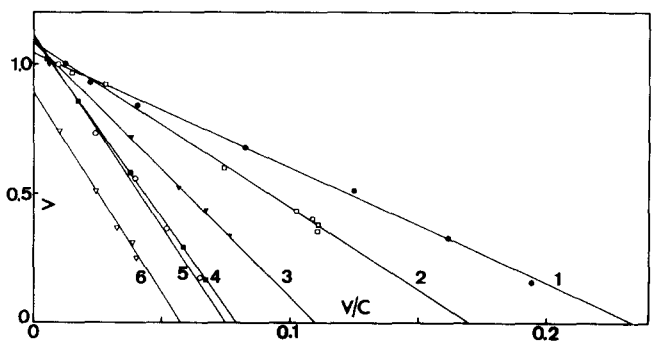

Fig. 2. Determination of K_m values for (1) 2-methoxyethyl acetate, (2) 7-aminocephalosporanic acid, (3) Celospor, (4) ethyl acetate, (5) cephalosporin C and (6) Boc-cephaloglycine at pH 8 and 25°C. Relative rates v plotted against v/substrate concentration (mM).

TABLE I

Values of K_m and V/enzyme unit at pH 8.0

Substrate	K_m (mM)	V (μmol/min per unit)
p-Nitrophenyl acetate	0.13 *	0.38 *
2-Methoxyethyl acetate	4.5	1.04
7-Aminocephalosporanic acid	6.5	1.09
Celospor **	10.0	1.07
Cephalosporin C ***	15.0	1.08
t-Boc-cephaloglycine †	16.0	0.89
Ethyl acetate	14.0	1.09

* at pH 6.9 in 10% v/v acetonitrile/90% v/v H_2O.
** $RNH\text{-}COCH_2CN$.
*** $RNH\text{-}CO\ (CH_2)_2\ CH(NH_2)\ (CO_2H)$.
† $RNH\text{-}COCH(C_6H_5)\ (NHCOOC_4H_9)$, where RNH_2 = 7-aminocephalosporanic acid.

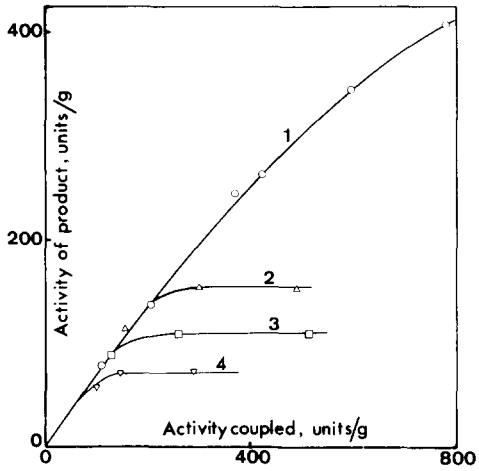

Fig. 3. Expressed activity (μmol · min^{-1} · g^{-1}) as a function of the activity coupled to 1 g carrier: (1) controlled pore glass and pure enzyme (specific activity 120), (2) controlled pore glass and crude enzyme (specific activity 13), (3) 0.2—0.3 mm brick particles and (4) 0.4—0.5 mm brick particles, both with nearly pure enzyme. Controlled pore glass assay in 10 mM phosphate, brick particles in 20 mM phosphate, both pH 8.

tate. A partial resolution of acetates of racemic alcohols into stereoisomers was observed.

Catalysis by immobilized preparations

The effect of increasing loading on the activity of controlled pore glass (0.2—0.8 mm particles) and that of brick particles of two different sizes is shown in Fig. 3. Curve 2 represents the results obtained with controlled pore glass and a crude preparation of esterase having a specific activity of 13 units/mg. The quantities of enzyme immobilized are based on the difference between the activity applied to the carrier and the free activity recovered from the washings after coupling, this quantity being small (<3%) except at the highest protein/carrier ratios *. Activity of the most heavily loaded 0.4—0.5 mm brick particles increased by more than a factor of 2 when they were ground to a fine powder. Activity of a controlled pore glass preparation, containing 300 units of esterase/g increased by about 15% when the phosphate concentration was doubled.

The pore size distribution of the brick particles is shown in Fig. 4 together with literature data [17] for the glass.

The following experiments were carried out with a controlled pore glass conjugate prepared by coupling 196 units of esterase/g and with 0.4—0.5 mm brick particles prepared with 93, 140 and 283 units/g carrier, their expressed activity in 20 mM phosphate pH 8 being 60, 49 and 24% of the activity immobilized.

* 7% with 520 units applied to 1 g of the 0.2—0.3 mm brick particles. When this carrier was derivatized with aqueous γ-aminopropyl triethoxysilane [14] in place of polyethylene imine, 26% of enzyme failed to couple under the same conditions, indicating that the polyfunctional polymer has a somewhat higher binding capacity. Otherwise practically the same results were obtained by both methods, and also with polyethylene imines having a smaller (1200) and higher (100 000) molecular weight.

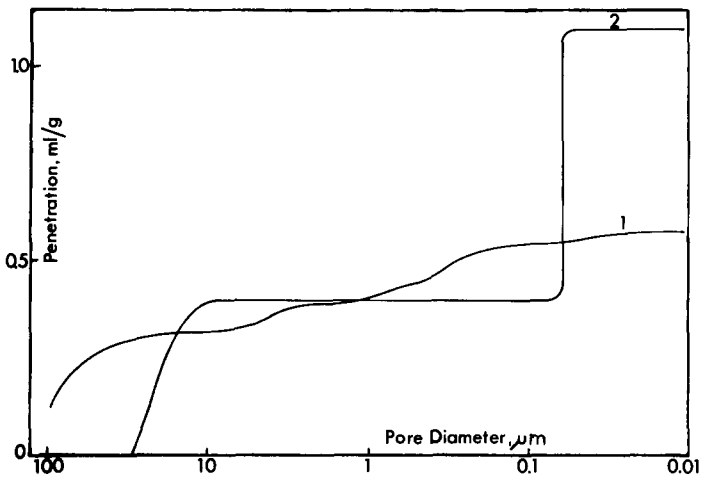

Fig. 4. Mercury intrusion data showing the pore size distribution in (1) brick particles and (2) controlled pore glass. The total internal surface of controlled pore glass is 82 m^2/g; of the brick particles 2 m^2/g, 80% of which belong to pores with a diameter larger than 550 Å.

They are designated as preparations A, B and C.

The effect of substrate concentrations on rates catalyzed by the controlled pore glass-esterase is shown in Fig. 5. The calculated values of K_m (app.) for 2-methoxyethyl acetate, Celospor and cephalosporin C are 5.0, 17 and 30 mM respectively, the relative values V (app.) 1.00, 0.98 and 1.24.

pH vs. rate profiles for the hydrolysis of 85 mM 2-methoxyethyl acetate by controlled pore glass esterase, measured in three different buffers, have been reported earlier [2]. Curves 1, 2 and 3 in Fig. 6 show the rates for preparation C in phosphate and in Tris. The broken line represents the normalized pH-profiles of preparations A, B and C as measured in a mixture of the following

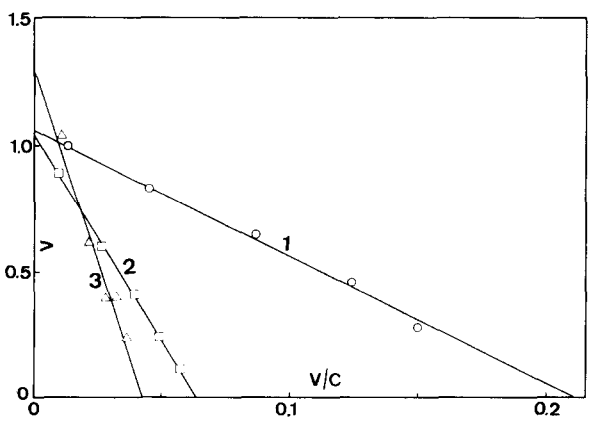

Fig. 5. Determination of K_m (app.) for the reaction of controlled pore glass-esterase with (1) 2-methoxyethyl acetate, (2) Celospor and (3) cephalosporin C. Relative reaction rates v plotted against v/substrate concentration (mM).

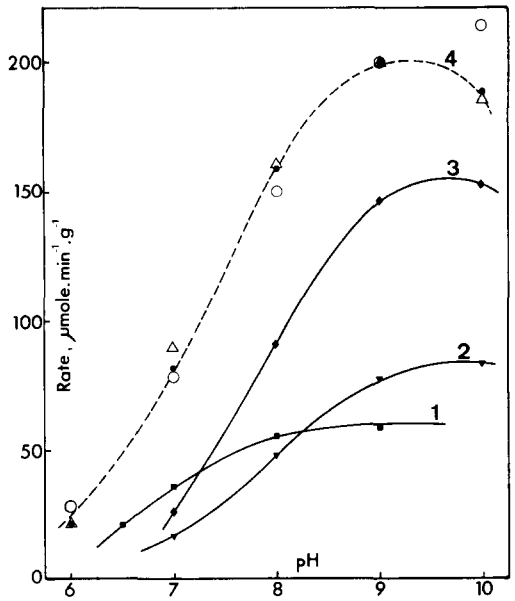

Fig. 6. pH vs. rate profiles for the hydrolysis of 85 mM 2-methoxyethyl acetate by preparation C in (1) 10 mM phosphate, (2) 10 mM Tris, and (3) 50 mM Tris. Curve 4 represent the normalized rates in the mixture of 20 mM buffers for preparations A (△), B (●) and C (○) on an arbitrary rate scale. The rates at pH 9 are 78, 114 and 148 μmol · min^{-1} · g^{-1}.

20 mM buffers, used to ensure a more or less constant buffer capacity over the experimental range: malic acid (pK 5.1), maleic acid (pK 6.2), phosphate (pK 7.2), Tris (pK 8.1) and borate (pK 9.2). Activities of these three preparations at pH 9 were 78, 114 and 148 μmol/min per g respectively, which correspond to 84, 81 and 52% respectively of the activity immobilized.

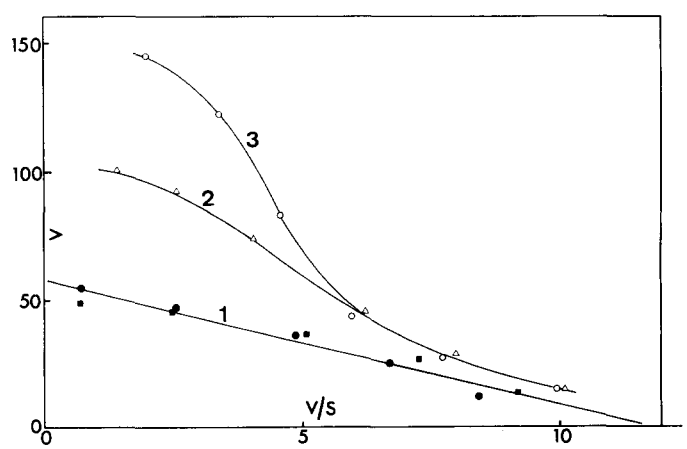

Fig. 7. Reaction rates v (μmol · min^{-1} · g^{-1}) as a function of v/substrate concentration (mM) for the following systems: (1) Preparation B in 10 mM phosphate (●) and Tris (■), both pH 8; (2) preparation C in 50 mM phosphate, pH 9; (3) preparation C in 50 mM Tris, pH 9.

The effect of 2-methoxyethyl acetate concentrations on rates was examined with preparation B in 10 mM phosphate and 10 mM Tris pH 8, and with preparation C in 50 mM phosphate and 50 mM Tris pH 9, as shown in Fig. 7. The two systems were chosen because at high substrate concentrations the rates in the two buffers are nearly the same in the first one, while they differ appreciably in the second.

Discussion

Catalytic properties of enzymes, such as their activity and specificity, are sometimes profoundly changed by chemical modification. When the chemical reagents are soluble polymers or solids, electrostatic and short range forces may also contribute to the changes. The expressed catalytic activity of insolubilized enzymes depends on the molecular properties of the immobilized species, on the partition of the mobile reagents between the catalyst and the liquid phase, and on the internal concentration gradients established in the particles as a result of diffusion limitations. Calculation for enzymes evenly dispersed in a uniform matrix shows that overall rates become proportional to the external substrate concentration when the lowest internal concentration $s << K_m^*$, the molecular Michaelis constant for the immobilized protein [18,19,20]. Experimentally, linear Lineweaver-Burke plots are obtained in that range, appreciable deviations being apparent in the region $s \geqslant K_m^*$ in well defined, diffusion-limited systems [21,22]. Furthermore, in reactions involving generation of hydrogen ions, the basic buffer component, B^-, is converted to the acid component, HB, by reaction with H^+. Thus, there is a flux of B^- from the periphery of the particles to the center, and a flux of the produced HB in the opposite direction. An internal pH gradient is established, acidity in any given region depending on the concentration ratio HB/B^- and the pK of the buffer. Large differences between the controlled external pH and internal pH manifest themselves as distorted pH vs. rate profiles [2] and are expected to lead to apparent deviations from Michaelis-Menten kinetics. Buffer rather than substrate diffusion is expected to be rate limiting under conditions usually employed for the pH-stat assay of immobilized enzymes, buffer concentrations being low in relation to substrate in order to ensure a sensitive response to acid and alkali.

Pores which impede mass transfer of small substrate molecules necessarily act as diffusion barriers in the coupling process. When coupling is fast, diffusion slow, and the carrier morphology non-uniform, poorly accessible pores and cavities are occupied only after the more readily available surfaces have been filled-up. In any event, crowding of the enzyme molecules will occur when the available internal surface is small.

Activity of the morphologically uniform glass, loaded with increasing quantities of pure enzyme, is seen to increase throughout the experimental range. However, an activity limit is reached sooner when crude protein with 1/10 of the esterase activity is coupled. The support used had a wide particle size distribution and the cut-off probably occurs at the point where the smaller grains are saturated with protein; at higher enzyme/carrier ratios immobilization takes place in the interior of large particles. Likewise, activity of the sieved brick particles levels off when immobilization begins to take place in the less

accessible cavities, or when mass transfer across the Nernst diffusion layer becomes rate limiting [18,23] on account of the high density of enzyme molecules on the available surface.

Under conditions of low to moderate diffusion limitations Michaelis-Menten kinetics hold, as shown by the results in Fig. 5. The relative values of V and V (app.) for the 3 substrates examined indicate that specifity of the enzyme was practically unchanged by immobilization. The ratios K_m (app.)/K_m increase with the molecular weight of the substrates and reflect perhaps the diffusivities of the species.

Large apparent losses of activity accompany immobilization on the brick particles at higher enzyme/carrier ratios and manifest themselves in grossly distorted pH vs. rate profiles. However, with decreasing loading levels or increasing concentrations of the buffer mixture all profiles approach curve 4 in Fig. 6 which may be taken to be the molecular profile of the immobilized enzyme.

When the data for preparation B in Fig. 7 are represented by a straight line, the value obtained for K_m (app.) $\simeq$ 5, which is practically the same as that for the controlled pore glass conjugate. Data for preparation C in the specified systems show the large deviations from Michaelis-Menten kinetics which must necessarily occur when diffusion limitations are severe. The curves, which diverge markedly at high substrate concentrations, are seen to converge at the lower end; when the rate of acid generation is slow enough, both buffers have sufficient capacity to cope with its production and to minimize differences between internal and external pH.

In concluding, a remark concerning the graphical representation of data extending over a wide range of rates (e.g. from 0.1 V to V) seems desirable. On a Lineweaver-Burk plot all points belonging to curves 2 and 3 in Fig. 7 fall on a single, nearly straight line (K_m (app.) $\simeq$ 10 mM) with some 'minor' scatter at the high concentration end. Data representing the three highest substrate levels are crowded in the lower left hand corner into a space corresponding to 1/5 of the reciprocal rate range.

While all linear transforms of the Michaelis-Menten equation have their drawbacks [24], visual distortion of the results, and of the relative weights of deviations and experimental errors, is particularly bad in the reciprocal plots.

Acknowledgements

The technical assistance of Mr. M. Sieber, J. Scheidegger, A. Schneider and K. Weiss is gratefully acknowledged.

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