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Kinetic Studies on Solid-Supported β-Galactosidase†

Peter S. Bunting and Keith J. Laidler*

ABSTRACT: A kinetic investigation was made of the action of β-galactosidase trapped in a polyacrylamide gel, the substrate being *o*-nitrophenyl β-D-galactopyranoside (NphGal). Rate measurements were made over a range of substrate concentrations, and k_c (app) and K_m (app) values were determined. The work was done with disks having thicknesses ranging from 25 to 1000 μ, and at various enzyme concentrations within the gel; under some of the conditions there was diffusion control, under others (thin slices and low enzyme concentrations) there was negligible diffusion control. The results are analyzed with reference to a theoretical treatment of Sundaram, Tweedale, and Laidler [(1970), *Can. J. Chem. 48*, 1498] which makes use of a function F which is related to the extent of diffusion control. It was found that for the thicker slices the rate at low substrate concentrations is proportional to the square root of the enzyme concentration, while for the thinnest slices, where there is little diffusion control, the rate is proportional to the first power of the enzyme concentration. These results are predicted by the theory. The values of *F* calculated from the experimental results were found to be in excellent agreement with the theoretical values.

During the past few years there has been considerable interest in the preparation and properties of solid-supported enzymes (for reviews, see Silman and Katchalski, 1966, Kay, 1968, Barker and Epton, 1970, Mosbach *et al.*, 1971, Volfin, 1971, Sundaram and Laidler, 1971, and Goldman 1971a). This work has application to a number of fields, such as automated analysis (Hornby *et al.*, 1970; Sundaram and Hornby, 1970) and clinical investigations (Chang, 1971). More recently, attention has been focussed on the use of solid-supported enzymes as model systems for the behavior of enzymes *in vivo* when they are attached to membrane surfaces or are embedded in membranes. It has been noted in particular that many enzymes have an increased apparent activity when separated from their natural membrane supports (de Duve, 1959). Experiments to investigate such aspects have included some qualitative studies on the rate of consecutive enzyme reactions when the enzymes are supported (Mosbach and Mattiasson, 1970; Mattiasson and Mosbach, 1971) and more quantitative studies on the effect of diffusion on enzyme systems (van Duijn *et al.*, 1967; Goldman *et al.*, 1968, 1971b).

In addition, a number of theoretical treatments of the kinetics of solid-supported enzymes have been given (Blum and Jenden, 1957; O'Sullivan, 1962; van Duijn *et al.*, 1967; Goldman *et al.*, 1968, 1971b; Sundaram *et al.*, 1970; Goldman and Katachalski, 1971; Kasche *et al.*, 1971; Kobayashi and Moo-Young, 1972). The present work was undertaken as a test of the treatment of Sundaram *et al.* (1970), who gave explicit solutions for the rate equations under the limiting conditions of low and high substrate concentrations. In addition they gave an approximate solution for the general case, and confirmed its reliability by computer calculations. This treatment led to predictions about the conditions under which the

† From the Department of Chemistry, University of Ottawa, Ottawa K1N 6N5, Canada. *Received June 6, 1972.* This work was supported by a grant from the National Research Council of Canada.

kinetics would become diffusion controlled, predictions that have been tested and confirmed in the present study.

The experimental work was done with disks of polymer in which the enzyme, β-galactosidase, was uniformly distributed. These disks were immersed in solutions of the substrate, *o*-nitrophenyl β-D-galactopyranoside. After various attempts to use carboxymethylcellulose, hydroxyethylcellulose, polyvinyl alcohol, and other polymers, cross-linked by γ irradiation, it was found most satisfactory to work with polyacrylamide gel; this is easy to prepare, and the product easy to handle in the kinetic experiments. This gel has previously been used as a support in a number of investigations (Bernfeld and Wan, 1963; van Duijn *et al.*, 1967; Updike and Hicks, 1967; Guilbault and Montalvo, 1969; Inman and Dintzis, 1969).

The present investigation has been particularly concerned with the kinetic effects of (1) varying the enzyme concentration (2) varying the substrate concentration, and (3) varying the thickness of the disks.

Experimental Section

Materials. The enzyme, β-galactosidase from *E. coli*, K12, was a partially purified preparation obtained from Worthington Biochemical Corp.; two lots were used, BG OHA for initial experiments and BG IEA for all Lineweaver–Burk plots. The substrate was *o*-nitrophenyl β-D-galactopyranoside (NPhGal) and was obtained from Sigma Chemical Co.

The cross-linking agents Temed and Bis[1] were obtained from Eastman Organic Chemicals; acrylamide was obtained from Eastman and from Matheson, Coleman & Bell. The ammonium persulfate used in the polymerizations was obtained from Fisher Scientific Co., and disodium hydrogen phosphate from J. J. Baker Chemical Co.

Methods. All solutions used to assay the enzyme and to prepare the gels were made up in 0.02 M Na_2HPO_4 buffer at pH 7.50 ± 0.05, and were adjusted with sodium chloride to an ionic strength of 0.10 M. The usual assay procedure for β-galactosidase (Wallenfels and Malhotra, 1960) has been shown by Shifrin *et al.* (1970) to result in a loss of enzyme activity over a period of several hours; on the other hand, the latter workers found that phosphate buffer resulted in constant activity over at least 6 hr. This was confirmed in the present work, but only if the buffer was degassed for 2 hr with nitrogen, which removes oxygen which may inactivate the essential thiol group of the enzyme.

Kinetic Procedure. Enzyme weighings were checked by ultraviolet absorption at 280 nm, in buffer at pH 7.5, with an $\epsilon_{280\,nm}^{1\,cm}$ value of 1.44 ± 0.04 l. g^{-1}; the latter value was determined on four separate occasions. NphGal was used as substrate and the rate of reaction followed on a Bausch and Lomb Spectronic 600 spectrophotometer, with a Sargent SRLG recorder attached, at 420 nm and a temperature of 25.0 ± 0.2°. Rates were calculated using $\epsilon_{420}^{1\,cm} = 3200\ M^{-1}$; this value was obtained by measuring the absorbance after hydrolyzing the substrate at pH 13 for 1 hr, after which there was no further change in absorbance; a p*K* of 7.10 was determined,[2] and $\epsilon_{420}^{1\,cm}$ values of 190 at pH 1.0 and 4350 at pH 13.0.

Kinetic measurements on the solid-supported enzyme preparations were carried out by suspending disks of the enzyme-containing gels in 10 ml of substrate buffer solution. By means of a Watson–Marlow flow inducer (Model MHRE 200) the solution was pumped through tygon tubing to a microvolume flow cell (obtained from Hellma Co.) to determine the absorbance. The volume of the solution in the flow cell plus tubing was less than 10% of the total solution volume. The flow rate was adjusted so as to be faster than the rate of formation of product. The solution was stirred with a small magnetic stirrer, which kept the slices moving around in the vessel. When necessary, to obtain a measurable rate, up to eight slices were suspended in the solution at one time. The rate was always directly proportional to the number of slices.

Preparation of Gels. The polyacrylamide gels were made by photopolymerization of acrylamide monomer solution (Davis, 1964; Reisfeld *et al.*, 1962); Bis was used as cross-linking agent, ammonium persulfate as photocatalyst, and Temed as catalyst and cross-linking agent. Since other work (Fawcett and Morris, 1966; Degani and Miron, 1970) had shown that the use of 5% of Bis (with reference to Bis plus acrylamide monomer) produces the smallest pore size in the gel, this percentage was used in our experiments. Stock solutions of 60% w/v acrylamide monomer (including the 5% Bis) were kept for 1 month at room temperature in the dark. Solutions of Temed (2% v/v) and ammonium persulfate (2.8% w/v) in degassed buffer were stable for 1 week at 4° in the dark. In most experiments 15% acrylamide solution was used, and the following indicates typical concentrations used to make the mixture: acrylamide (+ 5% Bis), 1.5 ml of 60%; Temed, 0.5 ml of 2%; persulfate, 0.1 ml of 2.8%; and enzyme and buffer 3.9 ml; the total is 6.0 ml. The amounts of acrylamide and enzyme were varied somewhat, but those of the other components, and the total volume, were held constant.

The polymerizations were carried out in glass tubes of constant 1.0 cm diameter. The tubes were first dipped into Fotoflow solution (Eastman-Kodak) diluted 1:20 with distilled water; this facilitated subsequent removal of the gels from the tubes (Davis, 1964; Reisfeld *et al.*, 1962). During polymerization the solutions were cooled by being placed in a beaker of water, covered with a plastic lid; the solutions were stirred and cooled to 0–4° in a Stir-Kool apparatus (from Thermoelectronics Unlimited Inc.). Irradiation was done using a fluorescent lamp about 2 m away from the tubes; complete gelation took about 45 min.

Slices were cut from the gels by use of a Model 880 American Optical Co. microtome (Bishop *et al.*, 1967; Weinberg *et al.*, 1967). The gel was first frozen by CO_2 which had been cooled by allowing it to escape from a cylinder through a nozzle. The slice thickness was varied by manual adjustment of the blade-to-sample distance. The slices were stirred in buffer for a few minutes to remove adsorbed enzyme; this procedure was repeated between runs to remove substrate and product from them.

The slice thicknesses were measured using a Zeiss optical microscope with a calibrated 12.5× magnification eyepiece and 10× and 40× magnification objectives. The slices were mounted on their sides between cover slips and were observed under water to prevent drying out and contraction. It was checked that there were no refraction errors. Each slice was examined at three points, and five measurements made at each point. The slices were found to be uniformly thick.

Partition and Diffusion Coefficients. Partition coefficients for the substrate and product were measured by placing a known volume of gel in a solution of known concentration. The system was left for 2 days to attain equilibrium, and the change in concentration of the solute (substrate or product) was deter-

[1] Abbreviations used are: Temed, *N,N,N′,N′*-tetramethylethylenediamine; Bis, *N,N′*-methylenebisacrylamide.

[2] This value is consistent with that quoted for $I = 0$ of 7.22 in the Handbook of Biochemistry (1968); our $I = 0.1$ will tend to decrease p*K*.

TABLE I: Partition and Diffusion Coefficients.

% (w/v) Acrylamide in Gel	Partition Coef	Diffusion Coef (cm^2 sec^{-1})
7.5	1.5	$(5.6 \pm 0.5) \times 10^{-6}$
15.0	0.89 ± 0.01	$(3.7 \pm 0.3) \times 10^{-6}$

mined. It was checked that there was no further change of concentration after 2 days.

Diffusion coefficients were measured by placing a cylinder of gel at the bottom of a tube and placing a solution of the diffusant (such as product) in the tube, in which there was a small stirrer; the whole was then placed in stirred buffer solution from which aliquots were withdrawn at regular intervals and analyzed. It was checked that there was no leakage of diffusant, and that a planar diffusion front was evident, by visually following diffusion of a blue dye; the linearity of diffusion with time confirmed this. The diffusion coefficient, D, was then calculated from the formula

$$\text{diffusion rate} = \frac{AD}{xV}([S_0] - [S]) \qquad (1)$$

where A is the cross-sectional area, x the length of the gel, V the volume of buffer solution, and $[S_0]$ and $[S]$ the concentrations of diffusant. Care was taken to deal with volume changes when the gels were placed in buffer solution. Stirring of the solutions on both sides of the gel was found to be essential.

The analysis of rate *vs.* substrate concentration data, to give K_m(app) and k_c(app) values, was carried out by computer using a program adapted from that given by Cleland (1967).

Results

Partition and Diffusion Coefficients. These are given, for the reaction product *o*-nitrophenol, in Table I.

Enzyme Activity. Initial experiments were concerned with obtaining a preparation of β-galactosidase in polyacrylamide gel of suitable activity, with no leakage of enzyme. Leakage of enzyme from the gels could not be checked by use of the normal protein assays such as the Folin–Lowry method (Lowry *et al.*, 1951) since residual acrylamide monomer interferes with the assay to such an extent that the small increase in color due to protein leakage could not be reliably determined. Instead, the enzyme activity was assayed as a function of time by removal of aliquots of surrounding buffer over a period of 6–8 hr. There was considerable enzyme leakage for 5 and 7.5% acrylamide gels, but negligible leakage for 10% and higher. These results were confirmed by assaying the supported enzyme slices.

Experiments were carried out to test the activity of the enzyme as a function of acrylamide concentration. Results are shown in Table II; there is necessarily a variation in slice diameter and slice thickness, and the last column of the table shows the rate normalized to a diameter of 1.0 cm and a thickness of 100 μ.

On the basis of this preliminary work, further details of which are given in a forthcoming paper, it was decided to carry out the remaining investigations with 15% acrylamide. Checks

TABLE II: Rates at Various Acrylamide Concentrations.[a]

% Acrylamide (w/v)	Slice Diameter (cm)	Slice Thickness (μ)	Rate (μmoles min^{-1}, per mg of Enzyme) (per l. of Soln)	Rate Normalized to 1.0 cm, 100-μ Slice
7.5	0.95		2.73	2.96
10.0	1.02	102	3.21	3.02
15.0	1.05	108	3.72	3.12
20.0	1.05	100	3.66	3.32
25.0	1.12	111	3.55	2.55
30.0	1.12	104	3.00	2.30

[a] Polymerization brought about photochemically.

were first made to ensure that the free and supported enzyme maintained constant activity over a period of several hours. The rate curves were linear for at least 2 min, so that reliable initial rates could be measured.

The influence of slice thickness on the rate of reaction, at constant enzyme concentration within the gel, is shown in Figure 1 for three different enzyme concentrations. Initially the curves are linear, with a leveling off when the reaction becomes diffusion controlled. It is to be noted that the higher the enzyme concentration the smaller is the thickness at which diffusion becomes rate limiting.

Figures 2 and 3 show the variations of rate with the enzyme concentration within the gel. Figure 2 shows a plot against the first power of the enzyme concentration, and it is to be seen that there is a linear dependence for the 100-μ slices but not for 200 μ and above; the latter show a square-root dependence (Figure 3). In this connection, a check was made to confirm that for the *free* enzyme in the presence of 15% monomer the rates were proportional to enzyme concentration.

Lineweaver–Burk plots of $1/v$ *vs.* 1/[NphGal] are shown for various slices in Figures 4 and 5. Such plots were also made for the free enzyme, yielding the following values for the kinetic parameters: $K_m = 1.73 \times 10^{-4}$ M (±4%) and $k_c = 273$ sec^{-1} (±2%). These values differ somewhat from those quoted in the literature (Wallenfels and Malhotra, 1960), but it is to be noted that assay conditions (*e.g.*, ionic strengths) are differ-

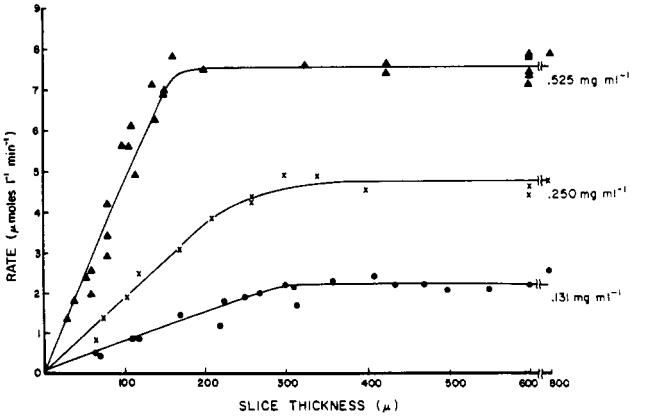

FIGURE 1: Influence of slice thickness on the rate (per liter of solution), at three enzyme concentrations. [NphGal] = 1.66×10^{-3} M; pH 7.5; $I = 0.10$ M; 0.02 M phosphate buffer; $T = 25.0°$.

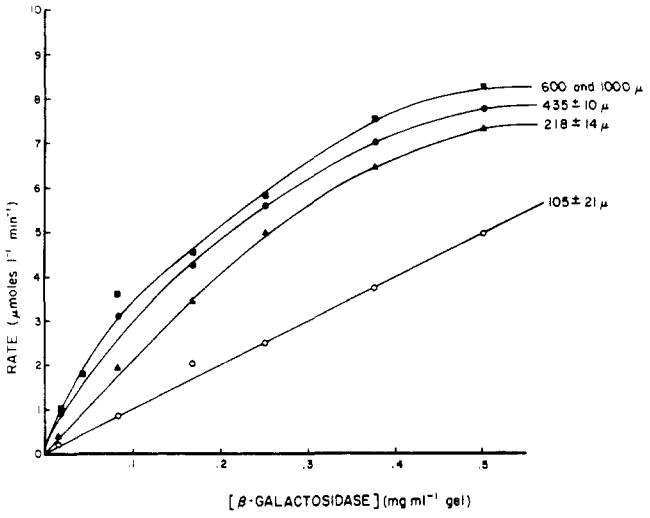

FIGURE 2: Plots of rate (per liter of solution) against enzyme concentration within the support, for four slice thicknesses.

ent and that Mg^{2+}, which activates the enzyme, was not used in the present experiments.

The kinetic parameters obtained in the solid-supported enzyme experiments are shown in Table III. With regard to these values, it is to be noted that the results with the 1000-μ slices probably do not correspond to attainment of the steady state. The theory (Sundaram *et al.*, 1970) predicts that roughly 12 min are required for establishment of the steady state under those conditions; a linear rate was observed for only a few minutes and probably corresponds to presteady-state conditions. For the 400-μ slices only about 2 min are needed to establish the steady state, so that the results are fairly reliable as representing steady-state conditions. For the 100-μ slices, steady state is probably established within a few seconds. It is possible that the curvatures in the Lineweaver–Burk plots (Figure 4) for the thicker slices (400 and 1000-μ) are related to this lack of establishment of the steady state. The theoretical treatment of Kobayashi and Moo-Young (1972) predicts that

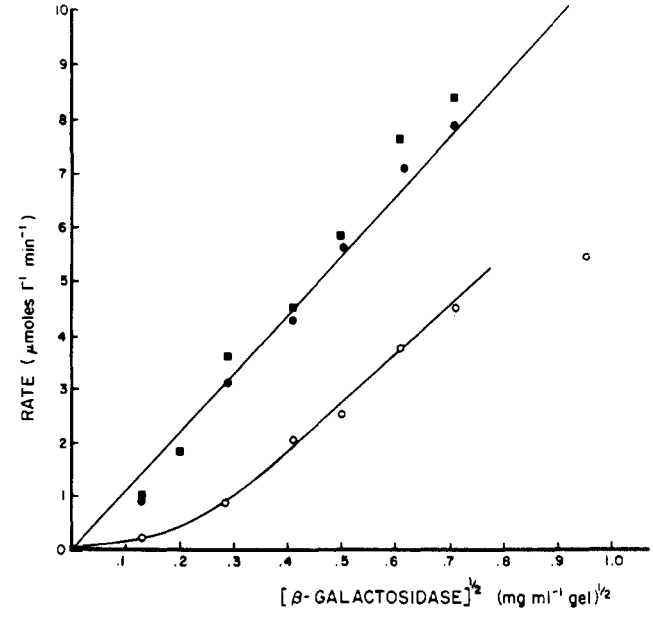

FIGURE 3: Plots of rate against the square root of the enzyme concentration; Lower line, 105 μ; upper line, 400–1000 μ thickness.

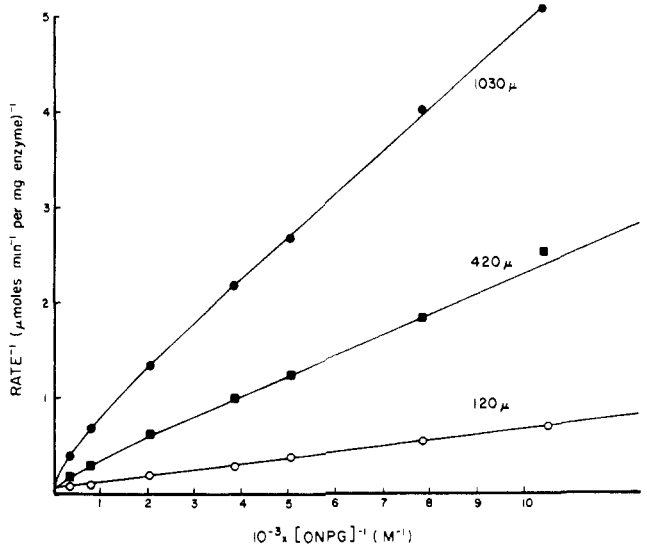

FIGURE 4: Lineweaver–Burk plots for 120-, 420-, and 1030-μ slices; each point is an average of six to seven determinations, for which the average error was ~12%; other conditions as before.

Lineweaver–Burk plots for supported enzymes should, when there is diffusion control, be somewhat convex to the 1/[S] axis; this is observed for all of the 100-μ slices in our experiments (Figure 5).

As a result of the curvatures at higher substrate concentrations, the V_{max}(app) values are very different for the different slices; this is shown in Table III. Extrapolation of these plots yields a *constant* value of 100 ± 20 sec^{-1} for k_c', as expected from the theory. However, this extrapolation procedure is unreliable; a more reliable k_c' value of 81.5 sec^{-1} (±5%) was obtained from the slope of a graph such as that for the 110-μ slice of Figure 2.

Since slices thinner than 100-μ could not be handled for repeated kinetic analysis, activity was reduced by lowering the enzyme concentration within the gel, in order to approach the diffusion-free limit. Figure 6 shows a plot of $1/K_m$(app) against log $[E]_m$.

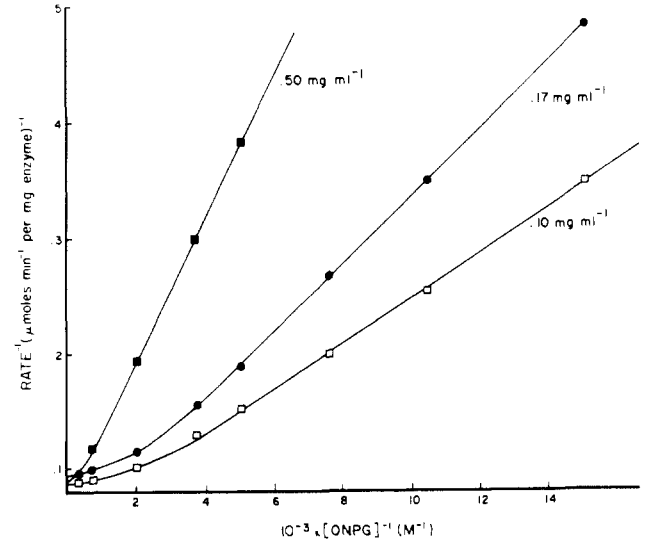

FIGURE 5: Lineweaver–Burk plots for the supported enzyme, for 110-μ slices and at three enzyme concentrations. Each point is an average of three determinations; conditions as previously.

TABLE III: Values of V_{max}(app) and K_m(app) for a Range of Enzyme Concentrations in the Gel.[a]

Slice Thickness (μ)	Slice Diameter (cm)	$[E]_m \times 10^7$ (moles l.$^{-1}$ of Gel)	V_{max}(app) (μmoles min^{-1} per mg of Enzyme)	K_m(app) $\times 10^4$ (M)
1030 ± 20	1.04 ± 0.01	9.26	2.5 ± 6%	10.0 ± 8%
420 ± 10	1.03 ± 0.01	9.26	5.6 ± 8	12.4 ± 10
120 ± 7	1.04 ± 0.01	9.26	14.1 ± 12	8.65 ± 14
110 ± 10	1.01 ± 0.01	3.09	16.9 ± 6	4.6 ± 10
110 ± 11	1.03 ± 0.004	1.85	17.9 ± 4	3.6 ± 6
105 ± 9	1.02 ± 0.02	1.31	10.8 ± 6	2.1 ± 13
110 ± 8	1.03 ± 0.005	0.77	9.9 ± 3	1.2 ± 8
110 ± 8	1.04 ± 0.005	0.31	7.9 ± 8	1.0 ± 10

[a] V_{max}(app) values are per liter of solution. The enzyme concentration was calculated assuming pure enzyme and a molecular weight of 540,000 (Craven *et al.*, 1965).

Discussion

There are four main reasons why the kinetics of supported enzymes are different from when the enzyme is present in free solution. (1) The enzyme may be conformationally different when supported, and so have modified k_c and K_m values. (2) In the support, the interaction between enzyme and substrate takes place in a different environment from that existing in free solution. This effect will also modify the k_c and K_m values. The values for these parameters in the support, as modified by effects (1) and (2), will be denoted as k_c' and K_m'. (3) There will be partitioning of the substrate between the support and the free solution, so that the substrate concentration in the neighborhood of the enzyme may be different from what it is in free solution. This effect is taken care of by the partition coefficient, P. (4) The reaction in the solid support may be to some extent diffusion controlled.

According to the treatment of Sundaram *et al.* (1970), the rate of a reaction catalyzed by a solid-supported enzyme is given to a good approximation by an equation of the Michaelis–Menten form

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

where [S] is the concentration of the substrate in the external solution, $[E]_m$ the concentration of enzyme in the support, and k_c(app) and K_m(app) are given by

$$k_c(\text{app}) = k_c' \tag{3}$$

$$K_m(\text{app}) = \frac{K_m'}{PF} \tag{4}$$

The function F relates to the extent to which the reaction in the solid support is diffusion controlled. The various theories (Goldman *et al.*, 1968, 1971b; Sundaram *et al.*, 1970) give the following expression for F

$$F = \frac{2(\cosh \alpha l - 1)}{\alpha l \sinh \alpha l} \tag{5}$$

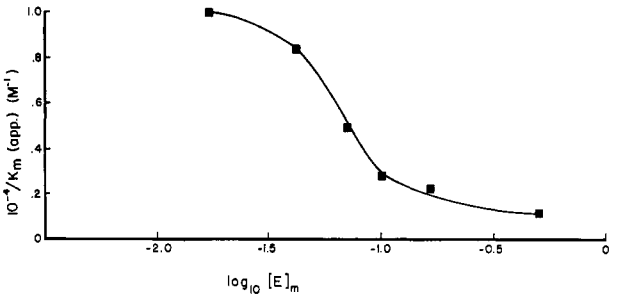

FIGURE 6: A plot of $1/K_m$(app) against log $[E]_m$.

where l(cm) is the thickness of the slice and α(cm^{-1}) is given by

$$\alpha = \left(\frac{k_c'[E]_m}{DK_m'}\right)^{1/2} \tag{6}$$

Here $[E]_m$ is the molar concentration of the enzyme in the support and D is the diffusion coefficient (cm^2 sec^{-1}). A more compact expression for F is obtained if we set

$$\gamma = \frac{\alpha}{2} = \left(\frac{k_c'[E]_m}{4DK_m'}\right)^{1/2} \tag{7}$$

when

$$F = \frac{\tanh \gamma l}{\gamma l} \tag{8}$$

The curve in Figure 7 shows the function F plotted against γl. For γl values up to about 0.2, F is close to unity; for γl values higher than 2, F is close to $1/\gamma l$.

Dependence of Rate on Enzyme Concentration. It was seen in Figure 3 that for the thicker slices, where there is diffusion control, the rate at low substrate concentrations is proportional to the square root of the enzyme concentration. This is readily explained by the theory; at low substrate concentrations eq 2 becomes

$$v = \frac{k_c'[E]_m}{K_m'/P}[S]F \tag{9}$$

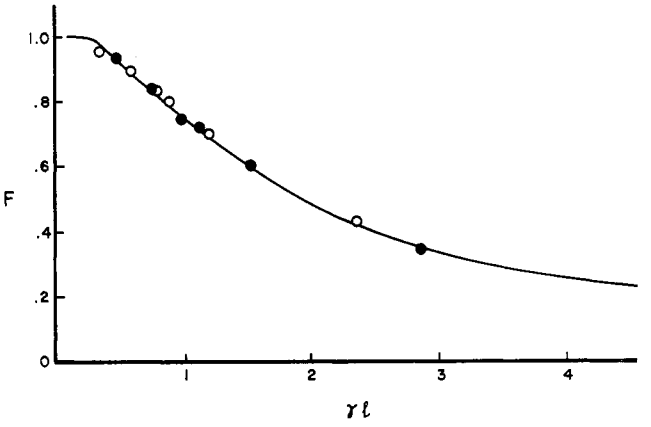

FIGURE 7: The solid line is a plot of the function F (see eq 2) against γl. The points are experimental (see text).

TABLE IV: Calculated F Values for a Range of Enzyme Concentrations in the Gel.[a]

Slice Thickness (μ)	Slice Diameter (cm)	$[E]_m \times 10^7$ (moles l.$^{-1}$ of Gel)	F	
			$K_m' = 0.90 \times 10^{-4}$ M	$K_m' = 1.46 \times 10^{-4}$ M
1030	1.04	9.26	0.04	0.05
420	1.03	9.26	0.10	0.13
120	1.04	9.26	0.35	0.45
110	1.01	3.09	0.60	0.70
110	1.03	1.85	0.77	0.84
105	1.02	1.31	0.72	0.80
110	1.03	0.77	0.84	0.90
110	1.04	0.31	0.93	0.96

[a] The enzyme concentration was calculated assuming pure enzyme and a molecular weight of 540,000 (Craven *et al.*, 1965).

Under these conditions $F \propto 1/\gamma l$, and since γ is proportional to $([E]_m)^{1/2}$ the overall rate is predicted to be proportional to $([E]_m)^{1/2}$, in agreement with experiment for the 400- and 1000-μ slices (Figure 3).

At lower values of γl, K_m(app) becomes very much smaller and [S] > K_m(app). The equation is now simply

$$v = k_c'[E]_m \qquad (10)$$

which is obeyed by the 100-μ slice (Figure 2). At low substrate concentrations the square-root relationship is again expected, since [S'] < K_m(app). Such behavior was in fact observed.

Values of k_c(app) and K_m(app). The combined effect of factors (1) and (2) on the kinetic parameters is readily seen from the results. The value of k_c is reduced in the supported enzyme from 273 sec^{-1} for the free enzyme to 81.5 sec^{-1}. The value of K_m is reduced from 1.73×10^{-4} to 0.90×10^{-4} M. These values for the supported enzyme are referred to as k_c' and K_m'.

The K_m' value for the supported enzyme (*cf.* eq 4) is of considerable importance in a comparison of theory with experiment, and corresponds to the case where F is unity, *i.e.*, where diffusion control is not evident. The shape of the curve in Figure 7 suggests that the results at the lowest enzyme concentration are very close to the diffusion-free limit, since the curve levels off. This particular plot was made because eq 4 shows that $F \propto 1/K_m$(app) and eq 6 shows that $\gamma l \propto [E]_m^{1/2}$, hence a plot of $1/K_m$(app) against log $[E]_m$ should show the same falling off as does a plot of F against γl (Figure 7); a comparison of Figures 6 and 7 shows that this is the case.

On the assumption that little diffusion control is involved at the lowest enzyme concentrations, a value of K_m' can be estimated from eq 6 as

$$K_m' = \frac{K_m(\text{app})}{PF} = 0.90 \times 10^{-4} \text{ M}$$

with $F = 1$ and $P = 0.89$ (Table I). With the use of this value, and the K_m(app) values at higher enzyme concentrations, it was then possible to calculate F values over the range of enzyme concentrations. The values obtained in this way are shown in the next-to-last column of Table IV. In Figure 7 these values are shown plotted as solid circles. The agreement is very satisfactory. There is still some slight diffusion control at the lowest γl value, the highest value of F being 0.93.

The open circles in Figure 7 correspond to F values calculated in an alternative manner, details of which will be given in a forthcoming paper by Kobayashi and Laidler; these F values are given in the last column of Table IV.

All in all, the main results obtained in the present experimental study are in very satisfactory agreement with the theory of Sundaram *et al.* (1970). This agreement is best exhibited by Figure 7.

Application of Biological Systems. In the light of the preceding discussion it is of interest to consider whether some measure of diffusion control is expected in biological systems. Muscle presents an obvious possibility.

According to Morales and Botts (1952) the density of muscle is ~1.0 and the fraction of it that is myosin (ATPase) is ~0.1. With a molecular weight of 2×10^7, 1 l. of muscle therefore contains 5×10^{-6} mole of myosin. The kinetic parameters for myosin at 25° are approximately (Ouellet *et al.*, 1952): $k_c \approx 100$ sec^{-1} and $K_m \approx 10^{-5}$ M. (It does not matter if the enzyme molecule has many active centers since we are concerned with $k_c[E]_m$). If $D = 10^{-6}$ cm^2 sec^{-1}, and the above parameters are valid for the supported enzyme

$$\alpha^2 = \frac{100 \times 5 \times 10^{-6}}{10^{-5} \times 10^{-6}} = 5 \times 10^7 \text{ cm}^{-2}$$

α is thus about 7×10^4 cm^{-1}, and diffusion control should thus become important if the thickness l is greater than about $0.1/7 \times 10^4 \approx 1.4 \times 10^{-6}$ cm = 140 Å.

It therefore appears that the muscle system is one for which there may well be substantial diffusion control under physiological conditions.

Acknowledgment

The authors are indebted to Dr. T. Kobayashi for valuable discussions and suggestions. They are also grateful to Mr. A. Marchetti and Mr. R. Larose for some preliminary work on this problem, and to the Departments of Biology and Geology of the University of Ottawa for loans of equipment.

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Mechanism of Activation of Bovine Procarboxypeptidase A S_5. Alterations in Primary and Quaternary Structure†

Jack R. Uren‡ and Hans Neurath*

ABSTRACT: A reevaluation of the chemical and physical properties of bovine procarboxypeptidase A S_5 has confirmed the majority of the properties described by Brown *et al.* (Brown, J. R., Yamasaki, M., and Neurath, H. (1963), *Biochemistry 2*, 877). A molecular weight of 63,000 was determined by self-association analysis of high-speed sedimentation equilibrium data and by sodium dodecyl sulfate gel electrophoresis. The effects of various salts on the proteolytic activation demonstrated that calcium ions greatly enhance the rate of activation of the zymogen and broaden the specificity of activation by other proteolytic enzymes. These effects may be related to the observation that calcium ions also promote subunit dissociation. Isolation of the products of activation demonstrated that both trypsin and chymotrypsin generate carboxypeptidase A_α with some of the β form present when trypsin is the activating enzyme. The activation of subunit II was analogous to the formation of π-chymotrypsin and 12 residues of the B chain of subunit II were sequenced and compared to those of other serine proteases.

Bovine pancreatic procarboxypeptidase A exists in two forms which differ from each other in sedimentation coefficient and chromatographic behavior on ion-exchange columns (Brown *et al.*, 1963). One of these, procarboxypeptidase A S_5 (PCP A S_5),[1] has a sedimentation coefficient of 5 S and is composed of two subunits (I and II). Subunit I is the immediate precursor of carboxypeptidase A whereas subunit II is the zymogen of an endopeptidase similar to chymotrypsin (Brown *et al.*, 1963; Peanasky *et al.*, 1969). Procarboxypeptidase A S_6 (PCP A S_6) has a sedimentation coefficient of 6 S and contains, in addition, a third subunit (III) which is believed to be an inactive derivative of subunit II (Behnke *et al.*, 1970).

† From the Department of Biochemistry, University of Washington, Seattle, Washington 98195. *Received June 21, 1972.* Taken in part from a dissertation submitted by J. R. U. to the Graduate School of the University of Washington in partial fulfillment of the requirements for the degree of doctor of philosophy. This work has been supported by research grants from the National Institutes of Health (GM 15731) and the American Cancer Society (NP-18N).

‡ Present address: Department of Pharmacology, Sterling Hall of Medicine, Yale University, New Haven, Conn. 06510.

[1] The following abbreviations are used: PCP A S_5, procarboxypeptidase A S_5; PCP A S_6, procarboxypeptidase A S_6; HPLA, D,L-hippuryl-β-phenyllactic acid; CGP, carbobenzoxyglycyl-L-phenylalanine; ATEE, acetyl-L-tyrosine ethyl ester; DFP, diisopropyl phosphorofluoridate.