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MODULATION OF MOLECULAR AND KINETIC PROPERTIES OF ENZYMES UPON IMMOBILIZATION

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Pea seedling glutamate dehydrogenase(EC 1.4.1.2) and Jack bean urease (EC 3.5.1.5) were immobilized on aminoethyl cellulose by cross-linking in a two-step reaction with glutaraldehyde. Specific activities of the immobilized dehydrogenase and urease were about 14% and 87%, respectively, of the original material in free solution. Both immobilized enzymes show no appreciable change in their pH dependence, whereas a less efficient binding of the substrates is suggested by the increased apparent Michaelis constants (K_m app.). Sigmoid kinetics were observed for both enzymes when reactions were carried out in a packed bed. Diffusional effects are considered responsible for producing these anomalous kinetics. The implications of these perturbations in terms of the catalytic efficiency of the enzymes, as well as the practical problems involved in the analysis of the kinetic data, are discussed.

INTRODUCTION

Because until recently efforts in enzymology have been concentrated upon the isolation and purification of enzymes to homogeneity, one of the first steps in the purification of a membrane-bound enzyme is usually the stripping of the enzyme from its membrane support. Studies of enzymes purified in this way cannot take into account effects that the membrane support may have on the catalytic properties of these enzymes. The situation becomes even more complicated if the enzyme possesses allosteric properties since subtle changes in effector concentrations can bring about appreciable changes in the enzymic activity (1). As already shown by Sundaram, Tweedale, and Laidler (2), substrates and other effector molecules can partition themselves between a membrane and its surrounding medium depending upon their relative solubilities in the two phases. Thus it is vitally

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important to study the properties of these enzymes in situations more closely resembling their natural state.

Thanks to the recent developments in the field of immobilized enzymes we are not only able to recognize this fact but are in a position to investigate the possible "membrane-support" effects by studying the properties of these enzymes in the immobilized form. However it is implicit that none of the polymers that have so far been used as solid supports for immobilizing enzymes, including the one used in this study, are as complex as the natural membrane in its chemical composition and structure.

This study was originally conceived as an investigation into the allosteric properties of a plant enzyme in solution and in the immobilized form. However, independent observations of sigmoidal kinetics with a nonallosteric enzyme in the immobilized form, by us and as reported elsewhere in the literature (3), prompted us to examine the cause and effect of the latter phenomenon.

EXPERIMENTAL

Materials

Glutamate dehydrogenase from roots of pea seedlings was purified about 150-fold, that is, up to and including the ammonium sulfate step, and free enzyme was assayed by the method of Pahlich and Joy (4). One unit of activity represents the amount of enzyme catalyzing the oxidation/reduction of 1 μmol of coenzyme (NADH, NADPH, or NAD^+) per minute.

Urease V was obtained from Sigma Chemical Co., St. Louis, Missouri (USA) with specific activity of 3500 Sigma units/g or 244 IU/g at pH 7.0 and 30°C.

2-Oxoglutaric acid (α-keto glutaric acid), glutamic acid, and urea were also obtained from Sigma Chemical Co. AE-cellulose[4] was supplied by Nutritional Biochemical Co., USA.

Immobilization of Glutamate Dehydrogenase

GDH was immobilized on AE-cellulose by the two-step method of Sundaram and Hornby (5). The first step consists of the activation of the AE-cellulose with glutaraldehyde, the cross-linking agent, followed by the coupling of the enzyme to the activated polymer during the second step.

The AE-cellulose was washed alternately in 0.5 M NaOH and 0.5 M HCl and finally with deionized water until all the chloride ions were

[4]Abbreviations used: AE-cellulose (aminoethyl cellulose), GDH (glutamate dehydrogenase), NADH (reduced nicotin adenine dinucleotid), NADPH, NAD^+.

removed. During the washing procedure an average of about 6% of fines was removed from the cellulose.

One gram of the washed AE-cellulose was added to 25 ml of 1% glutaraldehyde solution already adjusted to pH 9.4 with alkali and the mixture was stirred for 30 min at 22°C. The AE-cellulose, which had turned golden yellow by this time, was filtered in a buchner funnel and washed with deionized water. The washed polymer was suspended in 20 ml of ice cold water and 5 ml of enzyme solution at a concentration of 1 mg/ml was added. The pH was maintained constant by an automatic titrator. After 1 h of stirring at about 0°C little further change in pH was observed. The coupling mixture was transferred to the coldroom and left stirring overnight. The protein solution from the coupling mixture as well as the washings were then recovered quantitatively for protein determination by first washing the immobilized enzyme with 200 ml of 0.1 M $NaHCO_3$ and 1 M NaCl followed by a liberal wash with ice cold deionized water. The final preparations which had been washed free of any adsorbed protein were stored at 4°C in Tris buffer pH 7.75 (GDH) or in phosphate buffer pH 7.0 (urease).

Urease Preparation

Five milligrams of urease in 0.14 M mercaptoethanol, 1 mM EDTA, pH 7.2 was coupled to 100 mg of AE-cellulose maintained at the same pH during coupling for 1 h.

Determination of the Amount of Enzyme Coupled to the Matrix

The amount of protein attached to the matrix was estimated by a comparison of the amount of protein left in the residual coupling mixture with the initial amount of enzyme protein used in the experiment.

Both the Lowry method (6) and OD measurements at 260/280 nm as per Warburg and Christian (7) were used on the original and residual enzyme coupling solutions. The amount of enzyme immobilized was estimated by hydrolyzing 20-mg samples in 6 m HCl at 120°C for 24 h in glass tubes sealed under vacuum. The amount of (hydrolyzed) protein in the solution was determined by the Lowry method. Suitable blanks of the glutarated AE-cellulose were used.

Enzyme Assay Procedures

The activity of immobilized GDH was assayed by pumping mixed substrate solutions through a 3.5×1 cm water-jacketed column packed with the immobilized enzyme maintained at 25°C. The complete assay mixture

contained: Tris buffer, pH 7.75, 180 mM; ammonium sulfate, 300 mM; 2-oxoglutarate, 15 mM, and NADH, 0.5 mM. The column formed part of a closed circuit with the column effluent dropping into a stirred beaker containing the bulk of the substrate mixture from which it was recirculated to the column through a flow-through cell held in a Zeiss PMQ II spectrophotometer. OD at 340 nm was continuously recorded on a Sargent SRLG pen recorder. Flow rates were controlled (usually at 14 ml/min) by means of a Buchler peristaltic pump. Before each assay the column was first washed with a small volume of fresh assay mixture, and then a total of 20 ml was recirculated in the closed system. In some cases small volumes of substrates and coenzyme were slowly added to an already circulating system. This proved quite satisfactory, as long as time was allowed for the reaction rate to stabilize and correction made for the small changes in volume. Care was taken to keep the column height constant, taking the same volume of AE-cellulose–GDH suspension in the different experiments. The head of the substrate solution above the packed column was also kept constant. A final determination for activity calculations was made by weighing the dried material from the column at the end of each experiment.

AE-cellulose–urease was assayed using an open-ended column system by pumping 0.05 M urea, phosphate buffer ($I = 0.1$), pH 7.0, 10^{-3} M EDTA, at a flow rate of 0.9 ml/min through a column of 2×0.15 cm maintained at 25°C. The ammonia produced was estimated in aliquots of the effluent by the Berthelot reaction as adapted by Sundaram and Crook (8).

The choice of column dimensions as well as the flow rates and other conditions were based on subjective considerations. Thus a high flow rate for immobilized GDH columns was used to conserve the excessive consumption of the expensive cofactor. The low flow rate for the urease column was to achieve high catalytic rates.

Activities of immobilized preparations are expressed as μmol substrate converted per min/mg of dry preparation (U/mg) as recommended by the Committee for Standardization of Nomenclature in Enzyme Technology (9).

RESULTS

The exchange capacity of AE-cellulose was found to be 0.9 meq/g of dry material by titration in the presence of 0.1 M KCl. Taking this into consideration the concentration of glutaraldehyde in the activation step was arranged to be in slight excess of the available–NH_2 groups on the polymer. The polymer thus activated could be stored dry and used for the coupling of enzymes.

TABLE 1. **Influence of pH on the Coupling of Glutamate Dehydrogenase to AE-Cellulose**[a]

pH of coupling reaction	Milligrams of GDH bound to 1 g of AE-cellulose	% of GDH bound	AE-cellulose–GDH		
			Activity (mU/mg cellulose)	Specific activity (mU/mg bound protein)	% specific activity of free enzyme
6.0	1.29	25.8	0.274	212	14.2
7.0	1.25	25.0	0.231	185	12.36
8.0	2.4	48.0	0.564	235	15.7
9.0	1.85	33.3	0.361	195	13.05

[a]Five milligrams of partially purified pea root GDH was supplied in each coupling reaction, performed as described under Methods. The free enzyme had a specific activity of 1495 mU/mg of protein (for NADH activity).

Table 1 shows the pH-dependence of the coupling of GDH to AE-cellulose that has been activated with glutaraldehyde. The total and specific activities of the different immobilized GDH preparations are also included in the table.

Properties of the Immobilized Glutamate Dehydrogenase

In each activity determination with the enzyme preparation, the reaction rate became constant after about $1\frac{1}{2}$ min, and thereafter remained linear for at least 15 min. No activity was detected when any one of the substrates was omitted.

In spite of large differences in the amount of enzyme coupled, specific activities of the preparations are relatively close, varying within approximately ±10%. The pH 8.0 preparation shows the highest coupling yield with the maximum specific activity. Preparations coupled at this pH were used in the study of kinetics. The activity of the immobilized preparation was only about 14% that of the original enzyme.

The pH activity profiles of the different preparations of AE-cellulose–GDH are given in Figure 1, measured at optimum conditions for the forward reaction, that is, for the reductive amination with NADH. The pH optimum for all the preparations was around 7.8, although the shape of the pH profiles varied. The narrower profile of the pH 6 preparation resembled most closely that of the free enzyme, which has a pH optimum of about pH 8.0 (4).

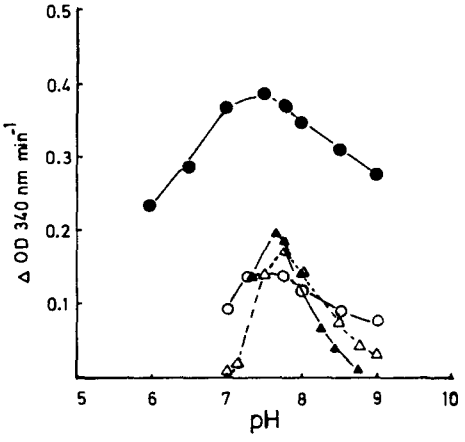

FIG. 1. The effect of pH of coupling on pH optima of immobilized GDH preparations. Coupling pHs were: 6.0 (▲); 7.0 (△); 8.0 (○); 9.0 (○). Activities for each preparation were measured as described under Methods, and the pH of the substrate solution was continuously monitored during each determination.

Figure 2 shows the effect of ammonium ion concentration on the activity of the immobilized GDH, with other substrates at saturation levels. The curve is markedly sigmoidal compared with the normal hyperbolic curve of the Michaelis–Menten type obtained when a similar experiment is performed with the purified free enzyme. This sigmoid form is unchanged by the addition of glutamate. Sigmoid curves are also obtained for the immobilized GDH when 2-oxoglutarate is the varied substrate and in this case the sigmoidicity is enhanced and K_m app. is increased by the addition of glutamate or NAD^+, the end products of the reaction (Fig. 3) both of which compete with the binding of 2-oxoglutarate to the enzyme. While the addition of glutamate during the assay does not show immediate changes, it depresses the activity on the subsequent addition of 2-oxoglutarate. The curve becomes strongly sigmoidal on the addition of NAD^+ to the substrate mixture before the start of the run. D-Glutamate produced a stronger effect than L-glutamate in altering the response to 2-oxoglutarate. EDTA (0.1 mM) and citrate (2.5 mM) had little influence on the activity, whereas aspartate produced a slight activation.

Lineweaver–Burk plots of these data do not produce a straight line graph; thus, dependable K_m values cannot be obtained. A value for the K_m may be obtained from the sigmoidal plots as the substrate concentration producing half maximal velocity, although this is only an approximate value and should be denoted as the "apparent K_m." Values for K_m app. for the various immobilized GDH preparations are given in Table 2. The values for the different preparations are similar, but vary considerably from the K_m values for the free enzyme. The K_m app. values for 2-oxoglutarate and

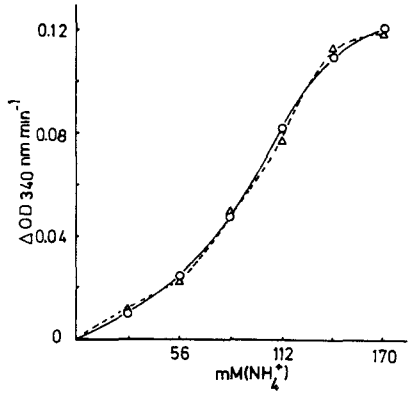

FIG. 2. The influence of ammonia concentration on the activity of immobilized GDH preparation, measured in presence (△) and absence (○) of 30 mM glutamate.

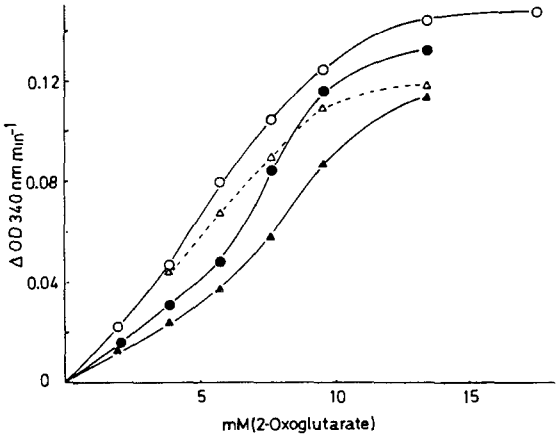

FIG. 3. The influence of 2-oxoglutarate concentration on the activity of immobilized GDH preparation (○–○) in the absence of glutamate; (△–△) 30 mM glutamate added during the assay; (▲–▲) 30 mM glutamate added at the start of the assay; (●–●) 0.15 mM NAD^+ added at the start of the assay.

ammonium ions are increased by about 2 to 4 times over the corresponding K_m values of the free enzyme.

When stored at 4°C, the immobilized GDH lost only about 10% of its activity over a month, during which time it was used several times a week for assays run at 25°C. Although the free enzyme is fairly stable (4), stability during longer storage is greatly increased by immobilization.

The results discussed refer to experiments carried out at a flow rate of about 14 ml/min. It was found that properties and activities did not change markedly in the range 11 to 16 ml/min for the size of the column employed. At higher flow rates activity decreased, since the higher pressures that were generated caused channeling and uneven flow in the columns.

In addition to NADH activity, the immobilized GDH retained NADPH and NAD^+ (deamination) activity. These activities were approximately of the same ratio as in the free enzyme (NADH:NADPH:NAD^+ was approximately 11:12:1).

Properties of Immobilized Urease

The coupling of urease to the AE-cellulose was somewhat more efficient than for GDH. Approximately 54% of the 5 mg of urease protein became bound to only 100 mg of glutaraldehyde treated AE-cellulose. The bound enzyme retained about 87% of the activity of the free enzyme.

TABLE 2. **Apparent Michaelis Constants of Various AE-cellulose–GDH Preparations Coupled at Different pH Values**[a]

pH of coupling of enzyme to cellulose	6.0	7.0	8.0	9.0	Data for free enzymes
K_m app. for 2-oxoglutarate	8.0×10^{-3} M	8.5×10^{-3} M	8.4×10^{-3} M	7.8×10^{-3} M	3.3×10^{-3} M
K_m app. for ammonia	1.36×10^{-1} M	1.3×10^{-1} M	1.4×10^{-1} M	1.4×10^{-1} M	3.8×10^{-2} M

[a] 3.5 × 1 cm columns of the immobilized-GDH preparations, coupled at different pH values, were all assayed at pH 7.75 as described under Methods. Free enzyme data from Pahlich and Joy (4).

Figure 4 shows the pH profiles of free and immobilized urease. The immobilized enzyme showed a much sharper curve, and the optimum was shifted by about 0.4 units to the acid side.

The effect of urea concentration on the activity of the AE-cellulose–urease preparation is shown in Fig. 5. This curve also deviates from the normal Michaelis–Menten hyperbola, but the effect is not so marked as is exhibited by the immobilized GDH. However, it is sufficient to make a Lineweaver–Burk plot useless, giving an intercept on the "wrong" side of the origin, as shown in the inset to Fig. 5. As described earlier, only apparent K_m values can be determined using the Michaelis–Menten plot and for the urease preparation values of 12–15 mM were obtained. This is about 5-fold greater than the value for the free enzyme.

Free urease is highly sensitive to end product inhibition and this sensitivity is retained by the immobilized preparation as shown in Fig. 5. Increasing concentrations of ammonia added initially to the substrate progressively inhibit the activity of the enzyme preparation and the apparent K_m for urea increases, although only slightly. The sigmoid portion of the curve is displaced toward higher substrate concentrations as the ammonia concentration is increased. At an ammonium ion concentration of 10^{-2} M the inhibition is almost complete and the sigmoidicity virtually disappears.

The storage stability of urease was increased appreciably by immobilization. A column of the urease preparation lost activity steadily over a 3-week period when it was used repeatedly at room temperature, and at the end of the period retained about 26% of its initial activity. A preparation of free urease in solution with similar activity was exposed to room

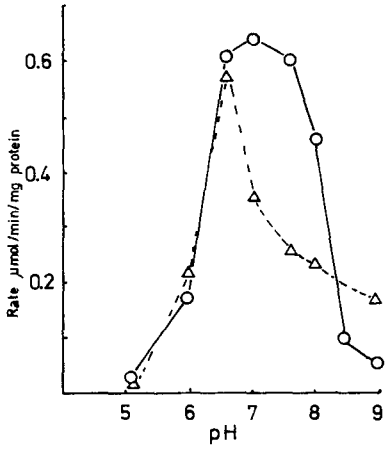

FIG. 4. pH Optima of free urease (○) and immobilized urease preparation (△).

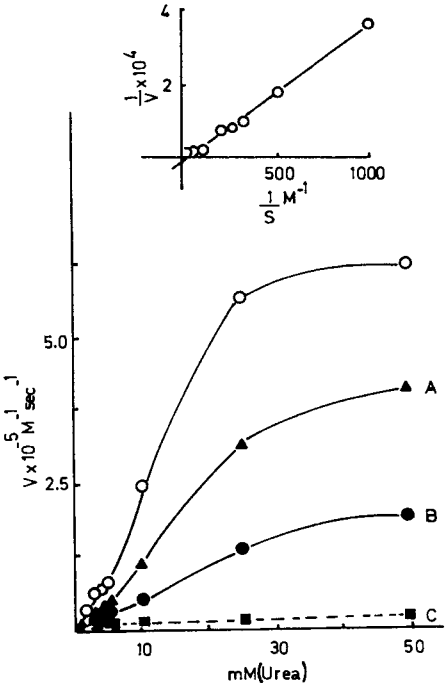

FIG. 5. Effect of urea concentration on the activity of immobilized urease preparation, and the influence of 10^{-4} M (A), 10^{-3} M (B), and 10^{-2} M (C) ammonia on the reaction. Inset: Lineweaver–Burk plot of data obtained in absence of ammonia.

temperature each time the column preparation was used; this free enzyme retained less than 15% of its original activity at the end of the 3-week period.

DISCUSSION

The coupling of an enzyme to insoluble support material by a one-step reaction where protein, polymer, and cross linking agents are mixed together is often an unsatisfactory procedure. When a one-step process was tried with the enzymes used in this work the GDH preparation was completely inactive, and immobilized urease showed only about 5% of the activity of a preparation made by the two-step process described. The two-step process is thus found to be useful for AE-cellulose as for nylon (5) and the intermediate glutaraldehyde-treated cellulose is stable. Both the first and second steps of the coupling procedure may be carried out at room temperature as long as the enzyme is relatively stable. Conditions for the

binding of the protein must be carefully defined to obtain the maximum activity of the final product, although it is clear that binding conditions do not cause significant variations in the behavior of different immobilized GDH preparations.

In this study it was our intention to work with small amounts of enzymes and it was not our aim to achieve maximum coupling yields.

The narrow pH profiles as seen in the pH 6 preparation widens with increasing coupling pH (Fig. 1). This suggests that the ionization constants of the active groups in the active site are altered in some way during the immobilization procedure. In fact it may be noticed that the ionization constants, that is, the pK_1 and pK_2 of GDH in the immobilized state obtained by the method of Dixon and Webb (10), changes in the different preparations. The pK_1 tends to go down whereas the pK_2 goes up in going from the pH 6 preparation to the pH 9 preparation.

The isoelectric point of GDH is about 5.0 and as the protein acquires more net negative charge with increasing pH during coupling, it is likely to have a stronger interaction with the positively charged supporting polymer. This could lock the enzyme into a more rigid conformation and be reflected in a noticeable change in the two pK values of the active site. Since GDH is an enzyme made of several subunits, it is easy to see how the introduction of a new degree of rigidity that is alien to the molecule in solution could affect its ionization characteristics and consequently its kinetic properties.

The other probability is that there is some kind of internal buffering within the microenvironment of the "polymer cage" as it were, which is different for the enzymes immobilized at various pH values.

This could explain the narrow pH profile of immobilized urease where it may be seen that pK_2 of the enzyme shifts to a lower value by about 1.25 pH units upon immobilization, whereas pK_1 remains unchanged (Fig. 4). It was observed earlier that when urease was immobilized on kaolinite by adsorption (7) pK_1 remained unaltered whereas pK_2 shifted by about 0.3 units toward the acid side. Similarly, it was recently observed with the enzyme covalently bound to CM-cellulose and CM-Sephadex (Sundaram, unpublished) by the EEDQ method (11) that again the pK_1 was unchanged whereas the pK_2 moved up by about 0.3 units. Thus it seems reasonable to conclude that the ionization of one of the ionizing groups at the active site of the enzyme is sensitive to the microenvironment provided by the polymer support.

Working with bovine liver GDH protected by substrates during coupling (12,13) obtained a higher specific activity with their immobilized enzyme preparations. Since we did not protect our enzyme during the coupling procedure it is not surprising that our preparations retained only 14% of the original activity.

A number of factors, including diffusion effects and electrostatic interactions have been suggested (14) to cause a shift in the pH optima of the immobilized enzymes as well as an increase or decrease in their K_m app. values. In the absence of any other major factors such as electrostatic interactions, effects such as diffusion or mass transfer may explain the 3 to 5-fold increases in the various K_m app. values obtained for both the enzymes in this study.

The K_m app. values for ammonium ions, 2-oxoglutarate, and the specific activity are almost the same for all the immobilized GDH preparations (Tables 1 and 2) even though the shape of the pH profiles and the amount of protein coupled to the polymer are different for the various preparations. This reveals that neither the binding constants, that is the K_m app. values, nor the specific activities of the immobilized enzymes, are affected by the ionization characteristics of the active groups of the enzyme.

While it may be convincing to invoke a regulatory effect to explain the sigmoid curves in the case of 2-oxoglutarate, the similar sigmoid response elicited by varying the concentration of ammonium ions appears less easily explained (Fig. 2) considering that the addition of 30 mM glutamate to the assay mixture at the start does not make any difference at all to the curve. Besides, the free enzyme does not display similar properties. The NH_4^+ ion-induced sigmoid curve very closely resembles that caused by the addition of 0.15 mM NAD^+ (●–● in Fig. 3).

It seems unlikely that charge effects are involved in producing the abnormal kinetics since pH optima are relatively unaffected; moreover both anionic and cationic substrates produce sigmoidal effects with immobilized GDH. Nor can it be due to any artefact in the form of a progressive substrate depletion in the columns, for it may be seen that not more than 10% of the substrate is converted even in the extreme case for each passage through the column for both GDH and urease. Thus, a combination of several factors, possibly interacting with different populations of bound enzyme molecules, may be responsible for the observed abnormal kinetics.

Double reciprocal plots of the Lineweaver-Burk type gives a nonlinear plot curving inward into the $1/v$ axis for 2-oxoglutarate variation (○–○, in Fig. 3) and similar curved lines in addition to a biphasic tendency for curves of assay with glutamate (▲–▲) and NAD^+ (●–●) added to the assay mixture. Both the lines in Fig. 2 also show inward curvature.

Julliard, Godinot, Gautheron (12) found that double-reciprocal plots lost their linearity for a reaction involving the deamination of glutamate in the presence of NAD^+. Havekes, Buckmann, and Visser (13) obtained biphasic double-reciprocal plots in some cases with a sharp change in the slope. Thus it is possible to obtain two different K_m app. values from their Lineweaver–Burk plots depending upon whether the curve is extrapolated

from very high or very low $1/s$ values. With their Sepharose-GDH preparations made at pH 9.0, the authors also obtained a negative intercept on the $1/v$ axis of their Lineweaver–Burk plots.

For the AE-cellulose–urease preparation, addition of the end product markedly inhibits activity with only small changes in sigmoidicity. A sigmoid curve of the general type found for the immobilized preparations would be produced by a process in which enzyme activity is proportional to some power (greater than one) of the substrate concentration ($[S]^n$, where $n > 1$) combined with limitation to some maximum value caused by saturation of the active sites. Since diffusion is directly proportional to substrate concentration (i.e., $n = 1$), diffusion itself cannot be controling the activity over the whole range of substrate concentration. However, a sigmoid curve could result from a combination of two separate limitations controling the activity at different substrate concentrations. We may imagine that an active site might have some "local" conditions that are separated by a nonstirred diffusion barrier from the "external" solution with known substrate concentrations flowing through the column. At low external substrate concentrations the activity (of the active site) might be diffusion limited, that is, the activity would be determined by the rate of the inward flux of the substrate to the active site. This would occur if the rate of substrate consumption was of a comparable magnitude to the rate of inward flux, and thus the concentration locally at the active site would remain considerably lower than that of the "external" solution. At higher external concentrations the flux of substrate molecules could surpass the rate of consumption by the active site. In this condition the local concentration would rise and begin more closely to approach the external concentration. This would allow the activity to approach normal Michaelian kinetics, with much less perturbation by diffusion limitations.

Nonlinear plots were also obtained for immobilized isocitric dehydrogenase by Chung (15). Kay and Lilly (16) in their study with chymotrypsin immobilized on DEAE-cellulose obtained unrealistic values for V_{max} from double-reciprocal plots. Thomas, Broun, and Selegny (3) have discussed similar problems based on their study with membrane-bound enzymes. In complex situations, the shape of the $[v]$ vs $[s]$ plots depends on at least two major factors—the range of substrate concentration in which diffusion has a significant effect ($s < K_m$, $s = K_m$, or $s > K_m$), and the extent of sigmoidicity of the curve.

Figure 6 is a collation of data from several findings that already exist in the literature. It is drawn stylistically to show the several possible variations of a $[v]$ vs $[s]$ curve that have been obtained with immobilized enzymes. The different K_m app. values are denoted as K_m^1 to K_m^6, the superscript 1 to 6 given to show which curves they correspond to (Table 3).

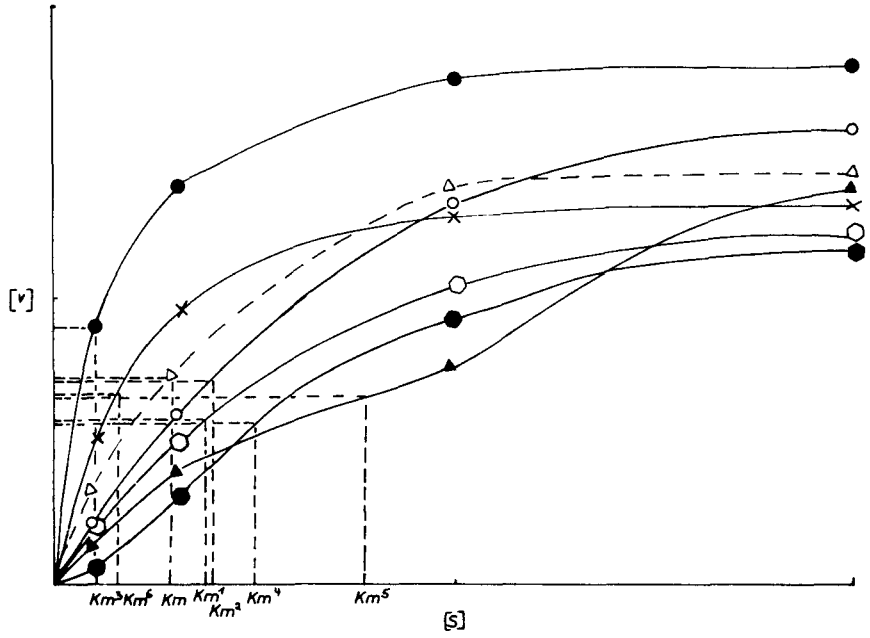

FIG. 6. A stylized plot to show the various kinds of $[v]$ versus $[s]$ plots obtained in the different studies. Curve △–△ denotes the normal curve of an enzyme in solution and others from curves 1 to 6 are for immobilized enzymes with their corresponding K_m app. value estimates denoted as K_m^1 to K_m^6. This figure is to be read along with Table 3 for more detailed information and analysis.

In dealing with anomalous kinetics it is prudent to analyze the data in greater detail over a wider range of conditions before arriving at any generalizations.

For an enzyme following the classical Michaelis kinetics it is sufficient to obtain the V_{max} and K_m values to define the pattern of the initial reaction velocity as a function of substrate concentration. However, for the sigmoid responses, although V_{max} still has the same implication, K_m does not sufficiently describe the situation. The recommendation of the Nomenclature Committee (9) to use the term K_m app. in the place of K_m seems to have been a prophetic coincidence in that, even in the case of classical allostery of reactions conducted in homogeneous solution, some workers suggested using "apparent K_m" to denote the substrate concentration at half-maximal velocity. Later, following a suggestion of Koshland, Nemethy, and Filmer (20), this quantity was designated as $(s)_{0.5}$. The fact that we now see that, in the case of immobilized enzymes, "sigmoid" curves may be caused by physical factors such as diffusion not only strengthens the choice

TABLE 3. **Anomalies Observed in Michaelis–Menten Kinetics of Immobilized Enzymes Due to Diffusion and Electrostatic Interaction and Their Significance**[a]

Enzyme support	Observation	Kinetic consequence	Reference
1. Ficin/CM-Cellulose	Increased K_m (K_m^1 app.) but decreased $k_{cat.}$	Poor binding and reduced $k_{cat.}$. Effect due to diffusion and electrostatic interaction	Many instances available in the literature: for example, Hornby et al. (17), Lilly et al. (18)
2. Urease/Kaolinite	Increased K_m (K_m^2 app.) and increased $k_{cat.}$	A true activation, that is, at high [s]	Sundaram and Crook (8)
3. GDH/AE-Cellulose	Increased K_m (K_m^4 app.) and sigmoid curve (sigmoidal portion at $s \gg K_m$)	Diffusion domination at $s \ll K_m$ leads to anomaly. Reduced $k_{cat.}$ may be due to manifold reasons	Present study
4. GDH/AE-Cellulose	Increased K_m (K_m^5 app.) and sigmoid curve (sigmoidal portion at $s \geq K_m$)	Probably due to a mixed population of polymers with widely varying physical properties leading to a wide range of K_m app. values. $k_{cat.}$ may asymptote to the same levels as free enzymes	Present study
5. G_6PDH-HK/Poly A·Am, Sepharose	Decreased K_m (K_m^3 app.) but increased $k_{cat.}$	Diffusion favors K_m and $k_{cat.}$ due to the higher concentration of substrates and products within the unstirred layer	Mosbach and Mattiasson (19)
6. Ficin/CM-Cellulose	Decreased K_m (K_m^6 app.) and decreased $k_{cat.}$	Reduced K_m due to diffusion and electrostatic interactions and reduction in $k_{cat.}$ due to kinetic and chemical and/or physical causes.	Hornby et al. (17), Lilly et al. (18)

[a]The different numbers appearing as superscripts to K_m app. values in various examples correspond with those given in Fig. 6. The numbering is for convenience of recognition of the various curves. The curve denoting K_m ($\triangle$–$\triangle$) may be assumed to be the normal Michaelis–Menten curve in Fig. 6. Abbreviations: CM-cellulose, carboxymethyl cellulose; poly A·Am, polyacrylamide; G_6, glucose-6-phosphate dehydrogenase; HK, hexokinase.

of the name K_m app. but also the method that was advised in obtaining that parameter. Thus in future work on immobilized enzymes it should be made clear what K_m app. values stand for and it is also absolutely essential to state whether the K_m app. values were obtained from the linear $[v]$ vs $[s]$ plots, the double reciprocal Lineweaver–Burk plot, or the single reciprocal Eadie–Hofstee plot.

Just as curvatures produced in Lineweaver–Burk plots due to sigmoid kinetics can give rise to the possibility of obtaining more than one value for V_{max} and K_m app., a similar dilemma can arise with the Eadie–Hofstee treatment as well. The data obtained with immobilized GDH give rise to atypical plots which range from a bell shaped curve to a shape ⌊⌒⌉, that is like the letter S turned 90° to the right. Here the dilemma lies in choosing between the slopes of the two vertical portions ⌊⌉. For obtaining $1/K_m$ values, the middle dotted line represents the discontinuity. The second vertical portion which corresponds to the high $[v]$ region of the plot usually gives a K_m app. value closer to that obtained from the linear plot as $[s]_{0.5}$. As pointed out by Engasser and Horvath (21) curvatures in Eadie–Hofstee plots indicate diffusion perturbation of reactions.

In a general analysis Sundaram and Pye (22) discuss the advantages and limitations involved in analyzing kinetic data of catalytic reactions at mixed interfaces by the various popular methods of kinetic analysis.

In an excellent review Engasser and Horvath (23) discuss the salient features of the various facets of diffusion and kinetics of immobilized enzymes that have been observed and characterized in the past decade and a half in various laboratories including ours.

The task at hand is to recognize a diffusion induced sigmoid kinetics and devise methods of obtaining realistic values for the kinetic parameters since, as shown in another paper (Sundaram, 24) diffusion effects through the unstirred layer can give high values of K_m app. and V_{max}.

The observations made in this paper raises some very important questions: (1) How does one differentiate between sigmoid kinetics produced by the molecular property of the enzyme and that caused by diffusion effects? and (2) what role does the support polymer or membrane play in the case of an immobilized or membrane-bound enzyme, that is, can the solid support or membrane act as an allosteric modifier, directly or indirectly? The latter can occur by inducing a substrate or a substrate analogue to act as an allosteric modifier of an immobilized or membrane-bound enzyme whereas in a homogeneous medium it does not possess that property? Or, similarly, can such a property be abolished by a membrane or solid support. If indeed the membrane can get directly or indirectly involved what are the implications? Little work has been done in this direction and it is worth a careful investigation.

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