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EFFICIENCY OF TWO ENZYMES IMMOBILIZED TO THE SAME SURFACE AND ACTING IN SEQUENCE
III. Criteria for Determining the Enhancement of the Sequential Rate by Enzymes Immobilized on the Same Surface

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To demonstrate that two enzymes immobilized to the same surface catalyzed their sequential reactions more efficiently than would be carried out if each of these two enzymes were immobilized on separate surfaces, a number of criteria were established and found to hold true. For these experiments the enzymes used were phosphoglucomutase and glucose-6-P-dehydrogenase. The carrier was comprised of cellulose particles activated with the linking reagent *s*-triazine trichloride. The criteria were (*a*) the presence of the intermediate, glucose-6-P, in the ambient solution at a concentration 33% lower than that expected by the amount of both enzymes present; (*b*) the sortening of the lag time; (*c*) the more efficient use of the initial substrate when both enzymes are immobilized to the same surface.

INTRODUCTION

In the previous article (2), we pointed out the importance of the Nernst layer in enhancing the overall rate of two enzymes immobilized to the same surface. The experiments concerned the effect on the overall rate of diluting the two enzymes under a number of conditions. In addition, we also emphasized the significance of the following factors in affecting this overall rate: enzyme purity and specific activity, binding methods, K_m, carrier, spacial arrangements, and reactor design.

In this paper we define three criteria for determining the degree to which the overall (sequential) rate of two enzymes immobilized to the same surface is enhanced. The theoretical basis of these criteria revolves around the view that by having the distance between the two immobilized enzymes

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shorter than the distance of the Nernst layer, the product of the first enzyme would have a better chance of becoming the substrate of the second enzyme before diffusing into the ambient environment away from those enzymes. The criteria are (*a*) the presence of the intermediate in the ambient solution at a concentration lower than that expected by the amount of both enzymes present, (*b*) the shortening of the lag time, and (*c*) the more efficient use of the initial substrate when both enzymes of the sequence are immobilized to the same surface.

MATERIALS AND METHODS

The materials used, the preparation of the *s*-triazine trichoride activated cellulose, the binding of the enzymes to the carrier, and the assays of soluble and immobilized enzymes are as described by Shimizu and Lenhoff (1,2).

Calculation of the Concentration of Glucose-6-P in the Bulk Solution

The assay solution was removed from the crucible containing the immobilized enzymes at some point during the sequential reaction. An aliquot of 1 ml was placed in a cuvette and the optical density at 340 nm was measured. Soluble glucose-6-P dehydrogenase was added and the enzyme allowed to react with the glucose-6-P still in the assay solution. After the reaction had reached completion the optical density was read again. The concentration of the glucose-6-P in the solution was calculated by dividing the change in optical density by the extinction coefficient of NADPH.

RESULTS

Concentration of the Intermediate During Early Lag Phase for Preparations Having Both Enzymes Immobilized on Separate Particles or on the Same Particle

Another method of evaluating the efficiency of sequential enzymes bound to separate particles involved measurements of the amounts of the intermediate substrate, glucose-6-P, in solution while the overall reaction was still in early lag phase (Fig. 1). The object was to determine if the concentration of glucose-6-P present in solution could account for the sequential rate observed at that point. Our assumption was that with the

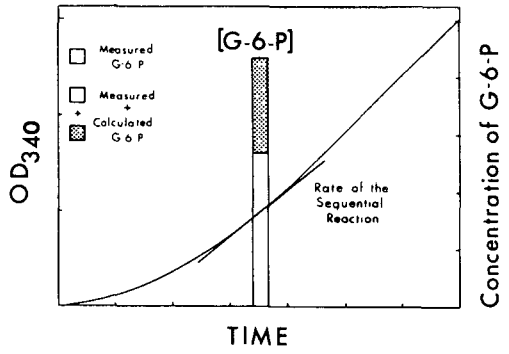

FIG. 1. Determination of the concentration of the intermediate in the bulk solution before the sequential reaction reaches the steady state. The sequential reaction was stopped and the concentration of glucose-6-P in the bulk solution (clear bar) was determined as described in the text under Materials and Methods. The calculation for determining the concentration of glucose-6-P required to give the observed rate (clear bar + shaded bar) is given in the text. The abbreviation G-6-P refers to glucose-6-P.

enzymes on the same particles, the level of glucose-6-P in the ambient solution would be less than that observed with the enzymes on separate particles, because in the first case the glucose-6-P would be consumed by the second enzyme before it could diffuse out into the ambient solution.

The experiment was carried out as follows. The sequential reaction for mixtures of glucose-6-P dehydrogenase and phosphoglucomutase was interrupted at points during the early lag period (see Fig. 1, tangent point). The concentration of glucose-6-P in the bulk solution at that time (Table 1, column *e*; Fig. 1, clear bar) was determined with soluble glucose-6-P dehydrogenase (see above) and compared to the concentration of glucose-6-P calculated from known apparent K_m values and a Lineweaver–Burk plot for immobilized glucose-6-P dehydrogenase to be required to give the observed rate (Tables 1 and 2, column *d*; Fig. 1, shaded plus clear bar). This calculation was made comparing the rate of the interrupted sequential reaction with the V_{max} of the glucose-6-P dehydrogenase (column *b*) of that preparation. For example, if that sequential rate, 4.76×10^{-4} μmol/min/ml (Table 1, column *a*) was only 0.04 (column *c*) of the V_{max} for glucose-6-P dehydrogenase of that preparation, 1.14×10^{-2} μmol/min/ml (column *b*) then, using the apparent K_m for glucose-6-P dehydrogenase, the estimated glucose-6-P concentration necessary to give 0.04 of the V_{max} rate was calculated to be 5.3 μM (column *d*). Column (*e*) expresses the actual concentration of glucose-6-P determined, 3.02 μM. Column (*f*), the ratio of the figures in columns *d* and *e*, shows that only 57% of the glucose-6-P needed to give the observed rate was free in the ambient solution.

TABLE 1. **Concentration of the Intermediate (Glucose-6-P) in the Ambient Solution for Preparations of Phosphoglucomutase and Glucose-6-P Dehydrogenase**

A. Enzymes immobilized to the same surface

(*a*) Sequential rate at time of interruption (μmol/min/ml $\times 10^4$)	(*b*) V_{max} of glucose-6-P dehydrogenase (μmol/min/ml $\times 10^3$)	(*c*) *a*/*b*	(*d*) [Glucose-6-P] estimated to give *a*/*b* (μM)	(*e*) Actual [Glucose-6-P] in ambient solution (μM)	(*f*) *e*/*d* $\times$ 100
6.35	23.6	0.0269	3.37	1.43	42.4
4.76	11.4	0.0416	5.3	3.02	57.0
2.7	9.87	0.0273	3.43	1.74	50.7
1.59	9.87	0.0180	2 20	1.43	65.0
1.59	8.59	0.0185	2.30	2.06	89.6
1.59	8.59	0.0.85	2.30	0.79	34.3
1.59	10.3	0.0154	1.91	1.35	70.7
0.79	6.83	0.0116	1.44	1.75	122.0
0.79	3.61	0.0220	2.75	1.90	69.1
					Mean = 66.8 $\pm$ 24.9

B. Enzyme immobilized to separate surfaces

3.49	22.2	0.0157	1.95	1.43	73.3
2.25	9.90	0.0284	2.84	2.54	89.4
1.90	11.7	0.0163	2.02	2.27	112.4
1.27	10.1	0.0126	1.55	1.67	108.7
0.79	4.11	2.41	2.41	2.22	92.1
					Mean = 95.2 $\pm$ 14.1

TABLE 2. **Lag Times for the Two-Enzyme Sequential Reaction**[a]

Amount of enzymes		Rates of reaction at low glucose-1-P levels		Lag time	
(*a*) Phosphoglucomutase (U/ml) × 10^4	(*b*) Glucose-6-P dehydrogenase (U/ml) × 10^4	(*c*) Phosphoglucomutase (μmol/min/ml) × 10^4	(*d*) Phosphoglucomutase +glucose-6-P dehydrogenase (μmol/min/ml) × 10^4	(*e*) Method 1 (min.)	(*f*) Method 2 (min.)
		Preparations with two enzymes immobilized to the same particles			
1. 4.76	36.06	2.3	1.48	2.04	4
2. 4.19	31.46	1.84	1.32	1.0	3
		Preparations with each of two enzymes on separate particles			
3. 3.93	41.06	1.58	1.31	7.26	16
4. 4.89	26.36	1.49	0.84	8.07	28
5. 36.34	222.22	7.26	7.04	3.24	8

[a]The lag time for preparations of immobilized phosphoglucomutase and glucose-6-P dehydrogenase were calculated was described in Shimizu and Lenhoff (1) in the presence of 5.2×10^{-5} M glucose-1-P (a concentration significantly lower than the K_m of immobilized phosphoglucomutase).

In summary, the overall results show that for preparations of enzymes immobilized to the same surface, the actual concentration of glucose-6-P in the bulk solution (column *e*) was generally lower than that estimated (column *d*) to account for the sequential rate. For the nine preparations studied, the average concentration of the glucose-6-P in solution was 67% of that calculated to be there. Preparations of the enzymes on separate particles (Table 1), on the other hand, had concentrations of glucose-6-P about 95% of the theoretical values. Hence, in the case of both enzymes bound to the same particles, it would appear that the concentration of the intermediate glucose-6-P immediately around the bound glucose-6-P dehydrogenase is significantly higher than found in the ambient solution.

Measurement of the Lag Times for Preparations Having Both Enzymes Immobilized on Separate Particles or on the Same Particle

The following experiments were carried out to determine whether or not the binding of the two enzymes to the same carrier (Table 2, preparations 1 and 2) would give sequential rates with shorter lag times that similar amounts of the same enzymes, each immobilized on separate carriers (Table 2, preparations 3 and 4). These measurements were done at phosphoglucomutase levels of about 4×10^{-4} U/ml because experiments measuring the sequential rates showed the greatest efficiency at these phosphoglucomutase levels, with the glucose-6-P dehydrogenase being in excess (see Ref. 2, Figs. 3 and 4).

Lag times were determined in two ways: first, by extrapolating the linear portion of the curve for sequential rates into the abscissa and noting the intercept (3) (Fig. 2, t_1) and, secondly, by approximating the time at which the sequential rate became linear (Fig. 2, t_2). For each preparation, the amount of phosphoglucomutase and glucose-6-P dehydrogenase were quantified by the usual method of assaying for each enzyme in the presence of high amounts of their respective substrates (Table 2, columns *a* and *b*). In addition, both the rates of phosphoglucomutase and the sequential rate were measured in the presence of 5.52×10^{-5} M glucose-1-P (columns *c* and *d*). This concentration was selected because it was significantly lower than the K_m determined for immobilized phosphoglucomutase.

The data (Table 2) show that in both experiments in which the enzymes were bound to the same particles (preparations 1 and 2), the lag times measured by either method were significantly shorter than the lag times of preparations in which the enzymes were bound to separate particles (preparations 3 and 4). It can be further noted that when the second enzyme, that is, glucose-6-P dehydrogenase, was not in great excess (preparation 4)

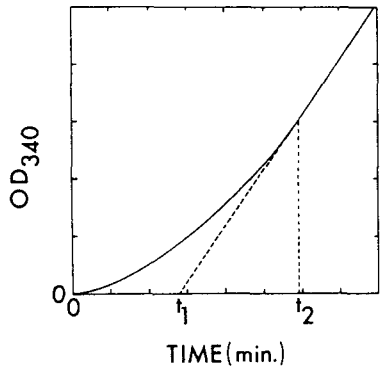

FIG. 2. Determination of the lag times for preparations of phosphoglucomutase and glucose-6-P dehydrogenase immobilized to the same surface and to separate surfaces. The sequential rates of the preparations were measured as described in Shimizu and Lenhoff (1). Two lag times, t_1 and t_2, where measured for each preparation: t_1 was calculated by extrapolating from the linear portion of the rate curve, t_2 from the point at which the rate became linear.

and when the enzyme were on separate particles, the lag time was even longer, especially when measured by the second method.

Lastly, with preparations in which the enzymes were bound to separate particles, the lag times were shortened significantly by increasing the amount of each enzyme on the particles about eight fold, although the ratio of the two enzymes to each other remained the same (e.g., compare the lag times for preparation 5 with those of preparation 4). Most likely the increase in the total amount of the enzymes present in solution resulted in a shorter distance existing between the enzyme molecules. Since the enzymes were in closer proximity, and the distance the intermediate must travel was presumably shorter, the lag time for the sequential rate decreased.

Effect of the Presence of the Two Enzymes Immobilized to the Same Surface Apparent Rate of Reaction of Phosphoglucomutase at Low Concentrations of Glucose-1-P

That the sequential enzymes immobilized to the same surface can react more efficiently than the same enzymes on separate particles as also observed while comparing the rates of utilization of low amounts of glucose-1-P by these two sequential systems. The relevant data to the above question come from two experiments in which excess soluble glucose-6-P dehydrogenase was added to the preparations. The added soluble glucose-6-P dehydrogenase guaranteed that we obtained accurate measurements of the rates of glucose-1-P consumption, because the soluble enzyme would pick

up the glucose-6-P as soon as it appeared in the bulk solution. With both preparations, the rates were measured after steady state had been reached.

In the first experiment, the steady-state rates of reaction of the phosphoglucomutase in these systems were measured at high concentrations of glucose-1-P (1.81×10^{-3} M) (Table 3, column 1) in order that the rate give an indication of the phosphoglucomutase present. In the second, the phosphoglucomutase was measured at low glucose-1-P concentrations (5.52×10^{-5} M); (Table 3, column 2) in order to tell us the rate at which these amounts of glucose-1-P were consumed in the sequential systems.

We noted that in all the cases in which the two rates were compared under these conditions, the rate of glucose-1-P consumption when its concentrations was low was always a greater percentage (Table 3, column 3) of the V_{max} of the phosphoglucomutase (i.e., the rate at high glucose-1-P concentration) in those preparations in which phosphoglucomutase and glucose-6-P dehydrogenase were immobilized to the same particles. For example, when phosphoglucomutase, giving maximal rates up to 39×10^{-4} μmol/min/ml while immobilized with glucose-6-P dehydrogenase to the same carrier, was assayed using low concentrations of glucose-1-P, the rate of the same phosphoglucomputase (Table 3, column 2) was about 37% (Table 3, column 3) that of the possible rate at which the phosphoglucomutase of these preparations could go at high glucose-1-P concentration (Table 3, column 1). In similar experiments using preparations of phosphoglucomutase bound to separate carriers and giving rates of 10 to 37×10^{-4} μmol/min/ml while the glucose-6-P dehydrogenase was also bound to separate particles, the phosphoglucomutase reacted at only 23% of the maximum rate possible under these conditions.

Another way to evaluate how low concentrations of the initial substrate (glucose-1-P) are handled by the two preparations is to look at the K_m's of the phosphoglucomutase under a number of conditions. The following experiments were carried out based upon two premises. First, the rate of the phosphoglucomutase is measured by the action of glucose-6-P dehydrogenase on the glucose-6-P formed by the phosphoglucomutase. Second, if low glucose-1-P is used to initiate the sequential reaction, and if the glucose-6-P dehydrogenase is either soluble or attached to another particle, then it would require more time for the glucose-6-P to build up in the ambient solution in sufficient amounts to react with the glucose-6-P dehydrogenase. Such would not be the case if the two enzymes were close to each other on the same particle, and as a result, small amounts of glucose-6-P would be picked up more readily.

These suppositions appear to be borne out by the K_m values shown in Table 4. The K_m's for soluble phosphoglucomutase for a preparation of only phosphoglucomutase immobilized to particles and for two preparations of

TABLE 3. **Rate of Reaction Immobilized Phosphoglucomutase at High and Low Concentrations of Glucose-1-P**[a]

Rate of reaction of phosphoglucomutase at High glucose-1-P μmol/min/ml $\times 10^4$	Low glucose-1-P μmol/min/ml) $\times 10^4$	$b/a \times 100$
Enzymes immobilized to separate surfaces		
48.8	8.7	18.6
45.9	8.5	18.0
37.4	7.1	18.9
36.3	7.3	20.0
35.9	6.3	17.6
34.5	8.5	24.7
21.8	4.6	21.0
17.8	5.2	29.2
13.8	3.4	24.6
10.1	2.9	28.9
		Mean = 25.4
Enzymes immobilized to the same surface		
39.2	10.9	27.9
34.4	11.6	33.8
32.5	9.6	29.5
28.1	9.5	33.7
26.3	9.5	36.2
23.2	8.4	36.2
21.5	7.6	35.1
16.7	6.5	38.9
15.9	6.3	39.9
15.0	4.8	32.2
14.8	5.8	38.9
9.8	5.8	58.2
9.7	3.8	39.6
		Mean = 36.9

[a] The rate of immobilized phosphoglucomutase was measured at 1.81×10^{-3} M and 5.52×10^{-5} M glucose-1-P. Both assays were done in the presence of excess soluble glucose-6-P dehydrogenase, as described in Shimizu and Lenhoff (1).

phosphoglucomutase and glucose-6-P dehydrogenase immobilized to the same particles were determined. Note that the apparent K_m for phosphoglucomutase immobilized alone on particles reacting with soluble glucose-6-P dehydrogenase is five times the K_m of soluble phosphoglucomutase. However, when the enzyme was immobilized with 1.43 U of glucose-6-P dehydrogenase to the same particles, the concentration of glucose-1-P

TABLE 4. **Effect of Increasing Amounts of Immobilized Glucose-6-P Dehydrogenase on the Apparent K_m of Immobilized Phosphoglucomutase**[a]

State of the phosphoglucomutase	K_m, or apparent K_m, values $\times 10^5$ (M)
Soluble	5.8
Immobilized	31.1
Immobilized to same particle as glucose-6-P dehydrogenase (at a ratio of 1:1.43)	17.2
Immobilized to same particle as glucose-6-P dehydrogenase (at a ratio of 1:16.1)	6.32

[a]The apparent K_m for immobilized phosphoglucomutase was determined for preparations having different ratios of glucose-6-P dehydrogenase to phosphoglucomutase activity. The apparent K_m's were determined using data from Lineweaver–Burk plots. The assays for immobilized phosphoglucomutase and soluble phosphoglucomutase were done as described in Shimizu and Lenhoff (1).

needed for $\frac{1}{2} V_{max}$ of phosphoglucomutase was only three times the K_m concentration of the soluble phosphoglucomutase. When the amount of the glucose-6-P dehydrogenase immobilized was increased to sixteen times the level of phosphoglucomutase immobilized on the same particle, the concentration of glucose-1-P needed for $\frac{1}{2} V_{max}$ of phosphoglucomutase decreased even further to 6.32×10^{-5} M, a value which approaches within 10% the K_m of the soluble phosphoglucomutase.

DISCUSSION

In this paper we have established and tested three criteria for testing whether or not two enzymes immobilized to the same surface catalyzed their sequential reactions more efficiently than would be if each of the two enzymes were immobilized on separate surfaces. Another criterion, described in our previous paper (2), was that the ratio of the sequential rate of the two enzymes bound to the same carrier to that of the V_{max} of the first enzyme did not diminish on dilution.

Concentration of the Intermediate in the Ambient (Bulk) Solution for Preparations Having Both Enzymes Immobilized on the Same Particles or Each Enzyme on Separate Particles

The results in Table 1 (part A) show that preparations of two enzymes immobilized to the same surface and acting in sequence give a concentration of the intermediate, glucose-6-P, in the bulk solution lower than that which can account for the rate of the second reaction of the sequence observed at that time. In contrast, preparations having the two enzymes on separate

particles (Table 1, part B) give a concentration of glucose-6-P in the bulk solution nearly equal to the concentration required for the observed rate. These results are consistent with the model presented earlier (2), in which the enzymes are present on the surface of the particle and are separated from the ambient solution by the Nernst diffusion layer. This layer will trap the intermediate at the particle surface, resulting in a higher concentration of glucose-6-P at the surface than in the ambient solution. By placing glucose-6-P dehydrogenase on the same surface it can react with the high level of glucose-6-P. In the preparations in which phosphoglucomutase and glucose-6-P dehydrogenase are immobilized to separate particles, the glucose-6-P redistributes itself by first diffusing into the ambient solution and then entering the Nernst layer of the particles, which has glucose-6-P dehydrogenase bound to it. As a result, there will be a lower concentration of glucose-6-P available to the glucose-6-P dehydrogenase bound separately than is available to the glucose-6-P dehydrogenase bound to the same surface with phosphoglucomutase.

This distribution of intermediates into areas of high and low concentrations was predicted by Davis (4) in his surface model for the channeling of metabolites. The model proposes that the segregation of intermediates will occur in the vicinity of a multienzyme aggregate if the affinity of the second enzyme for the intermediate is high and if the second enzyme is not saturated by the first enzyme with intermediate. In order to satisfy these conditions, we chose assay conditions and preparations such that the substrate concentration was less than the observed K_m for glucose-1-P and the immobilized glucose-6-P-dehydrogenase was present in excess over the phosphoglucomutase.

Our results suggest that a type of "channeling" is also taking place in the preparations having two enzymes on the same particle, although the mechanism involved may involve the Nernst layer rather than mechanisms that apply to the multienzyme complexes.

Shortening of the Lag Time for Preparations Having Both Enzymes Immobilized to the Same Surface

The data described in Table 2, preparations 1 and 2, are in agreement with the views of Goldman and Katchalski (5), the data of Mosbach and Mattiasson (6), Mattiasson and Mosbach (7), Bouin et al. (8), and Kock-Schmidt et al. (9); that is, the lag time for the sequential rate is shortened significantly (five to sevenfold) when the enzymes are bound to the same surface. Hence, it would appear that the second enzyme of the sequence is able to react with the intermediate substrate before it escapes from the Nernst layer.

The Apparent Rate of Reaction of Phosphoglucomutase at Low Concentrations of Glucose-1-P for Preparations Having Phosphoglucomutase Immobilized to a Surface and Phosphoglucomutase and Glucose-6-P Dehydrogenase Immobilized to the Same Surface

The results shown in Table 3 indicate that preparations having phosphoglucomutase and glucose-6-P dehydrogenase immobilized to the same surface enables the phosphoglucomutase to use low concentrations of glucose-1-P more efficiently than preparations in which only phosphoglucomutase is immobilized to the surface. Similarly, Table 4 shows that the apparent K_m's determined for preparations having phosphoglucomutase and glucose-6-P dehydrogenase immobilized to the same surface are lower than preparations having only phosphoglucomutase bound to the surface. That these results are in agreement is not surprising, because the determinations of the apparent K_m require measuring the rates at a wide range of glucose-1-P levels, including low ones.

For example, the results in Table 3 at low glucose-1-P concentrations can be interpreted to indicate that the product of the reaction with phosphoglucomutase, that is, glucose-6-P, was trapped in the Nernst layer and was utilized there by the glucose-6-P dehydrogenase immobilized to the same surface before it escaped into the ambient environment, where it would react with the excess soluble glucose-6-P dehydrogenase. That is not to say that equal amounts of phosphoglucomutase bound to particles either separately or with glucose-6-P dehydrogenase on the same surface have unequal rates. On the contrary, both preparations of phosphoglucomutase probably have equal rates of glucose-6-P formation, but with glucose-6-P dehydrogenase immobilized to the same surface, the glucose-6-P formed by the phosphoglucomutase is utilized more efficiently. A major feature of this system that must be recalled when interpreting these results is that the rate of the immobilized phosphoglucomutase is being measured in a coupled enzyme system, using excess levels of soluble glucose-6-P dehydrogenase. Hence, the rate of phosphoglucomutase is determined by the rate of appearance of glucose-6-P in the bulk solution.

A similar interpretation can apply to the observation of the change in the concentration of glucose-1-P needed to give a $\frac{1}{2} V_{max}$ for phosphoglucomutase immobilized to the same surface with immobilized glucose-6-P dehydrogenase. It is the rapid utilization of the glucose-6-P presumed to be trapped within the Nernst layer by neighboring immobilized glucose-6-P dehydrogenase that allows the apparent K_m of the immobilized phosphoglucomutase to approach that of the soluble enzyme. Another explanation for the results in Table 4 is that when glucose-6-P dehydrogenase and phosphoglucomutase are immobilized to the same surface, the interaction

between the two enzymes changes the affinity of phosphoglucomutase for its substrate, and that this affinity is affected by the amount of glucose-6-P dehydrogenase surrounding the phosphoglucomutase. While we do not have any data to eliminate this latter explanation, it seems unlikely that the interaction between adjacent phosphoglucomutase molecules should differ greatly from interactions between phosphoglucomutase and glucose-6-P dehydrogenase molecules.

Smith (10) has presented a theoretical discussion of the effect of the Nernst layer on the apparent K_m of an immobilized enzyme. The Nernst layer restricts the free diffusion of substrate to the surface bearing the enzymes, creating a gradient across the layer. Calculation of the true K_m could be obtained if knowledge of the thickness of the Nernst layer or the true V_{max} of the immobilized enzyme preparation could be determined. Kobayashi and Moo-Young (11) have also derived a model for determining the influence of the Nernst layer on the apparent K_m.

The effect of immobilization on the apparent K_m of immobilized enzymes was shown by Regan et al. (12), Hinberg et al. (13), and Kay and Lilly (14). They showed that by decreasing the size of the particles to which the enzyme was immobilized, the apparent K_m of the enzyme was lowered. The smaller size of the particles allowed the substrate to diffuse through the particles at the same rate as in solution, allowing complete access to substrate by all the enzyme molecules within the particles. Axen et al. (15) have shown that the high K_m of chymotrypsin immobilized to Sepharose could be lowered to almost the K_m of the soluble enzyme by digesting the Sepharose with dextranase.

Le Moullec and Thomas (16) have examined the kinetic properties of another two-enzyme sequential reaction. In their system, glucose-6-P isomerase and glucose-6-P dehydrogenase were cocross-linked to plasma albumin, and the activity of the first enzyme and the sequential rate were measured using a continuously stirred tank reactor. Their results showed that the value of the apparent Michaelis constant of the first enzyme decreased when the second unit's enzyme was functioning.

CONCLUDING REMARKS

In immobilized multienzyme systems, the number of enzymes that can be used in a sequential reaction may be limited, since the placing of three or more enzymes on the same surface will increase the distance between any two consecutive enzymes. This problem may be eliminated if the order of the placement could be controlled or if the enzymes were first arranged in a multienzyme complex prior to being immobilized.

The sequential enzymes do not necessarily have to act in a linear fashion, that is, going from A to B to C by enzymes E_a and E_b. Instead, the enzymes may act in a cyclical manner, with the two enzymes sharing a common cofactor such as NAD(P). During one cycle, NAD(P)H reacts with one enzyme and its substrate to form NAD(P) and a product; then the NAD(P) can react with a second enzyme and its substrate to regenerate the NAD(P)H and initiate another cycle. This system has been called by Lowry and Passonneau (17) "enzymatic cycling" and is another area of great potential use for immobilized enzymes. The use of enzymatic cycling with immobilized enzymes for diagnostic techniques and synthesis of rare organic molecules has been described by Lenhoff et al. (18), and several such systems have been prepared (19,20).

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