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# RELATIVE EFFICIENCIES OF A SOLUBLE AND IMMOBILIZED TWO-ENZYME SYSTEM OF GLUCOSE OXIDASE AND CATALASE

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## Summary

Dual catalysts of varying glucose oxidase (EC 1.1.3.4) and catalase (EC 1.11.1.6) activities were constructed by immobilization of the enzymes to silanized nickel silica alumina with glutaraldehyde. The amount of product formed and the efficiency of the systems were determined using either of the enzymes to initiate the cyclic reaction. The former depends on the activity of both enzymes in the system while efficiency describes how well the second enzyme in the sequence utilizes the intermediate of the reaction.

Efficiency was a function both of the ratio of the two activities and their absolute values. An increase in the activity of the second enzyme of the reaction sequence or a decrease in the activity of the first enzyme increased the efficiency. A higher efficiency was observed with higher absolute activities of the enzymes when the ratio of the enzymes was constant.

Dual catalysts were compared to mixed catalysts (each enzyme immobilized to separate particles) and to soluble, homogeneous systems at equal total activities. In general, the dual catalysts were superior to either of the others.

Computer simulations of the reaction rates indicated that all experiments were performed in the lag period of the two-step reaction.

## Introduction

In 1971 Katchalski and his associates [1,2] predicted that a membrane containing two enzymes, both following first-order kinetics, would, during the first stages of a reaction, produce end product at a faster rate than a homogeneous soluble enzyme system with the same two enzymes of the same activities. This phenomenon was attributed to the existence of an unstirred layer at the interface between the membrane and the bulk solution. They predicted that many factors would affect the relative improvement of the immobilized two-enzyme

system over the corresponding soluble one; these included the relative rate constants of the two enzymes, the thickness of the unstirred layer, the time of the reaction, the diffusion coefficient of the substrates, and the volume of the bulk solution.

Vasil'eva et al. [3] studied the kinetics of the coupled system of hexokinase and polynucleotide diphosphorylase in a homogeneous solution and in two nucleoprotein coacervates. In one case, the enzymes were included in different phases of the coacervate and in another case, the enzymes were both in the droplet phase. In the last case, the rate of coupled reaction was significantly increased over both the soluble system and the system in which one of the enzymes was in the droplet phase and one exterior to the droplet.

Mosbach and Mattiasson [4] examined the kinetics of the two-enzyme system of hexokinase and glucose-6-phosphate dehydrogenase immobilized to Sepharose and a copolymer of acrylamide-acrylic acid. The overall rate of the sequential reaction was greater with the immobilized system than with the same enzymic activities in homogeneous solution in the initial stages of the reaction. These authors attributed this to the increased concentration of the product of the first reaction (substrate of the second) in the microenvironment of the immobilized enzymes. The same workers [5] showed that a similar phenomenon occurred in a three-enzyme system in which β-galactosidase was incorporated into the two-enzyme system mentioned above except that the improved performance of the three-enzyme immobilized system compared to an equivalent soluble system lasted longer than the two-enzyme system. In another three-enzyme system of malate dehydrogenase, citrate synthase, and lactate dehydrogenase, Srere et al. [6] found that not only was the immobilized system superior to the soluble, but that the rate of a reaction which has an unfavorable equilibrium can be markedly improved in the immobilized system due to a decrease in distance between enzymes and a subsequent higher diffusion rate caused by the steeper concentration gradient of intermediate.

In the work reported here, we studied the two-enzyme system of glucose oxidase (EC 1.1.3.4) and catalase (EC 1.11.1.6) (Eqn. 1) immobilized to an inorganic support material. We determined how varying the activities and ratios of the two enzymes on the support material affected the efficiencies of the catalyst particles. The dual enzyme system, with both enzymes immobilized to the same particles, was compared to a mixed immobilized system where the enzymes were attached to different support surfaces and to a homogeneous two-enzyme soluble system.

$$\text{Glucose} + O_2 \xrightarrow[\text{oxidase}]{\text{Glucose}} H_2O_2 + \text{Lactone} \qquad (1)$$

$$H_2O_2 \xrightarrow{\text{catalase}} H_2O + \tfrac{1}{2}O_2$$

## Materials and Methods

*Materials.* Glucose oxidase (β-D-glucose : oxygen oxidoreductase: EC 1.1.3.4) from *Aspergillus niger* (110 I.U./mg) was purchased from Worthington Biochemical Corp. Lyophilized catalase (hydrogen-peroxide:hydrogen-peroxide

oxidoreductase: EC 1.11.1.6) from *A. niger* (9444 I.U./mg, glucose oxidase contaminant 0.00008%) was purchased from Calbiochem. Bovine serum albumin was obtained from Sigma. All enzymes were used without further purification. Glutaraldehyde was a product of Fisher Scientific Co. and glucose was obtained from Matheson, Coleman and Bell. The support used was a silica alumina impregnated with 5% nickel (No. Ni 0901-S, Harshaw Chemicals, Solon, Ohio). The silica alumina was ground and screened to a uniform size of 125—149 $\mu$m (100—120 mesh). The surface area and pore diameter of the support particles ranged from 0.5 to 1.0 $m^2/g$ and from 2 to 40 $\mu$m, respectively. All other chemicals used were the purest available commercially.

*Method of immobilization.* The support particles were first treated with $\gamma$-aminopropyltriethoxysilane to produce a functional amino group on the support by a procedure similar to that used by Weetall and Hersh [7]. The functional amino support material was then treated with a 2.5% aqueous glutaraldehyde solution containing 0.05 M citrate/phosphate buffer, pH 7.0. The reaction was allowed to proceed at room temperature for 30 min under vacuum followed by 30 min at atmospheric pressure. The particles were washed repeatedly with distilled water to remove unreacted glutaraldehyde, then several times with the same buffer used for immobilization. The enzyme(s) dissolved in 0.05 M citrate/phosphate buffer, pH 7.0, were added at the ratio of 1 ml/g support. Enzyme concentrations were varied to produce catalyst particles of variable activities as described below. Immobilizations were carried out in ice baths for 30 min under vacuum and then for additional 60 min at atmospheric pressure. The particles were thoroughly washed to remove excess enzyme(s).

*Methods of assay.* Both glucose oxidase and catalase were assayed by recording the changes in $O_2$ concentration in the reaction mixture as measured by a Clark-type electrode (Gilson Medical Electronics model KM Oxygraph). The electrode was attached to a jacketed cell having a 1.8 ml capacity which was agitated using a $2 \times 7$ mm magnetic stirring rod. All assays, unless otherwise stated, were done at pH 5.5 using citrate (22 mM)/phosphate (57 mM) buffer prepared according to the method of Gomori [8]. The concentration of the buffer used did not cause any significant change in oxygen solubility. All assays were carried out at 25°C. Repeated assays on the same sample of support were possible by allowing the agitated particles to settle and withdrawing the excess liquid by suction. Rinsing with water or buffer was repeated till all soluble reactants were removed before starting the next assay.

*Catalase.* The activity of catalase was assayed by following the increase in $O_2$ concentration using $H_2O_2$ ($5 \cdot 10^{-4}$ M) as substrate. The presence of glucose oxidase in the doubly immobilized preparations had no effect on the measurements as long as glucose was not added to the reaction mixture. All solutions were aerated with $N_2$ gas before use, to expel $O_2$. The reaction was started by the addition of substrate.

*Glucose oxidase.* Singly immobilized or soluble glucose oxidase activity was assayed by determining $O_2$ consumption in air-saturated buffer solution (0.25 mM $O_2$) using glucose (13.9 mM) as substrate. In the dual catalysts, the presence of immobilized catalase would result in the production of $O_2$ from the $H_2O_2$ produced by glucose oxidase. Thus, to measure glucose oxidase activity, excess soluble catalase was included in the reaction mixture. Such excess would

convert each mol of $H_2O_2$ produced back to one half mol of $O_2$ resulting in an observed rate of $O_2$ consumption exactly one half that of the actual glucose oxidase activity of the sample. In many cases, it was necessary to use excess catalase with samples of singly immobilized and soluble glucose oxidase as most commercial glucose oxidase samples contain some contaminant catalase activity. Inclusion of catalase inhibitors, such as $NaN_3$, in concentrations sufficient for complete inhibition resulted in partial inhibition of glucose oxidase.

After assaying with excess soluble catalase, it was necessary to remove all residual catalase activity left in the cell before assaying subsequent samples. Repeated washing with distilled water and/or buffer was not sufficient to eliminate adsorbed catalase. Incubation of a 2—3% sodium hypochlorite solution in the reaction compartment for 1—3 min followed by several rinses with distilled de-ionized water and buffer eliminated the adsorbed catalase activity. Most commercial bleach solutions contain 6.25% sodium hypochlorite and can be used after dilution.

*Order of assay in the dual system.* In the dual enzymic system, the pseudo first-order rate of the glucose oxidase-initiated cyclic reaction was determined first by adding glucose to the air-saturated reaction mixture. After rinsing the sample, catalase activity was assayed in the $N_2$-aerated buffer by adding $H_2O_2$. This was followed by assaying the catalase-initiated cyclic reaction by including glucose in the reaction mixture before addition of $H_2O_2$. Finally the rate of glucose oxidase activity was determined in the presence of excess catalase. This was done last since the excess soluble catalase could not be selectively removed from the system.

*Determination of efficiencies.* One advantage of studying a cyclic enzyme system, is that either enzyme can be used as the enzyme to catalyze the first reaction in the sequence depending on the substrate used. In either case, the first enzyme produces a product, $P_1$, which is then acted on by the second enzyme to produce the final product of the reaction, $P_2$. We define the efficiency of the system as how well the second enzyme in the overall reaction sequence utilizes the product of the first reaction compared to utilization by an excess amount of soluble second enzyme. It was shown for both these enzymes that an excess amount of soluble second enzyme was sufficient to completely utilize the product produced by the first enzyme in the sequence, no matter which enzyme was used to initiate the reaction sequence. This definition of efficiency is similar to that used by Goldman and Katchalski [2].

Typical data used to calculate the percent "catalase efficiency" in the two-enzyme system is shown in Fig. 1 for the glucose oxidase-initiated sequence. The top curve is the trace of $O_2$ consumption due to glucose oxidase action on glucose in the presence of excess soluble catalase. The bottom curve is that of $O_2$ consumption in the absence of any catalase activity. Theoretically, this curve should represent twice the rate in the top curve. Due to contamination or immobilized catalase activity, the bottom curve was in most cases calculated from the top one. The middle curve represents the depletion of $O_2$ in the dual enzyme system, that is, with glucose oxidase and catalase both present. All of these rates were pseudo first-order. The percent catalase efficiency was calculated as shown in Eqn. 2.

$$\text{Percent catalase efficiency} = \frac{K_{GOx} - K_{GOx\text{—overall}}}{0.5\, K_{GOx}} \times 10 \qquad (2)$$

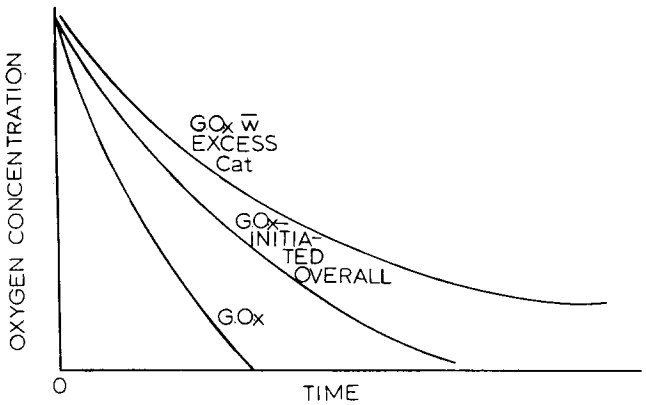


Fig. 1. Oxygen consumption traces by glucose oxidase (GOx) (bottom curve), glucose oxidase in the presence of excess catalase (Cat) (top curve), and glucose oxidase in the presence of a non-excess amount of catalase (middle curve) used to calculate efficiency and net product formation.

where $K_{GOx}$ is the pseudo first-order rate constant of the glucose oxidase reaction and $K_{GOx-overall}$ is that of the glucose oxidase-initiated overall reaction. The denominator in this equation is equal to the rate of the glucose oxidase reaction in the presence of excess catalase.

"Glucose oxidase efficiency" is measured in an analogous way (Fig. 2). In the dual system, the catalase activity is first measured with $H_2O_2$ as substrate. This is illustrated in the top curve. When glucose and excess soluble glucose oxidase are added to the reaction medium, there is no detectable oxygen production since the glucose oxidase utilizes the oxygen as fast as it is produced by the catalase. Thus the time course of the reaction would run along the abscissa in Fig. 2. The middle curve represents the catalase-initiated overall reaction when both catalase and glucose oxidase (not in excess) are present along with $H_2O_2$ and glucose. The amount of oxygen produced is less than that when only catalase is acting in the absence of glucose. The decrease in the measured oxygen production is due to the amount of oxygen that is used up by the glucose

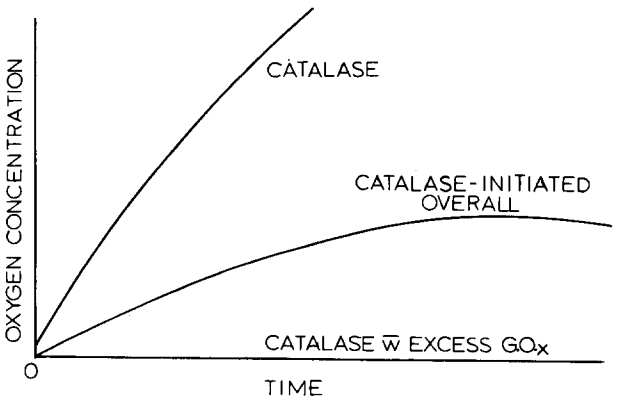


Fig. 2. Oxygen production traces by catalase (top curve), catalase in the presence of an excess amount of glucose oxidase (along abscissa), and catalase in the presence of a non-excess amount of catalase (middle curve) used to calculate efficiency and net product formation.

oxidase in the system. Oxygen, the intermediate in the reaction sequence, will reach a maximum and eventually decrease to 0 (Fig. 5). Both the catalase-initiated overall reaction and the catalase reaction obeyed first-order kinetics under the conditions used. The percent glucose oxidase efficiency of the system was calculated as shown in Eqn. 3.

$$\text{Percent glucose oxidase efficiency} = (1 - k_{\text{overall}}/k_c) \times 100 \quad (3)$$

where $k_{\text{overall}}$ is the pseudo first-order rate constant of the overall reaction and $k_c$ the first-order rate constant of the catalase reaction. The efficiencies of both enzymes are defined in terms of the initial rates of the reaction. In the determination of efficiency, it is important that the intermediate be equal to zero at the beginning of the reaction. This is a practical problem in the case of the catalase-initiated reaction where all $O_2$ must be removed.

*Comparison of dual, mixed, and soluble system.* Performance of the dual enzymic catalyst was compared to that in a system where each enzyme was bound to a different group of particles (mixed system) and a homogeneous soluble system. When the three systems were compared, the total units of activities of each enzyme in the reaction vessel were adjusted to equal values (±5%). Since the activities of the mixed and soluble systems could be adjusted by adding small quantities of either enzyme separatedly, the dual enzymic system was assayed first.

*Measuring net product of cyclic reaction.* One of the products of the glucose oxidase-initiated cyclic reaction with catalase is $O_2$. The net amount of the $O_2$ produced in the two-step reaction was measured at any time as the difference between the amount of $O_2$ in solution in the presence of the two enzymes (at the levels being studied) and the amount of $O_2$ in solution in the presence of just glucose oxidase. This represents one half the amount of $H_2O_2$ converted to oxygen by catalase.

The net production of $H_2O_2$ in the catalase-initiated cyclic reaction with glucose oxidase is determined at any time as two times the difference in $O_2$ content between the catalase reaction and the reaction catalyzed by the catalase/glucose oxidase two-enzyme system. This expresses the amount of product in terms of the fraction of substrate converted. The actual amount of product would be one half of this.

In both the above cases, the net product is the same compound as the initial substrate and can be used by the first enzyme. Thus, the methods used in this determination give approximate data which are valid only as long as the concentration of the net product formed does not affect the concentration of substrate compared to what it would be in the absence of regeneration of substrate by the cyclic reaction.

The error in measurement of product formed due to cycling will depend on the fractional conversion and also on the extent of build-up of intermediate (product of first enzyme). Since the latter is a function of the relative rate constants of the two enzymes, the error will depend on the ratio of the rate constants. An iterative computer program was used to determine the error involved in using our assumption (no cycling) in a cyclic reaction as a function of fraction substrate converted and ratio of rate constants (Table I).

In the catalase-initiated two-step reaction sequence, net product formed

TABLE I

PERCENT ERROR IN MEASUREMENT OF PRODUCT FORMED DUE TO RECYCLING

The percent error inherent in treating a cyclic enzymic reaction with two first-order steps as a non-cyclic reaction was computed for two levels of conversion at several ratios of rate constants; $k_1$ is the rate constant for the first reaction and $k_2$ that for the second.

| Fraction converted | Ratio of rate constants, $k_2/k_1$ | | | | |
|---|---|---|---|---|---|
| | 0.1 | 1 | 3 | 10 | ∞ |
| 0.02 | 29.6 | 10.0 | 6.0 | 3.6 | 1.4 |
| 0.05 | 46.5 | 16.7 | 10.5 | 6.9 | 4.4 |

could be measured by determining the amount of gluconate formed. This was not done because the assay procedure we used was simpler, more sensitive, and sufficiently accurate for our purposes.

## Results

Immobilized catalyst particles were constructed containing varying activities of catalase and glucose oxidase [9]. Three series of catalyst particles were prepared. In one, the catalase activity was kept approximately constant while the glucose oxidase activity was varied (samples 1, 2, and 3 in Table II). In a second, the glucose oxidase activity was kept constant and the catalase activity varied (samples 1, 6 and 7, Table II) and finally, a series of catalysts were prepared where the ratio of the catalase to glucose oxidase activities was kept constant, but the absolute activities of both varied (samples 1, 4 and 5, Table II). The activities of the catalyst particles and the respective percent efficiencies of both glucose and oxidase and catalase are summarized in Table II. As would be expected, an increase in the rate constant of the second enzyme in the reaction sequence leads to an increase in the efficiency with which that enzyme func-

TABLE II

EFFICIENCIES OF DUAL IMMOBILIZED SYSTEM

Enzyme concentrations in the immobilizing solutions were varied to produce catalyst particles of different activities [9]. The particles generally had glucose oxidase activities ranging from 1.5 to 6 units per g and catalase activities from 2 to 12 units per g. Adjustment of first-order rate constants was made by varying the amount of particles used, which varied from 40 to 80 mg per assay. Activity = first-order rate constant ($s^{-1}$). CAT, catalase; GOx, glucose oxidase.

| Sample | Activity ratio | Enzymic activities | | Efficiency (%) | |
|---|---|---|---|---|---|
| | CAT/GO | CAT | $GO_x$ | CAT | $GO_x$ |
| 1 | 1.7 | $3.2 \cdot 10^{-2}$ | $1.9 \cdot 10^{-2}$ | 89 | 42 |
| 2 | 7.0 | $3.8 \cdot 10^{-2}$ | $5.4 \cdot 10^{-3}$ | 99 | 13 |
| 3 | 27 | $4.0 \cdot 10^{-2}$ | $1.5 \cdot 10^{-3}$ | 98 | 1 |
| 4 | 1.8 | $1.1 \cdot 10^{-2}$ | $6.0 \cdot 10^{-3}$ | 47 | 29 |
| 5 | 1.6 | $1.5 \cdot 10^{-3}$ | $9.4 \cdot 10^{-4}$ | 5 | 39 |
| 6 | 0.4 | $7.8 \cdot 10^{-3}$ | $2.1 \cdot 10^{-2}$ | 24 | 81 |
| 7 | 0.05 | $1.0 \cdot 10^{-3}$ | $2.2 \cdot 10^{-2}$ | 14 | 100 |

tions, and a decrease in activity leads to a lowering of the efficiency. In samples 1, 2 and 3 (Table II), the percent glucose oxidase efficiency drops from 42 to practically 0 as the glucose oxidase rate constant of the particle decreases from $1.9 \cdot 10^{-2}$ to $1.5 \cdot 10^{-3}$ $s^{-1}$. With the same samples there is an increase in catalase efficiency from 89 to approx. 100%. However, since the catalase efficiency was very high to begin with, the change is not so striking.

In samples 1, 6 and 7, a decrease in percent catalase efficiency can be seen as the catalase activity is lowered. The glucose oxidase efficiency is increasing although the activity of glucose oxidase remains the same. The most likely reason for this is that with the lowering of the catalase activity on the particles, the glucose oxidase enzyme that is there can more fully utilize the lowered production of oxygen. When the ratio of the two activities is held constant but the absolute activities are decreased (samples 1, 4 and 5), the percent catalase efficiency decreases. No such decrease is observed with glucose oxidase efficiency under these same conditions.

The data in Table II were obtained for the dual system (both enzymes immobilized to the same particulate surface). The efficiencies of a mixed glucose oxidase/catalase catalyst and a homogeneous soluble system are shown in Table III. All systems were adjusted to the same total units of activity in the same total volume. In all three systems, the catalase efficiency increased as the ratio of catalase to glucose oxidase increased. The absolute values, however, are not the same. In general, the dual immobilized system was superior to either the soluble (as predicted by Goldman and Katchalski [2]), or to the mixed system. The exact relationship varied somewhat, depending on the ratio of rate constants involved. Under many of the ratios used, the soluble system functioned more efficiently than did the mixed catalyst. Glucose oxidase efficiencies in the catalase-initiated overall reaction were affected in an analogous manner by a change in ratio of enzymic activities in the three systems.

Experiments were performed in a similar way to determine net product ($P_2$) formation as for determination of efficiency, that is, rate constants for the indi-

TABLE III

COMPARISON OF EFFICIENCIES OF DUAL, MIXED AND SOLUBLE SYSTEMS OF GLUCOSE OXIDASE AND CATALASE

Dual and single enzyme catalysts of different activities were made by varying enzyme concentrations in the immobilizing solutions [9]. First-order rate constants of the dual system were established first and then those of the soluble and mixed systems were adjusted to match. The dual immobilized catalyst had glucose oxidase activities ranging from 3 to 6 units per g and catalase activities from6 to 12 units per g. 40—80 mg of catalyst particles were used in these assays. To obtain the same activities with single enzymic catalysts, 50—120 mg of total catalyst particles were used. The range of catalase and glucose oxidase activities on the single enzymic catalysts were comparable to those of the dual catalysts particles. Activity = first-order rate constant ($s^{-1}$). CAT, catalase; GOx, glucose oxidase.

| Activity ratio | Enzyme activities | | Glucose oxidase efficiency (%) | | | Catalase efficiency (%) | | |
|---|---|---|---|---|---|---|---|---|
| $CAT/GO_x$ | CAT | $GO_x$ | Dual | Soluble | Mixed | Dual | Soluble | Mixed |
| 0.3 | $4.4 \cdot 10^{-3}$ | $1.4 \cdot 10^{-2}$ | 91 | 76 | 61 | 14 | 8 | 7 |
| 0.5 | $2.6 \cdot 10^{-3}$ | $5.6 \cdot 10^{-3}$ | 81 | 73 | 51 | 56 | 31 | 19 |
| 1.7 | $3.1 \cdot 10^{-2}$ | $1.8 \cdot 10^{-2}$ | 46 | 39 | 37 | 65 | 54 | 53 |
| 2.3 | $2.8 \cdot 10^{-2}$ | $1.2 \cdot 10^{-2}$ | 40 | 40 | 26 | 67 | 56 | 57 |

vidual enzymes were determined in the dual immobilized system. The rate constants were then matched in both the mixed catalyst and the soluble system. The production of $P_2$ was measured as a function of time. In Fig. 3 is shown the rate of net product formation as a fraction of initial substrate ($S_0$) for the glucose oxidase-initiated sequential reaction in the soluble and the two immobilized systems. The rate constants for the glucose oxidase activity was $1.9 \cdot 10^{-2}$ $s^{-1}$ and that for the catalase reaction was $3.2 \cdot 10^{-2}$ $s^{-1}$. The dual immobilized system on the same support was superior to the enzyme immobilized to different supports as well as to the soluble system.

The same type of data is shown for the same systems but initiated with the catalase reaction (Fig. 4). Again, the net production of $P_2$ (expressed as the fraction of the original substrate, converted) is shown versus time. The dual immobilized system is superior, initially, but the soluble system improves relatively with time while the mixed catalyst is inferior to the other two.

The data in Figs. 3 and 4 reflect the error in interpretation caused by the assumption of a non-cyclic process for the cyclic process. The errors become very large for significant fractional conversions and for low ratios of $k_2/k_1$ (Table I), and are such as to give lower readings than actually occur. Thus in Fig. 4, the fraction of substrate converted actually appears to level off. This is not as obvious in Fig. 3 since the ratio of $k_2/k_1$ for the glucose oxidase-initiated reaction is larger than for the catalase-initiated reaction, and the error will be smaller. For the catalase-initiated reaction (Fig. 4), the mixed system plateaus at a lower value than either the dual or soluble systems. This is interpreted as being caused by diffusional effects. The slow diffusion of intermediate from one particle to another reduces the effective $k_2$ of the system, thus increasing the error.

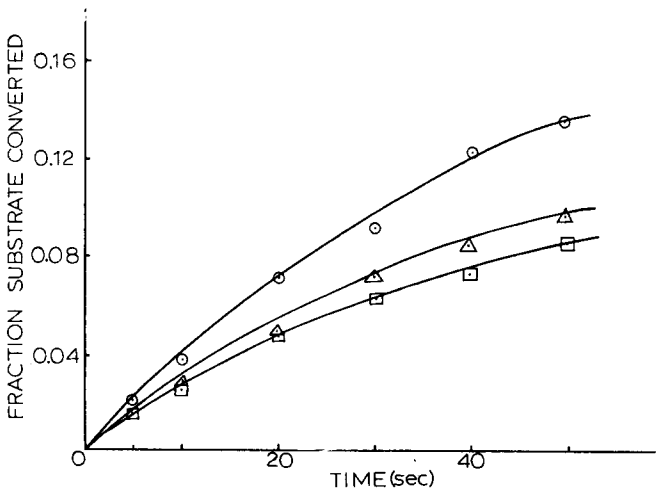


**Fig. 3. Fraction of substrate converted with time for the glucose oxidase-initiated reaction; $k$ for glucose oxidase was $1.9 \cdot 10^{-2}$ $s^{-1}$ and $k$ for catalase was $3.2 \cdot 10^{-2}$ $s^{-1}$. ○, dual enzymic system; □, mixed enzymic system; △, soluble enzymic system. All systems were adjusted initially to the same total units of activity for both enzymes (±5%).**

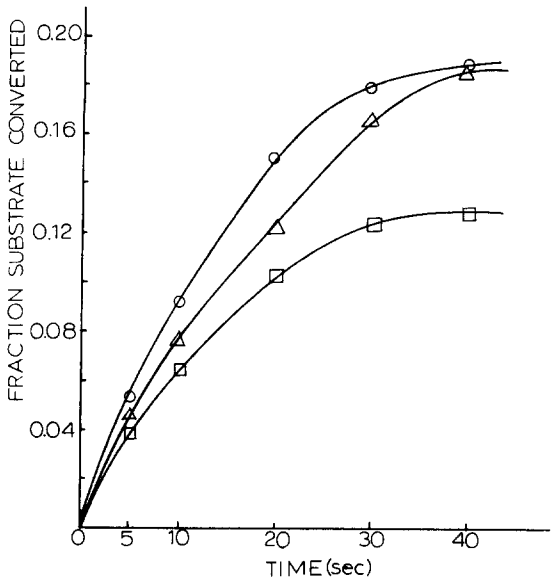


Fig. 4. Fraction of substrate converted with time for the catalase-initiated reaction; the first-order rate constants were the same as given in Fig. 3. ○, dual enzymic system; □, mixed enzymic system; △, soluble enzymic system. All systems were adjusted initially to the same total units of activity for both enzymes (±5%).

In Table IV is shown the time required for $P_2/S_0$ to equal 0.05 for the dual immobilized system with varying activities (expressed as rate constants of the two enzymes). This time is calculated for both the glucose-oxidase initiated and the catalase-initiated reaction sequences. The time required to reach $P_2/S_0$ = 0.05 is inversely proportional to the rate of production of $P_2$ (oxygen in the case of the glucose-oxidase initiated reaction and $H_2O_2$ in the case of the catalase-initiated reaction). Lowering the rate constant of either enzyme increases the time required for $P_2/S_0$ = 0.05.

TABLE IV

TIME REQUIRED FOR 5% CONVERSION OF SUBSTRATE IN DUAL IMMOBILIZED SYSTEM OF GLUCOSE OXIDASE AND CATALASE

The final product of the glucose oxidase-initiated reaction is $O_2$ while that of the catalase-initiated reaction is $H_2O_2$. The samples in this table are the same as given in Table II; the enzymic activities of the catalyst particles are given in Table II. CAT, catalase; GOx, glucose oxidase.

| Sample | Activity ratio | Time required (sec) | |
|---|---|---|---|
| | $CAT/GO_x$ | Glucose-oxidase initiated | Catalase initiated |
| 1 | 1.7 | 5 | 4 |
| 2 | 7.0 | 35 | 12 |
| 3 | 27 | >50 | >50 |
| 4 | 1.8 | 37 | 16 |
| 5 | 1.6 | ⩾50 | >50 |
| 6 | 0.4 | 14 | 7 |
| 7 | 0.05 | 50 | 50 |

## Discussion

In any reaction sequence catalyzed by two enzymes carried out in homogeneous solution, a lag time is observed before maximal rate of production of end product is reached [10,11]. The lag time has been defined arbitrarily as the time for the intermediate to reach its maximal concentration or the time when the rate of substrate removal by the first enzyme in the reaction sequence is just equal to the production of the second product by the second enzyme in the reaction sequence. The simulated course of our two-enzyme reaction sequence for the catalase-initiated reaction is illustrated in Fig. 5 and the lag time is indicated. These curves were obtained by an iterative computer technique. A similar procedure with similar results is obtained for the glucose oxidase-initiated two-step reaction. The curve for oxygen represents the intermediate which reaches a maximum and thereafter declines. There are three curves representing $H_2O_2$ content. The curve labeled $H_2O_2$ (no glucose oxidase) is the concentration of $H_2O_2$ that would be obtained in the absence of glucose oxidase. The curve labeled $H_2O_2$ represents the actual concentration of $H_2O_2$ in solution during the overall two-step reaction. The curve labeled $H_2O_2$ [1] represents the total amount of $H_2O_2$ that is being produced by glucose oxidase. It is equivalent to product that would be obtained in a non-cyclic reaction. It has, of course, no physical meaning in this system since the $H_2O_2$ that is produced is recycled and becomes substrate for the first enzyme, catalase, in the reaction sequence. The importance of knowing the lag time is that one can expect that the greatest differences between the dual immobilized enzyme and the soluble enzyme will occur early in the reaction sequence. The calculated lag times for the rate constants we used in our experiments were generally greater than the times of the experiments; therefore, we were dealing mainly with the lag period of the soluble enzyme system where differences between the immobilized and soluble systems should be maximal.

Calculation of some typical lag times for two consecutive first-order reactions are shown in Table V. Decreasing the rate constants gives increasing lag times. However, switching the rate constants between the two enzymes has no effect

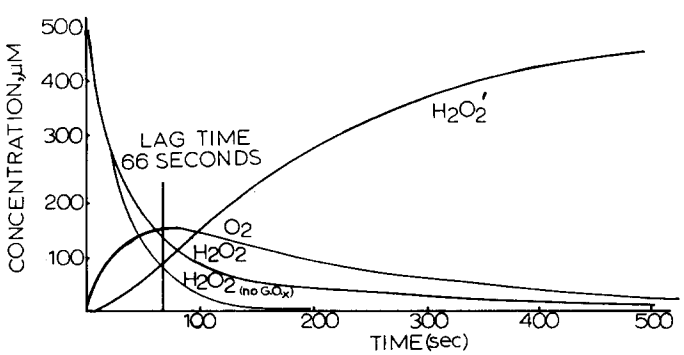


Fig. 5. Computer-simulation of the two-step reaction of catalase and glucose oxidase initiated by catalase. The rate constant used for catalase in this program was $2.8 \cdot 10^{-2}$ $s^{-1}$ and that for glucose oxidase was $1.2 \cdot 10^{-2}$ $s^{-1}$. The meaning of the curves is explained in the text.

TABLE V

RELATION OF LAG TIME TO ENZYME ACTIVITIES

The lag time of a sequential reaction of two first-order steps in solution was determined by an iterative computer program at different rate constants for the two steps. $k$ values are first-order rate constants ($s^{-1}$).

| $k_1$ ($s^{-1}$) | $k_2$ ($s^{-1}$) | $k_1/k_2$ | Lag time (s) |
|---|---|---|---|
| $10^{-3}$ | $10^{-3}$ | 1 | 1246 |
| $10^{-2}$ | $10^{-2}$ | 1 | 125 |
| $10^{-1}$ | $10^{-1}$ | 1 | 13 |
| $10^{-3}$ | $10^{-2}$ | 0.1 | 307 |
| $10^{-2}$ | $10^{-3}$ | 10 | 307 |

on the lag time. The significance of this is that with a given set of rate constants, the sequence of the reaction can be switched, that is, initiated with either the catalase or the glucose oxidase step, without changing the lag time of the overall sequential reaction. The initial substrate concentration has no effect on the lag time. However, the presence of any intermediate (product of the first enzyme) will decrease the lag period.

As observed by other workers [3,4], the efficiency of a dual immobilized system is superior to that of a soluble with similar activities. We have also observed in this work that the soluble system generally tends to perform more efficiently than the mixed immobilized system where the enzymes are on different support particles. The rationale for this is presumably that in the mixed immobilized system the product of the first reaction has to diffuse out of the particle containing the first enzyme and into the particle containing the second enzyme before it can be acted upon. The lack of very large differences between the soluble system and the mixed immobilized system was probably due in large part to the fact that the substrates which determined the first-order kinetics were small molecules, and thus their diffusion coefficients were relatively high.

Goldman and Katchalski [2] predicted that in systems in which the ratios of activities of two enzymes were constant, the efficiency of the system would increase with increasing activities. We have experimentally demonstrated this in this work (Table II) in the case of the glucose-oxidase initiated reaction where it is clearly shown. We propose that the increase in efficiency at the higher enzymic activities relates to the fact that there is more chance for the intermediate ($P_1$) to be acted on by the second enzyme in the sequence if a higher activity of that enzyme is present on the particle as long as the reaction is in the first-order regime. The increased efficiency with increasing activities was not clearly observed, however, with the catalase-initiated reaction. This is most probably due to the insufficient removal of all the intermediate reactant ($O_2$) from the solution before initiating the reaction sequence.

The difference between the homogeneous, soluble two-enzyme system and the dual immobilized enzyme depends on the rate constants of the individual enzymes. In efficiency (Table III) and in net product formation (Figs. 3 and 4), differences between the dual immobilized system and the soluble system are decreased as the rate constant of the second enzyme is decreased relative to that of the first enzyme. We interpret this as a diffusion effect. When the rate

TABLE VI

EFFECT OF PARTICLE SIZE ON CATALASE EFFICIENCY

The nickel silica alumina was ground and sieved to the sizes indicated. It was then silanized and the enzyme immobilized to it as described in the text. Activity = first-order rate constant ($s^{-1}$). CAT, catalase GOx, glucose oxidase.

| Particle size (μm) | Activity ratio $CAT/GO_x$ | Enzyme activities X $10^3$ | | Catalase efficiency (%) |
|---|---|---|---|---|
| | | CAT | $GO_x$ | |
| 105—125 | 2.8 | 6.30 | 2.21 | 26 |
| 149—177 | 2.1 | 4.36 | 2.06 | 34 |
| 250—420 | 1.4 | 4.45 | 3.13 | 66 |
| 500—707 | 0.87 | 2.06 | 2.38 | 65 |
| 707—1190 | 0.74 | 1.63 | 2.21 | 84 |

of the second enzyme in the sequence (either glucose oxidase or catalase) is low, a large proportion of the intermediate ($P_1$) can escape into the bulk phase. It does not tend to diffuse back into the particles due to the concentration difference of $P_1$ in the particle versus the bulk solution. Thus, the basic inherent advantage of the dual immobilized system is to a large extent negated, and both the efficiency and the net product formed in the soluble homogeneous system approaches, and in some cases equals, that observed in the dual immobilized system.

The results reported in these experiments are quantitative only for the conditions under which they are run. With higher activities of the catalyst particles, larger particles, and smaller pores, the dual immobilized enzyme should be relatively favored compared to either the soluble system or the mixed catalyst particles. Table VI summarizes some experiments relating percent catalase efficiency to particle size at constant pore diameter of the particles. The change in the ratio of glucose oxidase to catalase is caused by a relatively greater increase in catalase activity as the particles are crushed. The catalase efficiency increases with increasing particle size even though the activity of catalase relative to glucose oxidase decreases. The increase in efficiency is most probably due to the longer residence time of the intermediate in the larger particles.

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