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EFFICIENCY OF TWO ENZYMES IMMOBILIZED TO THE SAME SURFACE AND ACTING IN SEQUENCE

I. Preparation and Properties of Phosphoglucomutase and Glucose-6-Phosphate Dehydrogenase Immobilized to *s*-Triazine Trichloride Activated Cellulose

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Phosphoglucomutase and glucose-6-phosphate dehydrogenase were immobilized to *s*-triazine trichloride activated cellulose. The optimal conditions for binding the immobilized enzymes were determined and the kinetic and physical properties were investigated. The final ratio of the two enzymes immobilized to the surface was determined by the physical properties of the enzymes as well as by the ratio of the enzymes present in the attachment solution. The immobilized enzymes were found to retain at least 60% of the original activity for at least 40 days when stored at 4°C and in the presence of substrates and cofactors. Immobilized phosphoglucomutase and glucose-6-phosphate dehydrogenase also were much more stable at 58°C, retaining 28 and 13% of the original activity, respectively, after 75 min. The apparent K_m's were 5.4 to 1.5 times higher for immobilized phosphoglucomutase and glucose-6-phosphate dehydrogenase, respectively than for the soluble enzymes.

INTRODUCTION

In recent years research in immobilized enzymes has received much attention (1–6) because of the potential for examining the basic mechanism of enzymes in the solid state, that is, when attached to membranes, and because of the practical applications of immobilized enzymes to industry (7,8), food technology (9,10), and biomedical diagnostics (11). Most of the research to date, however, has focused on preparations having only one enzyme immobilized to the surface of the carrier (12–16). Research on two enzymes acting in sequence and immobilized on the same surface has received little

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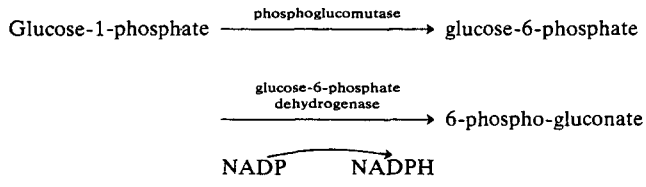
attention. Notable exceptions are the theoretical studies of Katchalski and Goldman (17), Hervagault et al. (18), Bouin et al. (19,20), and the pioneering work of Mosbach and Mattiasson (21,22).

We have undertaken the study of two enzymes immobilized to the same surface with the initial goal of determining the optimal conditions for the two enzymes to operate efficiently, and then to establish criteria for evaluating the efficiency of the immobilized enzyme systems compared to ones in which the enzymes are soluble or attached to separate carriers. The two enzymes we have selected are phosphoglucomutase (EC 2.7.5.1) and glucose-6-phosphate dehydrogenase (EC 1.1.1.49). The particular coupled reaction they catalyze is



These two enzymes were selected for study because the NADPH formed could be easily measured either spectrophotometrically or fluorometrically. Secondly, by using glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* we have a versatile system that can use either $NADP^+$ or NAD^+ (23). Lastly, this system is an example of a relatively reversible enzyme reaction pulled by an irreversible one. Immobilized sequential enzymes represent in the simplest form a model for studying how sequential enzymes that are components of biological systems might act.

We chose to link the enzymes covalently to the carrier in order to prevent their loss by desorption, as occurs in many other immobilization techniques (1,9,24,25). The immobilization method of Smith and Lenhoff (26) was used because of the ease in preparation of the carrier and for the high retention of activity of the immobilized enzymes. We chose to bind to the surface of cellulose particles, which are relatively nonporous, to avoid the complications due to diffusion within the carrier pores (27). Furthermore, it had also been shown by Goldman et al (1), using papain adsorbed to collodion membranes, that only the enzyme near the surface was active.

In this report we show that *s*-triazine trichloride activated cellulose irreversibly binds phosphoglucomutase and glucose-6-P dehydrogenase and we examine the properties of the immobilized enzymes. In addition, we show that the binding of the two enzymes to a surface introduces

complications not found with the binding of one enzyme to a surface and discuss possible explanations of these results.

EXPERIMENTAL PROCEEDURES

Materials

The following reagents were used: Phosphoglucomutase (rabbit muscle), glucose-1-P (disodium salt, Grade III), glucose-6-P (monosodium salt, Sigma grade), glucose-1,6-diP (tetracyclohexylammonium salt), $NADP^+$ (free acid, Sigma grade), bovine serum albumin (Fraction V powder), EDTA (disodium salt, Sigma grade), and Trizma base and Trizma HCl (reagent grade); all were purchased from Sigma Chemical Company (St. Louis, MO). Glucose-6-P dehydrogenase (from *L. mesenteroides*) was purchased from Microbic Operations, Beckman Laboratories (Carlsbad, CA) and $MgCl_2$ from Mallinckrodt Chemical Works (St. Louis, MO).

Methods

Calibration of the Concentrations of Substrates and Coenzyme. In order to accurately determine the concentrations of glucose-1-P, known volumes of a glucose-1-P solution were added to 1 ml of assay solution containing phosphoglucomutase, glucose-6-P dehydrogenase, glucose-1,6-diP and $NADP^+$. The reaction was allowed to go to completion and the final absorbance at 340 nm was measured spectrophotometrically. Using the extinction coefficient of 6.3×10^{-6} l mol^{-1} cm^{-1} for NADPH, the concentration of the glucose-1-P was calculated from the NADPH formed. Using glucose-6-P dehydrogenase alone, similar calibrations were made for glucose-6-P and $NADP^+$. As in the case with glucose-1-P, limiting amounts of glucose-6-P or $NADP^+$ were added in a fixed volume of 1 ml of reaction mixture containing an excess amount of glucose-6-P dehydrogenase and either excess amounts of $NADP^+$ or glucose-6-P; the final absorbance at 340 mm was measured.

Preparation of [^{14}C]-Labeled Bovine Serum Albumin. The radioisotope iodo[^{14}C]acetamide (2.85 mCi/mol) was purchased from Swartz/Mann (Orangeburg, NY). The iodo[^{14}C]acetamide was diluted in 95% ethanol to 0.2 mCi/ml, and 0.1 ml was added to 3 ml of 10% bovine serum albumin in 50 mM phosphate buffer, pH 7.3, and incubated overnight at room temperature. The mixture was then passed over a Sephadex G-25 column. The protein concentration was determined by a modification of the method of Lowry et al. (28). The radioactivity was measured using a Beckman LS-250 Liquid Scintillation Spectrophotometer. The scintillation fluid

contained 125 g of napthalene and 7.5 g of 2, S-diphenyloxazole per liter of dioxane. The counting efficiency of the scintillation fluid was calculated to be 86.5%.

Attachment of Phosphoglucomutase and Glucose-6-P Dehydrogenase. Phosphoglucomutase and glucose-6-P dehydrogenase were covalently bound to the carrier, chromatographic cellulose (Whatman cellulose powder, CF 11, 105–210-μm particles), by the linking reagent *s*-triazine trichloride, as described by Smith and Lenhoff (26). The enzymes were placed in 200 μl of 0.05 M citrate buffer, pH 5.5, and about 25 mg of the *s*-triazine trichloride activated particle were added. The activated particles were left in the attachment solution over night at room temperature. At the end of the binding period the particles were transferred to a sintered glass crucible of coarse porosity, and washed with 100 ml each of distilled water, 1 M NaCl, and again with distilled water. The particles were then assayed for the activity of the enzymes immobilized to them. At the end of the assay, the particles were removed and the remaining solution was tested for enzyme activity that may have desorbed from the particles. In most cases little if any desorbed enzyme was found. The particles were then transferred to a screw-cap vial and stored in the assay solution at 4°C.

Standard Assay Solution. The following standard assay solution was used for all experiments: 0.1 M tris buffer at pH 7.6, 10^{-3} M $MgCl_2$, 5×10^{-5} M EDTA, 2×10^{-4} M histidine, 2×10^{-2} % bovine serum albumin, and 3×10^{-4} M $NADP^+$. For the assays of phosphoglucomutase and the sequential reaction, glucose-1,6-diP was added to give a concentration of 2.65×10^{-8} M. The glucose-1-P, glucose-6-P, and $NADP^+$ were added at concentrations approximately 15 to 20 times their respective apparent K_m concentrations (see Table 3 below). For all assays involving an immobilized enzyme, the reaction was initiated by the addition of 20 μmol of glucose-1-P to 10 ml of the assay solution.

Assay of Immobilized Enzymes in a Stirred Tank Reactor. The immobilized enzymes, in 10 ml of assay solution, were placed in a 15-ml Gooch, low form, fritted disk crucible (Kimax Brand, coarse porosity). The opening at the bottom of the crucible was plugged using a #1 or #0 rubber stopper. An 18-gauge needle was inserted into the rubber stopper with the needle tip extending into the small cavity remaining between the rubber stopper and the wall of the crucible. A 3-ml disposable syringe was then connected to the needle. A teflon-coated magnetic spin bar, $\frac{1}{2}\times\frac{5}{16}$ in., was placed in the crucible and the entire assembly mounted over a magnetic stirrer.

For all immobilized sequential enzyme assays, the reaction was started by turning on the magnetic stirrer and adding 20 μg of glucose-1-P to 10 ml of the assay solution (Fig. 1). The *overall rate* of the two enzymes acting in

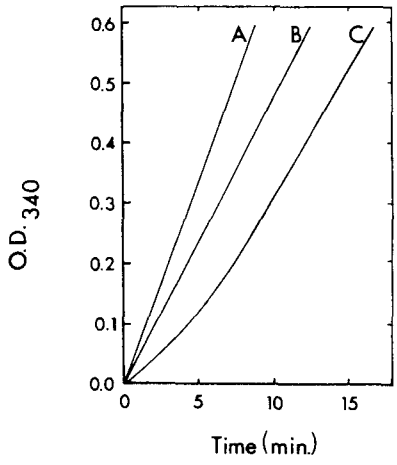


FIG. 1. Method for measuring the overall rates of phosphoglucomutase and glucose-6-P-dehydrogenase acting in sequence and of the individual enzymes in such a mixture. For each sequential enzyme mixture the following three rates were measured: (A) glucose-6-P dehydrogenase, (B) phosphoglucomutase, and (C) the overall rate. The assay solution and assay conditions are described in the text. The volume of the reaction was 1 ml for the soluble enzymes and 10 ml for the immobilized enzymes.

sequence (hereafter called the *sequential rate*) was determined simply by adding glucose-1-P to the standard assay solution containing the enzymes and measuring spectrophotometrically the amount of NADPH formed. To determine the maximum rate of the immobilized phosphoglucomatase, about 0.2 units of soluble glucose-6-P dehydrogenase were added to the standard solution prior to the addition of glucose-1-P. The activity of the glucose-6-P dehydrogenase was determined after addition to 50 μmol of glucose-6-P to 10 ml of the standard assay solution. For example, a typical reaction mixture for assaying the sequential reaction consisted of 3×10^{-4} M NADP, 2.65×10^{-8} M glucose-1,6-diP and 1.81×10^{-3} M glucose-1-P.

In a typical assay, the magnetic stirrer was turned on and the appropriate substrates added. At intervals, 1-ml aliquots of assay solution were removed by withdrawing the plunger of the syringe, leaving behind the major portion of the assay solution with the particles bearing the enzymes. The aliquot was placed in a cuvette and the absorbance at 340 nm was read in a Beckman Acta CIII spectrophotometer. The sample was then returned to the crucible. The entire time for removing a sample from the reaction mixture, reading the absorbance, and replacing the sample was less than 1 min.

Assay For Soluble Enzyme. The activity of phosphoglucomutase was measured by following the formation of NADPH at 340 nm using glucose-6-P dehydrogenase as an auxilliary enzyme and $NADP^+$ to measure the product glucose-6-P. The rate of the glucose-6-P dehydrogenase was also measured by the formation of NADPH at 340 nm. All assays were done at room temperature in 1-ml cuvettes using a Beckman Acta CIII spectrophotometer.

RESULTS

Determination of the Optimal Time for Binding of the Enzymes to the Activated Carrier

To determine the optimal time to achieve maximum binding of phosphoglucomutase and glucose-6-P-dehydrogenase to the carrier, four identical attachment solutions (see above) were made, in which about 10 mg

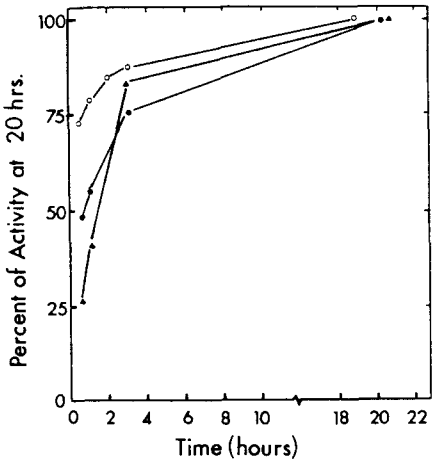


FIG. 2. Kinetics of the binding of [^{14}C] bovine serum albumin (open circles), phosphoglucomutase (solid triangles), and glucose-6-P dehydrogenase (solid circles) to *s*-triazine trichloride activated cellulose. The attachment solutions for phosphoglucomutase and glucose-6-P dehydrogenase were made up as described in the text. For the binding experiments using [^{14}C] bovine serum albumin, the attachment solutions contained 180 μg of protein N in a final volume of 280 μl. The conditions for the attachment were the same as for the attachment of phosphoglucomutase and glucose-6-P dehydrogenase. The particles having [^{14}C] bovine serum albumin were washed to remove any adsorbed protein, dried, and weighed before being counted in a Beckman LS-250 liquid scintillation spectrophotometer. The radioactivity and the activities of the preparations are expressed as a percent of the level measured at 20.5 h taken as 100%

of *s*-triazine trichloride activated cellulose was added to 0.6 units of phosphoglucomutase and 5.4 units of glucose-6-P dehydrogenase. At 0.5, 1, 3, and 20.5 h, the particles were removed from the attachment solutions and the activities of the immobilized enzymes were determined. Similar immobilization experiments were done with attachment solution containing [^{14}C] bovine serum albumin in place of the enzymes. These experiments (Fig. 2) showed that 20.5 h sufficed for maximal binding to occur.

From the amount of soluble enzyme originally placed in the attachment solution (see Note to Table 1), the data show that after 20.5 h, 31.3% of the phosphoglucomutase and 2.0% of the glucose-6-P dehydrogenase was immobilized. The results with glucose-6-P dehydrogenase showed that most of the enzyme activity originally in the attachment solution could be accounted for on the washed particles, in the solutions used to wash those particles, and in the remaining attachment solutions. The recoveries for the 0.5, 1, 3, and 20.5-h incubations were 113, 88, 121, and 73% respectively. Similar attempts to quantify phosphoglucomutase were not successful because of complications resulting from activation of the enzyme when it is transferred from pH 5.5 (the pH of the attachment solution) to pH 7.6 (the pH for the enzyme assay) in the presence of histidine and magnesium (29).

Desorption of Nonimmobilized Enzyme From Cellulose Particles

Before using the immobilized enzyme preparation, it was necessary to remove any enzyme that had adsorbed but was not covalently linked to the surface of the particles. Table 1 shows that regardless of which enzyme was used or of the time during which the activated particles were incubated in the attachment solution, approximately four washes with 1 ml of assays solution were sufficient to remove most of the nonimmobilized enzyme from 10-mg preparations of activated cellulose. Usually each wash removed 90% of the nonimmobilized enzyme remaining from the previous wash. The enzyme activity in the fourth wash was 0 to 0.4% of the activity found in the first wash. The data also indicate that of the enzymes adsorbed to the activated cellulose, a greater percentage of the phosphoglucomutase remained covalently linked compared to that of the glucose-6-P dehydrogenase.

The Effect of Increasing the Amount of Carrier on the Relative Activities of Immobilized Phosphoglucomutase and Glucose-6-P Dehydrogenase Bound

To determine the effect of the increased surface area on the relative activity of the individual enzymes, preparations of phosphoglucomutase and

TABLE 1. **Treatment of Phosphoglucomutase and Glucose-6-P Dehydrogenase Particles**[a]

	Attachment time (h)			
	0.5	1	3	20.5
A. PHOSPHOGLUCOMUTASE [Enzyme Activity ($U \times 10^4$)]				
Soluble enzyme recovered from:				
1st wash	5,396.9	7,308.1	7,449.7	926.7
2nd wash	450.6	592.6	486.8	72.9
3rd wash	62.3	54.0	23.8	13.6
4th wash	0	3.0	0	3.7
Total activity recovered in wash	5,909.8	7,957.7	7,959.5	1,016.9
Activity of washed immobilized enzyme preparation	584.1	912.4	1,851.0	2,234.5
Percent of activity recovered as immobilized enzyme after four washes	9.0	10.3	18.9	68.7
B. GLUCOSE-6-P DEHYDROGENASE [Enzyme Activity ($U \times 10^4$)]				
Soluble enzyme recovered from				
1st wash	10,634.9	10,199.0	13,240.4	6,481.5
2nd wash	605.1	965.8	1,014.8	363.4
3rd wash	116.2	94.0	54.0	103.4
4th wash	0	17.2	39.8	15.0
Total activity recovered in wash	11,456.2	11,276.0	14,349.0	6,963.3
Activity of washed immobilized enzyme preparation	520.0	583.2	808.1	1,065.6
Percent of activity recovered as immobilized enzyme after four washes	4.3	4.9	5.3	13.3

[a] Adsorbed nonimmobilized enzyme removal at the end of the attachment reaction. Four attachment solutions were made, each consisted of ca. 0.6 U of phosphoglucomutase, ca. 5.4 U of glucose-6-P dehydrogenase, and 10 mg of activated particles (see legend to Fig. 1). At 0.5, 1, 3, and 20.5 h, the attachment solution was removed from one of the preparations and 1 ml of standard assay solution was added to the particles. The particles were mixed and the 1 ml of the standard assay solution was removed after the particles had settled. The activities of the desorbed phosphoglucomutase and glucose-6-P dehydrogenase in the assay solution was then determined (see the text under Materials and Methods). This wash and assay procedure was repeated three more times.

glucose-6-P dehydrogenase immobilized to the same surface were made using different amounts of *s*-triazine trichloride activated cellulose in the attachment solution. The activities of the preparations are given in Table 2. When only phosphoglucomutase was present in the attachment solution, the activity on 15 mg of carrier was found to be 265.1×10^{-4} U/ml. When glucose-6-P dehydrogenase was added to the attachment solution, preparation B, the resulting activity of the immobilized phosphoglucomutase

TABLE 2. **Activities of Preparations After Incubation in Attachment Solutions Containing Different Amounts of Carrier or Varying Amounts of Glucose-6-P Dehydrogenase**[a]

	Attachment solution			Immobilized enzyme activity	
Preparation	Carrier (mg)	Glucose-6-P dehydrogenase (U)	Bovine serum albumin (μg PN)	Phospho-glucomutase ($U/ml \times 10^4$)	Glucose-6-P dehydrogenase ($U/ml \times 10^4$)
A	15	—	—	265.1	—
B	15	5.5	—	50.9	76.1
C	25	5.5	—	51.4	207.1
D	50	5.5	—	9.2	281.8
E	100	5.5	—	4.8	290.0
F	100	4.3	468	21.6	245.9

[a]These data measure the effect of changing the amount of carrier and the amount of glucose-6-P dehydrogenase in the attachment solution on the activity of immobilized phosphoglucomutase and glucose-6-P dehydrogenase. All attachment solutions for the preparations of immobilized phosphoglucomutase and glucose-6-P dehydrogenase contained 3.0 U of phosphoglucomutase in 250 μl of 0.04 M citrate buffer, pH 5.5. The total amount of protein N in the attachment was about 215 μg. The preparations were made by adding different amounts of *s*-triazine trichloride activated carrier, bovine serum albumin, or glucose-6-P dehydrogenase to the attachment solution. The preparation of the carrier, attachment solutions, wash procedure, and the method for assaying the immobilized enzymes are given in the text under Methods. A known volume of a standardized bovine serum albumin solution was added to the attachment solution for preparation F.

was lower but glucose-6-P dehydrogenase activity was now present. In preparations C, D and E greater amounts of carrier were added to the attachment solution containing both phosphoglucomutase and glucose-6-P dehydrogenase. In these three preparations the activity of the immobilized glucose-6-P dehydrogenase was about threefold higher than that in preparation B. The activity of the immobilized phosphoglucomutase, however, decreased as the amount of carrier was increased beyond 25 mg. In preparation F, bovine serum albumin was added to the attachment solution in addition to the phosphoglucomatase and glucose-6-P dehydrogenase. The activity of the immobilized glucose-6-P dehydrogenase was about the same as in preparations C, D, and E. The activity of the immobilized phosphoglucomutase was now greater than either D or E, although less than in B or C.

It should be noted that the data presented in Fig. 2 and Tables 1 and 2 indicate that phosphoglucomutase and glucose-6-P dehydrogenase, when used separately, differ in the rates at which they adsorb and bind to the carrier. In addition, in the presence of both enzymes together, these rates are altered even more.

The Effect of pH on the Activity of Soluble and Immobilized Preparations of Phosphoglucomutase and Glucose-6-P Dehydrogenase

The following preparations were used: (*a*) soluble phosphoglucomutase and glucose-6-P dehydrogenase; (*b*) phosphoglucomutase and glucose-6-P dehydrogenase immobilized to separate particles; and (*c*) phosphoglucomutase and glucose-6-P dehydrogenase immobilized to the same particle. The effect of pH on the activity of either enzyme was the same whether it was soluble or immobilized either on separate particles or on the same particles.

K_m Values for the Soluble Enzymes and Apparent K_m Values for the Immobilized Enzymes

With the soluble enzymes, the K_m of phosphoglucomutase for glucose-1-P and glucose-6-P dehydrogenase for NADP are within the range determined by Bocchini and Najjar (30) and Olive and Levy (23), whereas the K_m of glucose-6-P dehydrogenase for glucose-6-P (Table 3) is about twofold higher than given by Olive and Levy (23). All of the apparent K_m's determined for preparations of immobilized phosphoglucomutase and glucose-6-P dehydrogenase were from 1.5- to 5-fold higher than the soluble enzymes.

Comparing Long-Term Stability of Soluble and Immobilized Enzymes at 4 and 23°C

To determine the effects of temperature and the presence of substrates and cofactors on the retention of enzymatic activity, preparations of soluble

TABLE 3. **K_m Values for Soluble and Immobilized Enzymes**[a]

	Soluble enzyme (M)	Immobilized enzyme (M)
Phosphoglucomutase for glucose-1-P	5.8×10^{-5}	3.11×10^{-4}
Glucose-6-P dehydrogenase for glucose-6-P	8.71×10^{-5}	1.29×10^{-4}
Glucose-6-P dehydrogenase for NADP	9.35×10^{-6}	5.09×10^{-5}
Glucose-6-P dehydrogenase for NAD	1.00×10^{-4}	3.07×10^{-4}

[a]The apparent K_m values are listed for immobilized phosphoglucomutase and immobilized glucose-6-P dehydrogenase and K_m values for soluble phosphoglucomutase and soluble glucose-6-P dehydrogenase. The assay for the immobilized and soluble enzymes is given in the text under Methods. The apparent K_m and true K_m values were calculated using Lineweaver-Burk plots.

phosphoglucomutase, soluble glucose-6-P dehydrogenase, and phosphoglucomutase and glucose-6-P dehydrogenase immobilized on the same particles were incubated at 4 and 23°C for periods up to about one month. The preparations were kept in the standard assay solution, pH 7.6, containing glucose 1,6-diphosphate and $NADP^+$ at the stated temperature. Glucose-1-P and glucose-6-P were added during the incubations to the preparations of immobilized phosphoglucomutase and glucose-6-P dehydrogenase. At the end of the incubation periods, each sample was brought to room temperature (23°C) and assayed with the respective substrates.

The results show that the immobilized enzymes were more stable than the soluble enzymes regardless of the temperature (Fig. 3). At 23°C, the immobilized enzymes maintained more activity in the presence of substrates

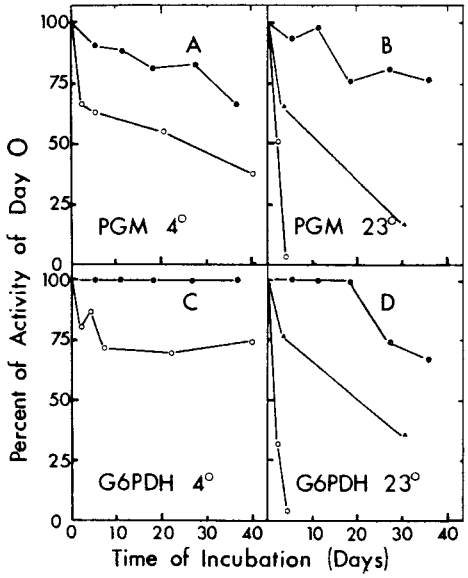


FIG. 3. Long-term stability of soluble and immobilized enzymes at 4 and 23°C. Preparations of soluble phosphoglucomutase and glucose-6-P dehydrogenase (open circles) were placed in the standard assay solution containing $NADP^+$ and glucose 1,6-diphosphate at 4 and 23°C. Aliquots of 1 ml were removed at various times and the enzymes were assayed at room temperature as described in the text. One preparation of phosphoglucomutase and glucose-6-P dehydrogenase (solid triangles) immobilized to the same particle was incubated in the assay solution without any additions. The other preparation (solid circles) was incubated in the standard assay solution containing $NADP^+$, glucose 1,6-diphosphate, and glucose 1-P using the same concentrations as in the enzyme assays. At the end of the incubations, the buffer solutions were removed, the particles washed with distilled water, and the immobilized enzymes were assayed as described in the text under Materials and Methods. All results are expressed as percent of the activity the respective enzyme preparations had before being incubated.

and cofactors than they did in their absence (Fig. 3B, D). Not shown in Fig. 3 are comparable experiments carried out at pH 9.14. Essentially similar trends were obtained at that pH as were observed at pH 7.6, except that (*a*) immobilized phosphoglucomutase was less stable at both temperatures, and (*b*) immobilized glucose-6-P dehydrogenase was less stable at 23°C.

Stability of Immobilized and Soluble Phosphoglucomutase and Glucose-6-P Dehydrogenase Kept at 58°C for Varying Periods

A preparation of phosphoglucomutase and glucose-6-P dehydrogenase immobilized to the same surface and preparations of soluble phosphoglucomutase and soluble glucose-6-P dehydrogenase were placed in a 58°C water bath for varying periods to determine the stability of the enzyme at high temperature. The soluble enzymes showed no activity after 2 min incubation at 58°C, whereas after 5 min incubation, immobilized phosphoglucomutase retained 91.5% of its original activity and immobilized glucose-6-P dehydrogenase 97% (Fig. 4). After a second incubation of 10 min the immobilized phosphoglucomutase and the glucose-6-P dehydrogenase retained 53.9 and 41.5% of their original activity. Further incubations of 20 and 40 min did not result in further major losses of activity.

DISCUSSION

We have described in this paper the preparation and properties of phosphoglucomutase and glucose-6-P dehydrogenase immobilized to the same surface. A discussion of each parameter investigated is presented below. A key factor to be kept in mind is the influence of the amount of soluble enzyme and of carrier present in the attachment solutions on the final activity of each enzyme immobilized. Such considerations are especially important in sequential enzyme systems since the distance between adjacent active enzyme molecules must be kept as short as possible to ensure that the intermediate of the sequential reaction is consumed as soon as it is produced and does not escape into the surrounding solution. These experiments also point out the complications that arise when immobilizing more than one enzyme to the same surface.

Rate of Immobilization of Proteins to the Surface of s-Triazine Trichloride Activated Cellulose

The results (Fig. 2) show that of the proteins bound (phosphoglucomutase, glucose-6-P dehydrogenase, and [^{14}C] bovine serum albumin)

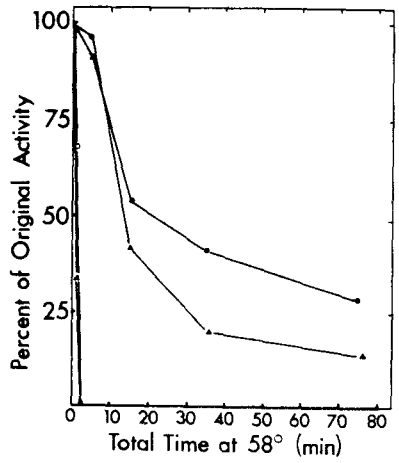


FIG. 4. Stability of soluble and immobilized enzymes at 58°C. One preparation of immobilized phosphoglucomutase and glucose-6-P dehydrogenase was used throughout the experiment and was incubated at 58°C for four separate periods of 5, 10, 20, and 40 min respectively (phosphoglucomutase, 37.7×10^{-4} U/ml, and glucose-6-P dehydrogenase, 78.8×10^{-4} U/ml). During the incubations the immobilized phosphoglucomutase (solid circles), the immobilized glucose-6-P dehydrogenase (solid triangles), the soluble phosphoglucomutase (open circles), and the soluble glucose-6-P dehydrogenase (open triangles) were kept in standard assay solution containing glucose 1,6-diphosphate and NADP. The test tubes containing the soluble enzymes were removed from the water bath at different times, chilled in ice for 5 min, and then brought to room temperature for the enzyme assays. The preparation of immobilized enzymes was kept in a fritted glass crucible in 10 ml of assay solution and placed in the heated water bath. At the end of the the incubation the crucible was removed, the warm assay solution was withdrawn, and the remaining particles were washed with distilled water. The particles were assayed for phosphoglucomutase and glucose-6-P dehydrogenase as described in the text under Methods. The time is expressed as the total time the particles had been exposed to 58°C.

nearly 90% was bound after being exposed to the activated carrier for 3 h. Nonetheless we chose to incubate the enzyme with the *s*-triazine trichloride cellulose for 20-h periods so as to bind as much enzyme as possible. The longer exposure did not appear to inactivate any of the enzyme that was bound (Fig. 2). In addition, under these conditions, a 20-h incubation guaranteed that any remaining active groups on the *s*-triazine trichloride cellulose would be hydrolyzed to give a carrier with inert hydroxyl groups rather than with the active triazine nuclei.

When both enzymes were present in the attachment solution the rate of binding of phosphoglucomutase was faster than that for glucose-6-P dehydrogenase. Since phosphoglucomutase and glucose-6-P dehydrogenase are competing for a limited number of sites on the surface of the activated carrier, any physical property which would allow one enzyme to reach the

surface faster than the other would permit it to bind more rapidly. The higher rate for phosphoglucomutase binding may be due to the lower molecular weight of phosphoglucomutase (31) which will enable it to diffuse to the surface of the carrier faster than the larger (23) glucose-6-P dehydrogenase molecule. The differences in rate of attachment may reflect differences in the charge of the proteins, although this is unlikely because Smith and Lenhoff (26) have shown that all proteins bind equally well to *s*-triazine trichloride activated cellulose at pH 5.5, regardless of the isoelectric point of the protein or the ionic strength of the buffer used for the attachment solution. The effect of the charge of the protein is reduced even further by use of an uncharged carrier.

Desorption of Nonimmobilized Enzyme From the Surface of the Carrier

The desorption experiments (Table 1) show that more enzyme was initially adsorbed to the *s*-triazine trichloride cellulose than eventually became covalently linked to them. Of the adsorbed phosphoglucomutase about 10–20% of the active enzyme remained covalently linked to the particles after four washes. The figure of 68.7% activity retained after 20.5 h incubation is probably erroneous and may reflect inactivation of absorbed phosphoglucomutase. Such inactivation of adsorbed enzyme is reflected in (Table 1, part A) by the row indicating total activity recovered; note that the amount recovered after 20.5 h incubation is about one-third that recovered after 3 h incubation. Such an activation of adsorbed enzyme has been demonstrated by Goldman and Lenhoff (27).

A similar pattern of desorption was also observed for glucose-6-P dehydrogenase (Table 1 part B). Approximately 5% of the adsorbed activity was retained as covalently bound enzyme after four washes. The 13.3% figure at 20.5 h, like the 68.7% figure for phosphoglucomutase after the same incubation time, also appears to be an artifact of inactivation of adsorbed enzyme (again, note that after 20.5 h, only about half of the desorbed activity was recovered when compared to that recovered after 3 h. Four washes were adequate to remove virtually all of the adsorbed noncovalently linked enzyme from the particles. Subsquent use of the preparations showed no further release of enzyme activity into solution.

Effect of Increasing the Amount of the Carrier on the Relative Activity of Immobilized Phosphoglucomutase and Glucose-6-P Dehydrogenase

A high level of activity of the immobilized enzyme was observed when only phosphoglucomutase and a small amount of carrier were placed in the

attachment solution (Table 2). The addition of glucose-6-P dehydrogenase to the attachment solution resulted in lower activity of phosphoglucomutase immobilized. The presence of glucose-6-P dehydrogenase in the attachment solution appears to be competing with the phosphoglucomutase for points of covalent attachement on the surface of the carrier.

Increasing the amount of the carrier to greater than 15 mg per preparation and thereby increasing the surface area available for enzymes to bind resulted in an increase in the amount of glucose-6-P dehydrogenase activity immobilized. The amount of glucose-6-P dehydrogenase activity immobilized did not increase any further when the amount of the carrier was increased from 50 to 100 mg. This finding suggests that the amount of active glucose-6-P dehydrogenase that can be immobilized to the surface had reached a maximum, and with 50 and 100 mg there was probably more surface area available than enzyme that could be immobilized. The glucose-6-P dehydrogenase activity immobilized using 25 mg of carrier (preparation C) was intermediate between that immobilized between 15 and 50 mg of carrier; in preparation C, therefore, the amount of soluble glucose-6-P dehydrogenase was still in excess of that which could be immobilized.

The activity of immobilized phosphoglucomutase did not increase as the amount of the carrier was increased. At values greater than 25 mg of carrier the activity of the immobilized phosphoglucomutase began to decrease. The loss of phosphoglucomutase activity may have been the result of denaturation due to the increased distance between adjacent enzyme molecules. This denaturation was partially overcome by the addition of bovine serum albumin to the attachment solution (preparation F). The bovine serum albumin may stabilize the phosphoglucomutase through protein–protein interactions. The presence of bovine serum albumin may have also minimized the number of free triazine molecules surrounding the phosphoglucomutase.

The presence of a large excess of *s*-triazine trichloride carrier in the attachment solution may result in covalent bonds being formed between amino acid residues in phosphoglucomutase other than those normally involved in the binding of phosphoglucomutase. These added points of attachment may change the conformation of the phosphoglucomutase or may alter the active site, resulting in loss of activity. It is possible that the dephosphorylated form of the enzyme may be bound. It was shown by Bocchini et al. (32) that this form is more reactive to thiotitration than the phosphorylated form of phosphoglucomutase.

It is difficult to determine why glucose-6-P dehydrogenase activity is not affected by the increased amount of carrier, whereas phosphoglucomutase activity is. One possible explanation is the absence of cysteine residues in glucose-6-P dehydrogenase. The reactivity of *s*-triazine

trichloride to sulfhydryls is high (26) and may be even higher when the carrier is present in excess. Since phosphoglucomutase contains cysteine residues and glucose-6-P dehydrogenase does not (32, 33), the possibility for the formation of additional covalent bonds with the carrier is much higher for phosphoglucomutase.

The Effect of pH on the Activity of Preparations of Soluble and Immobilized Phosphoglucomutase and Glucose-6-P Dehydrogenase

The activity for both the immobilized and soluble enzymes was determined at different pH values and found to be nearly identical for enzymes in both states, regardless of whether or not the enzymes were bound on the same or separate particles. Such similarities of pH ranges suggest that none of the factors usually responsible for changes in pH optima apply here.

The K_m of Soluble Phosphoglucomutase and Glucose-6-P Dehydrogenase and the Apparent K_m for Immobilized Phosphoglucomutase and Glucose-6-P Dehydrogenase

The apparent K_m values determined for phosphoglucomutase and glucose-6-P dehydrogenase immobilized to separate particles were consistently higher than the K_m values for the soluble enzymes (Table 3). Increased apparent K_m values have been observed in many other preparations of immobilized enzymes (1,3,16,26,34–41). These increases are believed to be due to (*a*) the presence of a diffusion layer surrounding the enzyme-bearing matrix, (*b*) altered affinity of the enzyme for its substrate as a result of the immobilization process, (*c*) electrostatic interaction between the matrix and the product or substrate. Many solutions have been proposed to determine the true K_m of preparations of immobilized enzymes (42–49).

Long-term Stability of Soluble and Immobilized Phosphoglucomutase and Glucose-6-P Dehydrogenase at 4° and 23°C

Comparisons of the stability of immobilized and soluble enzymes at 4° and 23°C after treatments as long as 40 days showed that in all cases the immobilized enzymes were much more stable (Fig. 3). In addition, it was shown that the presence of all substrates and cofactors enhanced the stability of both phosphoglucomutase and glucose-6-P dehydrogenase at 23°C. Similar experiments with other immobilized enzymes show enhanced retention of activity (2,14,16,41,50–63). It is thought that the immobilization of the enzyme imparts thermal stability by allowing thermal energy

adsorbed by the enzyme to be distributed throughout the matrix, thereby stabilizing the protein conformation (41). Immobilization may also increase the stability of the enzyme by providing a microenvironment of high protein concentration (16).

Stability of Soluble and Immobilized Enzymes at 58°C

The enzymes immobilized to the same particles were relatively stable at 58°C for over an hour, compared to the soluble enzymes, which were inactivated within 2 min at that temperature (Fig. 4). Such stability of immobilized enzymes at high temperature has been noted by others (37,40,50,53,55,58,59,64–67), although heat stability is not a general attribute of immobilized enzymes. Goldman and Lenhoff (27) suggest that in those cases in which thermal stability is not observed, the enzyme may be prevented from returning to its original conformation because of restrictions caused by the immobilization procedure. In the experiments reported herein, we feel that the *s*-triazine trichloride is covalently linked to a number of sites such that much of the enzyme retains its native conformation during most of the heat treatment.

CONCLUDING REMARKS

These results indicate that the binding of the two enzymes to the same surface introduces complications not found when binding only one enzyme to a surface. The ratio of the two enzymes bound to the surface is not as easily controlled as in preparations of soluble enzymes or when both enzymes are immobilized to separate particles. The rate of binding of each enzyme is determined by the physical properties of the enzyme and will influence the ratio of the two enzymes that will be bound to the surface. In certain cases it may be necessary to bind the enzymes in a stepwise fashion: that is, allowing one enzyme to bind first, then transferring the preparation to another solution containing the other enzyme.

That more enzyme is adsorbed to the surface than is bound may be due to the attraction of nonbound protein to the high concentration of protein present at the surface of the carrier. The loss of activity of the adsorbed enzyme after 20 h did not seem to affect the activity of the immobilized enzyme, since the level seen at that time was higher than the level after 3 h of binding. If the longer incubation inactivated the enzyme, we would have expected a lower amount of bound-enzyme activity. The increase in activity of phosphoglucomutase and glucose-6-P dehydrogenase bound after 20 h is close to the percent increase observed in the amount of [^{14}C] bovine serum

albumin bound between 3 and 19 h. The latter is an indication that binding of protein is taking place during this time.

The amount of enzyme present in solution is important for making preparations with a high activity of immobilized enzyme. The idea of keeping the two enzymes in close proximity is important for the sequential reaction to be most efficient; this will be discussed elsewhere. The properties of the immobilized enzymes point out their potential usefulness in analytical procedures; in addition to being easily removed after an assay, they are more stable than the soluble enzymes (Fig. 3).

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