Matrix-Bound Enzymes

Part II:* Studies on a Matrix-Bound Two-Enzyme-System

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The present investigation describes the binding through covalent linkages of a two-enzyme-system, consisting of hexokinase and glucose-6-phosphate dehydrogenase, to individual polymer particles. The polymers used as matrices were Sepharose 4 B and a copolymer of acrylamide-acrylic acid. The enzymes were attached to the matrices using cyanogen bromide and a water-soluble carbodiimide, respectively. The enzymic activity of the matrix-bound two-enzyme-system was determined in the coupled test after addition of glucose, ATP and $NADP^+$, as moles of NADPH formed per minute. Subsequently the separate enzymic activities of bound hexokinase and glucose-6-phosphate dehydrogenase were determined. The formation of NADPH in the coupled test with free hexokinase and glucose-6-phosphate dehydrogenase was then measured using the same total number of enzyme units/ml for both enzymes as obtained from the matrix-bound two-enzyme-system. Taking the amount of moles of NADPH formed per minute/ml in the soluble system as reference, an increase in the formation of NADPH per minute with the matrix-bound two-enzyme-systems was measured amounting to 40—100 % for the Sepharose-system and 140 % for the copolymer (acrylamide-acrylic acid)-system, respectively.

In addition glucose-6-phosphate dehydrogenase as well as hexo-kinase+glucose-6-phosphate dehydrogenase were entrapped in cross-linked acrylamide polymers. The enzyme-polymer granules obtained were repeatedly used in enzymic tests as "NADPH-generators" showing no loss of activity for the former preparation after a period of 80 days and only about 10 % for the latter preparation during the same length of period. Finally a simple method for spectro-photometric assays found suitable for matrix-bound enzymes is described.

Since we now have a variety of methods at our disposal which can be applied for the binding of enzymes to matrices, a further step in this field should be taken with the aim of preparing matrix-bound multi-enzyme-systems.

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To the authors' knowledge, the only steps hitherto taken in this direction have been a study on several entrapped glycolytic enzymes, with each enzyme arranged in a separate section within a column [1] and kinetic investigations on the two-enzyme-system pyruvate kinase and lactate dehydrogenase, with each individual enzyme bound to separate filter-paper disks.[2] However, no reports have yet appeared on the binding of more than one enzyme on the same matrix surface. A situation like the latter offers a more realistic model for the conditions occurring *in vivo*, in which enzymes are arranged in sequences on membranes or other cellular material.

Furthermore, provided a tight coupling of enzymes acting in sequence can be accomplished on one matrix, it may be possible to demonstrate, that the intermediate substrates formed either do not, or only partially, diffuse out into the surrounding medium. They will rather, due to the close proximity of the coupled enzymes, immediately be transformed by the enzyme next in sequence, the net effect being a higher yield of product formed per minute as compared to soluble enzymes. To demonstrate the latter effect, a two-enzyme-system consisting of hexokinase (HK) + glucose-6-phosphate dehydrogenase (G-6-PDH) has been studied. The choice of these two enzymes has partially been influenced by the fact, that matrices carrying these enzyme activities might be of practical use as "NADPH-generators" in, *e.g.*, enzymic assays requiring reduced nicotinamide adenine dinucleotide phosphate.

MATERIALS AND METHODS

Materials. Acrylamide, N,N,N',N'-tetramethylethylenediamine was purchased from Eastman, N,N'-methylenebis(acrylamide) from Fluka, Buchs SG, acrylic acid from Ugilor, and 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluenesulfonate from Aldrich Chemical Co.

Sepharose 4 B was obtained from Pharmacia, Uppsala. NADP, ATP, and glucose-6-phosphate were purchased from Sigma Chem. Comp. The enzymes used, hexokinase (cryst., from yeast, ammonium sulfate susp., 100−130 U/mg) and glucose-6-phosphate dehydrogenase (cryst. from yeast, ammonium sulfate susp., 300 U/mg) were also obtained from Sigma.

Abbreviations used: Acrylamide=AAm, acrylic acid=AA, N,N'-methylenebis(acrylamide)=Bis, N,N,N',N'-tetramethylethylenediamine=TEMED, 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluenesulfonate=CMC, cross-linked copolymer of acrylamide-acrylic acid=copoly-(AAm-AA), glucose-6-phosphate=G-6-P, glucose-6-phosphate dehydrogenase=G-6-PDH, hexokinase=HK, 0.05 M tris-HCl, 0.007 M $MgCl_2$ buffer (pH 7.6)=tris-$MgCl_2$. Other abbreviations used, as in part I.

Preparation of entrapped glucose-6-phosphate dehydrogenase in 10 % (w/w) gel granules. The polymerization procedure applied followed except for slight modifications, the method given in part I. Two stock solutions were prepared. Solution I: 0.5 ml of 0.1 M phosphate buffer (pH 6.5) containing 95 mg of AAm, 2 mg of ammonium persulfate and 15 μl of TEMED. Solution II: 0.5 ml of the above buffer containing 5 mg of Bis and 16.6 U of G-6-PDH (333 U/ml). Both solutions were kept at 0° for a few minutes prior to mixing in a small test tube. The solution was then gassed with nitrogen and kept at 0°. Polymerization proceeded within one hour. The preparation of granules from the gel obtained followed the description given in part I. The enzyme-granules were subsequently washed for 30 min each in cold 0.1 M $NaHCO_3$, 0.001 M HCl and 0.5 M NaCl. After a final wash in tris-$MgCl_2$ buffer, they were ready for use.

Preparation of both entrapped and covalently bound glucose-6-phosphate dehydrogenase to cross-linked copolymer of acrylamide-acrylic acid, 10 % (w/w). The procedure given in part I was slightly modified. The amounts of monomers used for the preparation of 1 ml of gel were: 48 mg of AAm, 48 mg of AA and 5 mg of Bis. The catalyst system

consisted of 2 mg of ammonium persulfate and 20 μl of TEMED. 96 U of G-6-PDH (the enzyme had been dialyzed against 0.1 M phosphate buffer (pH 7.0) at 4°, 227 U/ml) were added followed by the addition of 4 mg of water soluble carbodiimide (=CMC). After 30 h at 4° the formed gel block was granulated and the enzyme granules obtained washed for 2 h in 0.1 M $NaHCO_3$ followed by a 30 min wash in each of the above mentioned solutions.

Preparation of coentrapped hexokinase and glucose-6-phosphate dehydrogenase in 10 % (w/w) gel granules. The procedure given for the preparation of entrapped G-6-PDH was followed. The total number of enzyme units added to stock solution II was 100 U for HK (333 U/ml) and 33.3 U for G-6-PDH (333 U/ml).

Simultaneous coupling of hexokinase and glucose-6-phosphate dehydrogenase to Sepharose 4 B. 1 ml of Sepharose 4 B suspension (20 mg/ml) was activated using 2 ml of BrCN solution (50 mg/ml water). Activation proceeded under slight stirring during 8 min, keeping the pH of the suspension at 11 by continuous addition of 4 M NaOH. The gel was then washed on a glass filter with cold water and 0.1 M $NaHCO_3$. The Sepharose was then transferred to a small beaker containing 1 ml of cold 0.1 M $NaHCO_3$. After addition of 15.9 U of HK (the enzyme had prior to coupling been dialyzed for 20 h against 0.1 M phosphate buffer (pH 7.0) at 4°, 497 U/ml) together with 2.5 U of G-6-PDH (dialyzed as above, 227 U/ml) coupling proceeded under slight stirring for 12 h at 4°. The enzyme gel was subsequently washed as described above (Sepharose I). For the other preparation (Sepharose II), a higher ratio HK *versus* G-6-PDH was coupled in order to have the soluble system reach V_{max} (equal to that of the matrix-bound system) after only a short lag-period.

Simultaneous coupling of hexokinase and glucose-6-phosphate dehydrogenase to cross-linked copolymer of acrylamide-acrylic acid, 20 % (w/w). The coupling to copoly-(AAm-AA) of HK together with G-6-PDH followed largely the procedure given in part I. 20 mg of dry gel granules in a small beaker were permitted to swell for 30 min in 1 ml of 0.1 M phosphate buffer (pH 6.5). 12.4 U of HK (dialyzed as above, 497 U/ml) together with 3.4 U of G-6-PDH (dialyzed as above, 227 U/ml) were added to the suspension. After slight stirring for 5 min, 50 mg of CMC was added to the suspension and stirring continued for 12 h at 4°. The enzyme-gel granules were washed as described for the entrapped enzymes.

Assay procedure for matrix-bound enzymes. The following general procedure was applied. A 25 ml E-flask, containing the incubation mixture, was kept in a water-bath (25°), the latter being placed above a magnetic stirrer. A speed setting was chosen to keep the suspension stirred at a rate found optimal for the enzymic reaction to be studied (120 rpm, dimensions of Teflon bar used: 0.4×2.7 cm). Using a peristaltic pump (LKB Perpex, type 12001-2) the incubation solution was continuously pumped out of the system (2 ml/min) by way of a plastic tubing, the inlet of which had been covered with a piece of nylon net to keep off any gel particles. The incubation solution was passed through a flow-cuvette (Zeiss MT 4 D), placed in a spectrophotometer (Zeiss P M Q II), and back again to the reaction vessel.

The incubation solution present in tubing and cuvette and thus not being in contact with the enzyme gel granules amounted in the present studies to 2 ml out of a total of 12 ml of incubation mixture.

Determination of enzymic activity of matrix-bound hexokinase. The enzymic activities of both entrapped and covalently bound HK preparations were determined in the above described assay unit. Excess of soluble G-6-PDH was added to the incubation mixture and the amount of NADPH formed as determined at 340 $m\mu$ used as a measure of the enzymic activity of the matrix-bound HK. The incubation mixture consisted of matrix-bound HK (100 mg entrapped prep. or 20 mg of dry polymer) suspended in 11.6 ml of tris-$MgCl_2$ buffer containing 26.64 μmoles of glucose, 5.23 μmoles of $NADP^+$ and 3.3 U of G-6-PDH (333 U/ml). The enzymic test was started by addition of 7.26 μmoles of ATP dissolved in 400 μl of buffer.

Determination of enzymic activity of matrix-bound glucose-6-phosphate dehydrogenase. The procedure followed the description given above. The amount of NADPH formed was measured at 340 $m\mu$. The incubation mixture consisted of matrix-bound G-6-PDH (100 mg entrapped prep. or 20 mg of dry polymer) suspended in 11.6 ml of tris-$MgCl_2$ buffer in which 5.23 μmoles of $NADP^+$ was dissolved. The enzymic test was started by addition of 11.18 μmoles of G-6-P dissolved in 400 μl buffer.

Determination of enzymic activity of matrix-bound hexokinase + glucose-6-phosphate dehydrogenase. The determination of the enzymic activities of gel particles carrying both HK and G-6-PDH proceeded in the following way. First the formation of NADPH was determined in the coupled reaction in an incubation mixture containing 26.64 μmoles of glucose, 5.23 μmoles of NADP$^+$ and 7.26 μmoles of ATP in 12 ml of tris-MgCl$_2$ buffer. Subsequently the incubation solution was sucked off and the enzyme gel particles washed thoroughly with tris-MgCl$_2$ buffer. Immediately thereafter the separate G-6-PDH activity was determined by incubation with G-6-P and NADP$^+$. Finally, after thorough washing of the gel particles as above, the separate HK activity was determined through incubation of the same gel particles with free G-6-PDH, glucose, ATP and NADP$^+$ as described above. No loss of activity of neither bound HK nor G-6- PDH was observed through the different assays following each other.

To check whether the polymers applied as two-enzyme matrices, Sepharose 4 B and copoly-(AAm-AA), affect the enzymic reactions studied through adsorption of substrate or cofactor, incubations were carried out with soluble HK and G-6-PDH in the presence of varying amounts of "blank-gel". No such interference, however, was observed.

Determination of enzymic activity of free hexokinase + glucose-6-phosphate dehydrogenase in the coupled reaction. In order to permit a comparison between the enzymic activity of free HK and G-6-PDH in the coupled reaction and that of the corresponding matrix-bound two-enzyme-system, the following assay was run. The same total number of enzyme units of free HK and G-6-PDH per ml, corresponding to the obtained separate enzymic activities (determined as nmoles of product formed/min/ml of HK and G-6-PDH in the matrix-bound two-enzyme-system), has been added to an incubation solution containing 6.66 μmoles of glucose. 1.83 μmoles of ATP and 1.31 μmoles of NADP$^+$ in 3 ml of tris-MgCl$_2$ buffer. The formation of NADPH was determined at 340 mμ.

Stability tests of entrapped glucose-6-phosphate dehydrogenase and coentrapped hexokinase and glucose-6-phosphate dehydrogenase. The enzymic activities of both preparations were determined as described in the previous section. After carefully washing the enzyme containing gel particles with tris-MgCl$_2$ buffer, they were stored at −15°. These preparations were thawed at intervals and tested for enzymic activities.
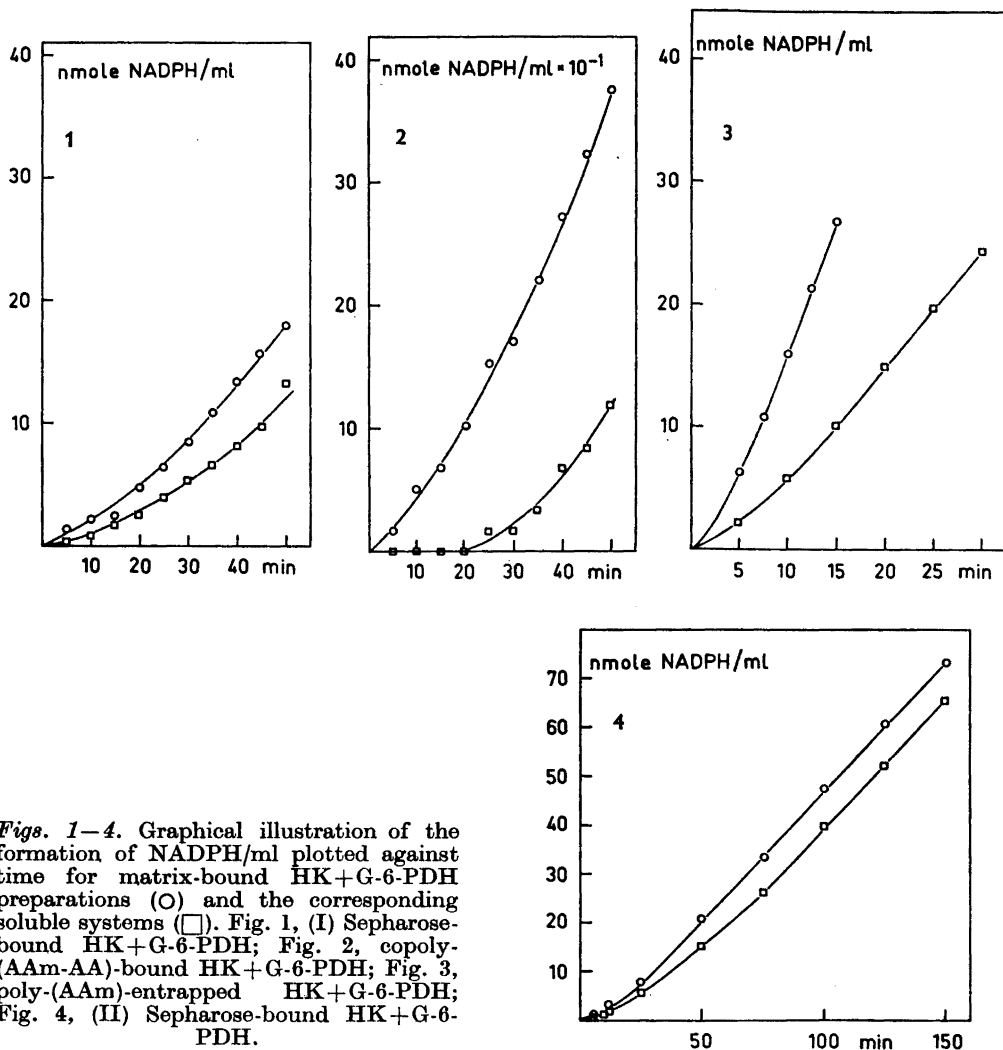

RESULTS AND DISCUSSION

The two enzymes studied in the present investigation, HK and G-6-PDH, were bound through covalent linkages to two different matrices, Sepharose and a cross-linked copolymer of acrylamide-acrylic acid. Except for a recent note [3] on the coupling of HK to Sephadex G 200, which appeared while this work was in progress, no reports have yet appeared in the literature on the binding of these enzymes to insoluble matrices. The two enzymes were chemically attached to Sepharose using the cyanogen halide method as developed by Porath and Axén, and to the copoly-(AAm − AA) with the aid of a water-soluble carbodiimide as described in part I.

After preliminary studies on the proper coupling conditions for each individual enzyme, the two enzymes were bound simultaneously to the above matrices. The activity of the gel particles carrying the two-enzyme-system, HK + G-6-PDH, was determined in the coupled reaction as number of moles of NADPH formed per minute and ml after addition of excess of glucose, ATP and NADP$^+$. The separate enzymic activities of these matrices could be determined by addition of G-6-P and NADP$^+$ for the assay of bound G-6-PDH and for the determination of bound HK activity by incubation with glucose, ATP, NADP$^+$ and free G-6-PDH. The formation of NADPH in the coupled test with free HK and G-6-PDH was then measured using the same total number of enzyme units/ml for each enzyme as obtained from the matrix-

Table 1. Formation of NADPH (nmole/min/ml) in the coupled reaction of the matrix-bound two-enzyme-system, hexokinase + glucose-6-phosphate dehydrogenase. The enzymic activities, expressed as nmoles of product formed/min/ml of bound HK and G-6-PDH have also been determined separately. In the assays with free enzymes, the above obtained separate activities have been used and the number of nmoles of NADPH/min/ml formed in the coupled test measured. To permit a comparison of the two systems, the activities of the soluble systems were determined at the same time at which the matrix-bound enzyme systems had reached their V_{max} and been taken as reference ($= 100$ %).

Sample	Enzyme activity			
	nmole G-6-P/ min/ml	nmole NADPH/ min/ml	nmole NADPH/ min/ml in coupled reaction	ratio bound/ free in coupled reaction (per cent)
(I) Sepharose-bound				
HK+G-6-PDH			0.50 (30th min)	200
HK	1.1			
G-6-PDH		1.3		
Free				
HK+G-6-PDH			0.25 (30th min)	100
HK	1.1			
G-6-PDH		1.3		
(II) Sepharose-bound				
HK+G-6-PDH			0.55 (40th min)	140
HK	2.4			
G-6-PDH		1.1		
Free				
HK+G-6-PDH			0.40 (40th min)	100
HK	2.4			
G-6-PDH		1.1		
Copoly-(AAm-AA)-bound				
HK+G-6-PDH			0.085 (30th min)	240
HK	0.25			
G-6-PDH		0.94		
Free				
HK+G-6-PDH			0.035 (30th min)	100
HK	0.25			
G-6-PDH		0.94		
Poly-(AAm)-entrapped				
HK+G-6-PDH			0.20 (15th min)	210
HK	1.3			
G-6-PDH		14		
Free				
HK+G-6-PDH			0.095 (15th min)	100
HK	1.3			
G-6-PDH		14		

Figs. 1—4. Graphical illustration of the formation of NADPH/ml plotted against time for matrix-bound HK+G-6-PDH preparations (O) and the corresponding soluble systems (□). Fig. 1, (I) Sepharose-bound HK+G-6-PDH; Fig. 2, copoly-(AAm-AA)-bound HK+G-6-PDH; Fig. 3, poly-(AAm)-entrapped HK+G-6-PDH; Fig. 4, (II) Sepharose-bound HK+G-6-PDH.

bound two-enzyme-system. Taking the number of moles of NADPH formed per minute/ml in the soluble system as reference (= 100 %), an increase in the formation of NADPH/min/ml with the matrix-bound two-enzyme-system was found amounting to 40 — 100 % for the Sepharose-systems and 140 % for the copoly-(AAm-AA)-system, respectively (Table 1).

The following interpretation of these results could be made. G-6-P, the product of the enzymic reaction of HK, will immediately be oxidized in the matrix-bound system to gluconolactone-6-P through the enzymic reaction of G-6-PDH sitting in close proximity to HK on the matrix. With other words,

all or part of the G-6-P formed will be transformed before equilibration through diffusion with the surrounding medium can take place, or, put in another way, the actual concentration of G-6-P in the microenvironment of the G-6-PDH molecules will be higher as compared to the corresponding soluble two-enzyme-system, where the distance for G-6-P from the site of its formation to G-6-PDH is far greater, thus leading to dilution.* The possibility, that the gel matrices used may absorb substrate or cofactors participating in the two-enzyme-assay, thus accounting for the effect observed, can be excluded since no interference has been observed in assays with soluble enzymes to which "blank-gels" had been added. The values listed in Table 1 have been chosen from a series of experiments; it has been found difficult to reproduce exactly the coupling conditions.

In Figs. 1 − 4 the formation of NADPH/ml in the coupled reaction has been plotted against time. As can be seen, all the matrix-bound systems reach their V_{max} while the corresponding soluble systems are still in their lag-phase. The length of the lag-phase, however, varies for each preparation. Of the enzyme systems bound by covalent linkages to different matrices, Sepharose (I) (Fig. 1), Sepharose (II) (Fig. 4) and copoly-(AAm-AA) (Fig. 2), this effect is most pronounced in the last mentioned preparation. This result is in line with the interpretation given above, since here the ratio of enzymic activity of HK *versus* G-6-PDH is small. In the Sepharose-bound enzyme system (II), a high ratio in the enzymic activity of HK *versus* G-6-PDH has been chosen in order to shorten the lag-phase for the corresponding soluble system. As is illustrated in Fig. 4, the curve for the soluble system reaches a linear increase of NADPH formation/ml/min ($= V_{max}$) which is equivalent to that of the matrix-bound system.

In the coentrapped enzyme-gel preparations, the formation of NADPH was also higher, by 110 %, than in the soluble system. This finding is easily explained by the fact that the diffusion out into the medium of the G-6-P formed within the gel-lattice is impeded, thus increasing the probability of its

Table 2. Activity and stability of G-6-PDH as well as of HK+G-6-PDH entrapped in cross-linked polyacrylamide. The enzyme-gel granules were tested at the below given intervals, between the different tests they were stored at −15°.

| Sample | Enzymic activity (μmole NADPH/min/g dry polymer) | | | | |
	0	5	10	20	80 days
Polyacrylamide-entrapped G-6-PDH	0.40	0.41	0.40	0.40	0.39
Polyacrylamide coentrapped HK+G-6-PDH	0.088	0.083	0.083	0.082	0.078

* Another factor that might have influenced the obtained results is the fact that particles in a stirred solution are surrounded by an unstirred layer that may impede diffusion of products formed at a matrix surface.

reaction with G-6-PDH precent in the gel. The preparation of the matrix-bound two-enzyme-system described here is a first step in the construction of models of structurally organized enzyme systems.

The entrapped G-6-PDH as well as HK + G-6-PDH preparations have been used as "NADPH-generators". The enzyme-gel granules could simply be added to the NADPH-requiring enzyme system together with the appropriate substrates and cofactors and after completion of the reaction filtered off. They were stored frozen at − 15° and used repeatedly without any major loss of activity (Table 2). Preparations in which G-6-PDH was kept within the gel both entrapped and covalently bound showed an enzymic activity of 2 μmoles of NADPH formed per minute and g of dry polymer. Using this method any interference of the auxiliary enzymes with the other enzymes present in the incubation mixture, is avoided. In addition, considering the rather high cost of the auxiliary enzymes or NADPH, it appears to be an economic procedure.

A continuous spectrophotometric assay of matrix-bound enzymes gives, as is easily understood, rise to a number of problems. They may be circumvented by either carrying out the enzymic reaction to be studied in a column by passing the matrix-free eluent through a spectrophotometer,[4] or by letting the enzyme reaction take place in a special type of cuvette permitting stirring of the suspension.[5] The first procedure is laborious and requires a special "column-kinetics", while in the latter procedure, optical interference on the part of the carrier may occur. The assay procedure in the present study eliminates these limitations. It appears well suited for the study of any one-enzyme reaction provided the substrate is in excess and no product inhibition takes place, as typified by the trypsin reaction studied in part I. When dealing with multi-enzyme systems, low substrate concentrations or when product inhibition is occurring, the fraction of the total volume present in cuvette and tubing ("dead volume") and thus not in contact with the enzyme-granules, has to be accounted for. However, provided the ratio total volume/"dead volume" is large enough it may be neglected. This has been shown to be the case in the assays described here. Controls run batchwise on the above polymerbound two-enzyme-system yielded the same number of moles of NADPH after 30 min of incubation as in the continuous assay procedure described.

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