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# Polymeric Thiols as Enzyme Activators of Serum Creatine Phosphokinase

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## ABSTRACT

Several hydrophilic polymeric thiols were prepared from amino-activated polymeric supports by reaction with *N*-acetylhomocysteine-ethiolactone. Supports include agaroses, cellulose, Glycophase™ controlled-pore glass, and Matrex™ acrylic beads. Thiol content in these polymers was 3–72 μmol SH/g dry polymer. Several were effective solid-phase activators of the sulfhydryl-dependent enzyme creatine phosphokinase at concentrations comparable to that of monomeric thiol required for enzyme activation. The kinetic activation curves for the polymeric and the monomeric (thioglucose) activators were similar, suggesting unhindered interaction of the enzyme with the polymeric activator.

**Index Entries:** Polymeric thiols; polymeric mercaptans; creatine phosphokinase; solid-phase enzyme activators; sulfhydryl-dependent enzymes.

## INTRODUCTION

Polymers with mercapto (thiol) groups are of interest because of their potential utility as ion-sequestering agents, redox catalysts, and free-radical scavengers (*1*). Less attention has been given to the preparation and use of mercapto polymers as enzyme activators (i.e., in lieu of low-molecular-weight thiol activators for enzymes such as cysteine proteases or creatine phosphokinase). The preparation of such polymers requires the

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use of an enzyme-compatible solid-phase support, whereas much work in the past has centered around the use of polystyrene as the polymeric mercapto carrier. More hydrophilic, biocompatible carriers have been used in mercapto polymers, e.g., thiol agarose (2). Such polymers are effective solid-phase reducing agents for protein disulfide units (3).

Many other classes of potential sulfhydryl carriers may be envisioned to be effective biocompatible protein activators. Glasses, polysaccharides, polyamines, and others, may be converted to thiol-containing polymers by chemical modification to yield hydrophilic materials. Furthermore, the degree of thiol substitution may be varied in a controlled fashion and the effect on reducing power and activation may be directly monitored.

We report here the synthesis of polymeric thiols with a wide range of thiol content and varying degrees of hydrophilic character. Applications center around the use of such polymers as potential enzyme activators. Specifically, serum creatine phosphokinase was chosen for such studies because it is known to be activated by thiol compounds such as mercaptoethanol, *N*-acetylcysteine, dithioerythritol, and thioglucose (4). Several of the polymers reported here are efficient activators of creatine phosphokinase at a thiol concentration similar to that for thioglucose activation.

## MATERIALS AND METHODS

### Materials

Chemicals and solvents were obtained from Kodak Laboratory Chemicals unless otherwise noted. Glycophase controlled-pore glass was obtained from Pierce. Microcrystalline celluloses ($< 20\ \mu$) were from Sigma (Sigmacell 20) or FMC (Avicel). Matrex 101 microbeads (1–3 $\mu$) from Amicon had a surface area of 6 $m^2/g$. Polyethyleneimine (MW 40,000–60,000) was from Polysciences. *N*-acetylhomocysteinethiolactone was from Aldrich. Agaroses were supplied by Pharmacia and Biorad and were less than 50 $\mu$ in diameter. Sodium cyanoborohydride was an Alfa product.

### Assay for Mercapto Groups

Ellman's method was used to determine the sulfhydryl content of the polymers (5).

### Synthesis of Polymeric Thiol Reagents— Thiolation of AH-Sepharose 4B with *N*-Acetylhomocysteinethiolactone

AH-Sepharose 4B (15 g; activated) was suspended in a mixture of 60 mL of dioxane and 15 mL of water. *N*-acetylhomocysteinethiolactone (2.0

g) was added to the suspension, and the pH was adjusted to 7.5. The reaction mixture was stirred under nitrogen at room temperature for 4 d. The product was collected on a sintered-glass funnel and washed with 800 mL of 50% aqueous dioxane (negative SH test found on last few mL of wash). The product was then washed with 100 mL of water and freeze-dried, giving 1.5 g of material with 0.7% sulfur by combustion analysis.

In a duplicate run, the activated product was freeze-dried, giving 0.9 g of material with 12 μmol/g of sulfhydryl.

## Glycophase 1350 Å/Diaminodipropylamine/ *N*-Acetylhomocysteine Thiolactone

Glycophase 1350 Å (1 g) was suspended in 20 mL of 0.1 M sodium periodate, and the suspension was stirred at room temperature for 2 h. The oxidized glass was washed twice with water by centrifugation and then added to a solution of 1 M diaminodipyropylamine (50 mL), adjusted to pH 8, containing 3 g of sodium cyanoborohydride. The suspension was shaken at room temperature for 4 d and then washed thoroughly with water. The glass was resuspended in a solution of 2 g of *N*-acetylhomocysteine thiolactone in 50 mL of 1:1 tetrahydrofuran (THF)/water. Shaking was continued at room temperature for 3 d; the glass was washed thoroughly with 1:1 THF/water, then with water, and lyophilized to give the product. Spectrophotometric analysis for thiol groups with Ellman's reagent showed 3 μmol SH/g.

## Affi-Gel 701/*N*-Acetylhomocysteine Thiolactone

Affi-Gel 701 (50 mL) was washed by centrifugation and resuspended in a solution of 3 g of *N*-acetylhomocysteine thiolactone in 50 mL of 1:1 THF/water. The gel was shaken at room temperature for 4 d, washed with 1:1 THF/water and with water, and stored refrigerated as a slurry. Thiol analysis gave 3.5 μmol SH/mL.

## Glycophase 1350 Å/Polyethyleneimine/ *N*-Acetylhomocysteine Thiolactone

Glycophase 1350 Å (1.5 g) was suspended in 30 mL of 0.1 M $NaIO_4$ and shaken at room temperature for 3 d. The glass was washed with water and resuspended in a solution of polyethyleneimine (1 mL of a 33% solution of 40,000–60,000 MW PEI) in 50 mL of water (pH adjusted to 8) containing 1 g of sodium cyanoborohydride. The glass was shaken at room temperature for 1 d, washed, and resuspended in a solution of 2 g of *N*-acetylhomocysteine thiolactone in 50 mL of 1:1 THF/water (pH 7.6). Stirring was continued for 3 d, followed by washing and lyophilization to give the product (560 mg) containing 15 μmol SH/g.

## Glycophase 550 Å/Polyethyleneimine/ *N*-Acetylhomocysteine Thiolactone

The procedure was identical to the above except that Glycophase 550 Å was used as the support. The recovered solid had a thiol content of 39 μmol SH/g.

## Avicel TG-101/Polyethyleneimine/ *N*-Acetylhomocysteine Thiolactone

Avicel TG-101 (15 g) was suspended in 300 mL of 0.1 M sodium metaperiodate and shaken at room temperature for 1 h. The cellulose was washed and added to a solution of polyethyleneimine (20 mL of a 33% solution of MW 40,000–60,000) in 100 mL of water (pH 8). Stirring was continued overnight. Sodium borohydride (4 g) was added, and shaking was continued for 28 h. The cellulose was washed and then added to a solution of 6 g of *N*-acetylhomocysteine thiolactone in 100 mL of 1:1 ethanol/water, with pH adjusted to 8.5. The suspension was shaken at room temperature for 18 h, then washed with ethanol/water (1:1) and lyophilized (14.3 g, 38 μmol SH/g). By this procedure, another run gave a modified polymer with 18.5 μmol SH/g.

## Matrex™ 101/Polyethyleneimine/ *N*-Acetylhomocysteine Thiolactone

Matrex 101 (30 mL, 0.40 meq COOH/g) was added with stirring to 10 mL of polyethyleneimine (33%, 40,000–60,000 MW). The mixture was heated at 80°C for 2 h and washed thoroughly with water by centrifugation. The activated Matrex 101 was then added to a solution of 2 g of *N*-acetylhomocysteine thiolactone in 25 mL of 0.2 M sodium carbonate (pH 9) and 25 mL of ethanol. Stirring was continued at room temperature for 36 h, followed by washing with 1:1 ethanol/water and water. Lyophilization gave the product containing 38 μmol SH/g.

## Agarose/Adipic Acid Dihydrazide/ *N*-Acetylhomocysteine Thiolactone

Adipic acid dihydrazide (18.0 g, 10.0 mol) was suspended in 200 mL of sodium carbonate buffer (0.1 M, pH 9.0). To the suspension was added 1.5 g of CNBr-activated Sepharose 4B (Pharmacia, 15 g of powder washed with 3 L of distilled water, pH 2.8, followed by 200 mL of water). The suspension was shaken at 11°C for 72 h, and the solids were washed with 4 L of water on a sintered-glass funnel.

This resin was suspended in a mixture of 60 mL of dioxane and 15 mL of water. *N*-acetylhomocysteine thiolactone (1.0 g, 6.2 mmol) was added, and the pH was adjusted to 7.5. The reaction mixture was stirred under

nitrogen at room temperature for 18 h. The product was collected on a sintered-glass funnel and washed with 800 mL of 50% dioxane/water followed by 100 mL of water.

This material was suspended in a solution of 1.0 g of *N*-acetylcysteine in 100 mL of water and stirred under nitrogen for 1 h. The resin was collected on a sintered-glass funnel, washed with 1 L of water (negative nitroprusside test on final few mL), suspended in 30 mL of water, and freeze-dried, giving 0.5 g of material with sulfhydryl content of 11 μmol/g.

## 3,3-Diaminodipropylamine/CL Sepharose 4B/ *N*-Acetylhomocysteine Thiolactone

CL-Sepharose 4B (25 mL) was activated with 3,3-diaminodipropylamine by the procedure above. *N*-acetylhomocysteine thiolactone (1.0 g, 6.2 mmol) was added to the stirred suspension, and the pH was adjusted to 7.5 with 0.3 N sodium hydroxide. The reaction mixture was stirred at room temperature under nitrogen for 72 h. The product was isolated as described above, giving 0.24 g of polymer with sulfhydryl content of 72 μmol/g.

## AH-Sepharose 4B/3-Mercaptopropionic Acid

A 4-g sample of AH-Sepharose 4B (Pharmacia) was washed with 800 mL of 0.5 M sodium chloride followed by 80 mL of water. The resin was suspended in 50 mL of water, and the pH was adjusted to 7.5. To the suspension were added 0.53 g (1.3 mmol) of 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluenesulfonate (Aldrich) and 0.53 mL (6.2 mmol) of mercaptopropionic acid. The pH of the suspension was readjusted to 7.5, and the reaction mixture was shaken at room temperature under nitrogen for 72 h. The product was isolated as described above, giving 0.3 g of polymer with sulfhydryl content of 11 μmol/g.

## AH-Sepharose 4B/Mercaptosuccinic Acid

A 7.5-sample of AH-Sepharose 4B was washed and suspended in 100 mL of water at pH 7.5. To the suspension were added 1.0 g (2.4 mmol) of 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate and 1.0 g (6.6 mmol) of mercaptosuccinic acid. The pH was readjusted to 7.5, and the reaction mixture was stirred at room temperature under nitrogen for 4 d. The product was isolated as described above, giving 0.85 g of material with sulfhydryl content of 13 μmol/g.

## AH-Sepharose 4B/*N*-Acetylcysteine

The sulfhydryl-containing polymer was prepared as described for AH-sepharose 4B/mercaptosuccinic acid, using 1.0 g (6.13 mmol) of *N*-acetylcysteine. The product (0.56 g) had a sulfhydryl content of 20 μmol/g.

### AH-Sepharose 4B/Cysteine

The polymer was prepared as described above, except that 2.4 g of carbodiimide was used. The product (0.47 g) had a sulfhydryl content of 24 μmol/g.

### Screening Polymeric Thiols for Activation of Creatine Phosphokinase

A weighed amount of polymer was added to a known volume of a pool of human serum with high normal creatine phosphokinase level. The serum with the polymer was incubated on ice for 15–30 min to activate serum creatine phosphokinase. The polymer was removed by centrifugation and discarded. A 10-μL aliquot of the activated serum was then used for creatine phosphokinase activity determination.

### Measurement of Creatine Kinase Activity

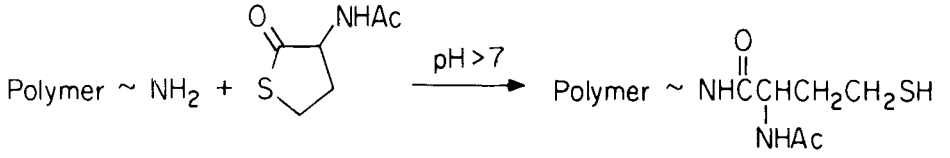
The reaction mixture contained in a total volume of 1 mL: 10 U hexokinase, 10 U glucose-6-phosphate dehydrogenase, 5 mM magnesium chloride, 10 mM glucose, 2 mM nicotinamide adenine dinucleotide phosphate, 30 mM creatine phosphate, 2 mM adenosine diphosphate, and 100 mM imidazole-acetate buffer at pH 7.0. This reaction mixture was equilibrated at 37°C, and reaction was initiated by the addition of 10 μL of the preactivated serum. The rate of NADPH formation at 340 nm was measured on a Beckman model 25 recording spectrophotometer fitted with temperature control. Any modification of this standard procedure is mentioned in the text.

## RESULTS AND DISCUSSION

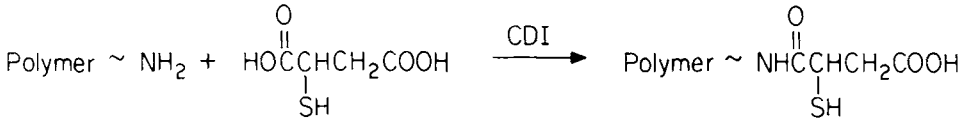
### Preparation of Polymeric Thiols

Polymeric thiols can be prepared by free-radical addition of blocked monomers followed by removal of the blocking group to liberate the mercapto group or introduction of mercapto groups by chemical modification of existing polymers. Polymeric carriers in the latter approach could conceivably be either soluble or insoluble (solid-phase). Soluble polymers, however, would be expected to be prone to oxidation to disulfides with crosslinking and precipitation. Our approach has centered about the second method, and we have restricted the selection of potential carriers primarily to insoluble, hydrophilic polymers (such as agaroses, modified glasses, and polysaccharides).

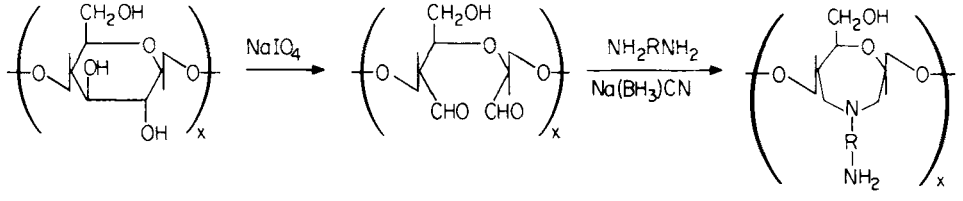
Chemical modification generally involved treatment of an amino-containing polymer with a thiol-containing ligand to generate the polymeric thiol. For example, incubation of an amino-functionalized polymer with *N*-acetylcysteine thiolactone gave ring opening and direct replacement of the amino group by a thiol group.



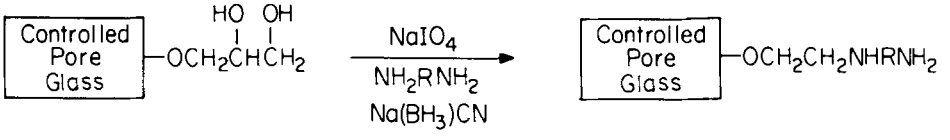

This process is facilitated by the addition of $Ag^+$. Alternatively, the amino-bearing polymer may be treated with a thiol acid (e.g., mercaptosuccinic) in the presence of a condensing agent (carbodiimide) to give a polymeric thiol.




Amino-containing agaroses are available commercially (AH-Sepharose, Affi-Gel 701) and may be directly modified by the above methods (*3*). Cellulose may be converted to amino-functionalized derivatives by periodate oxidation followed by reductive animation with diamines or polymeric amines:

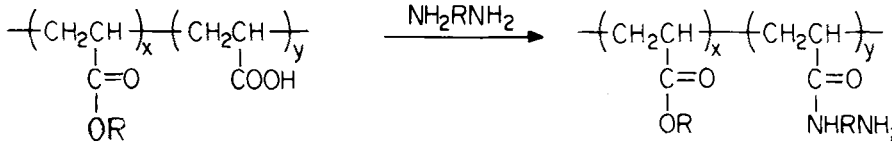



Similarly, controlled pore glass (Glycophase) may be activated by the same method to give the amino derivative:




These amino polymers may then be directly converted to their respective thiol derivatives by the methods outlined for agarose. This method of amino derivatization and thiol replacement also has been used to convert agarose in a two-step sequence to thiol agarose.

Carboxyl-containing acrylic polymers (e.g., Matrex 101) were converted to amino derivatives by direct amination with a polyamine material:

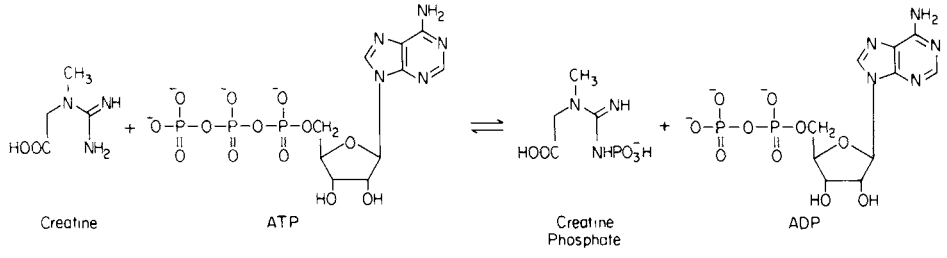


Reaction with *N*-acetylhomocysteine thiolactone then gave the corresponding polymeric thiol.

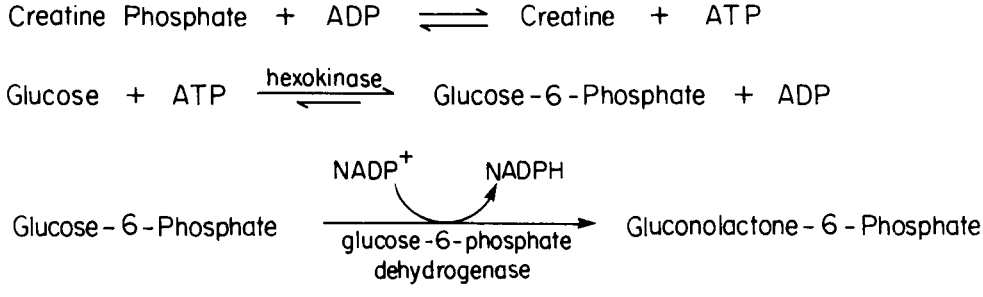
By the methods outlined, a variety of hydrophilic insoluble polymeric thiols were prepared with thiol substitution of 3–72 μmol SH/g dry polymer, as measured by reaction with Ellman's reagent (*5*). Activation of polysaccharides with polyethyleneimine (MW 40,000–60,000) rather than diaminodipropylamine, followed by thiolation, generally gave higher thiol substitution. Selected polymeric thiols prepared and their corresponding mercapto substitution values (μmol SH/g) are compiled in Tables 1 and 2. Table 1 lists agarose-derived materials, and Table 2 lists polythiols derived from other hydrophilic supports.

## Activation of Creatine Phosphokinase by Polymeric Thiols

Creatine phosphokinase (E.C. 2.7.3.2) is the enzyme that catalyzes the reversible phosphorylation of creatine by adenosine triphosphate (ATP):




Because creatine phosphokinase is activated by thiol compounds (*4*) (e.g., *N*-acetylcysteine, mercaptoethanol), it served as a model for testing the efficiency of polymeric thiols as enzyme activators. Creatine phosphokinase was incubated with a known concentration of selected polymer, and the enzyme activity was then monitored in a coupled assay using hexokinase and glucose-6-phosphate dehydrogenase, monitoring NADPH formation at 340 nm.




Activation of creatine phosphokinase by a series of agarose-derived polymeric thiols is shown in Table 1. The activation is expressed as the ratio of enzyme activity in the presence of polymer activator (at a given mM thiol concentration) to that with no activator present. Several

Table 1
Serum Creatine Phosphokinase Activation by Agarose Based Polymeric Thiols[a]

| Polymeric thiol | | μmol SH/g polymer | mM Polymeric SH tested in serum | Activation[b] |
|---|---|---|---|---|
| AH-Sepharose-4B/cysteine·HCl | | 24.0 | 0.60 | 3.0X |
| AH-Sepharose/*N*-acetylcysteine | | 19.7 | 0.61 | 2.8X |
| AH-Sepharose/mercaptosuccinic | | 12.9 | 0.30 | marginal |
| AH-Sepharose/*N*-acetylhomocysteine | | 12.9 | 0.40 | 3.2X |
| AH-Sepharose/3-mercaptopropionic | | 10.9 | 0.28 | marginal |
| CL Sepharose 4B-$CH_2NH(CH_2)_3NH(CH_2)_3NHC(=O)CHCH_2SH$ with $NHC(=O)CH_3$ | (A) | 72.0 | 1.44 | 4.0X |
| | | | 7.2 | 5.0X |
| Sepharose-$OCNHC(CH_2)_4CNHCCHCH_2CH_2SH$ with $NHCOCH_3$ | (A) | 11.0 | 0.44 | 1.6X |
| | | | 1.1 | 2.0X |
| | | | 1.76 | 1.5X |
| Sepharose-$OCNHNHC(CH_2)_4CNHNHCCHCH_2CH_2SH$ with $NHCOCH_3$ | (B) | 17.0 | 0.34 | marginal |

[a] A serum sample was incubated with a known concentration of sulfhydryl for each polymeric thiol. A 10-μL sample of the supernatant after centrifugation of the serum polymer mixture was used in 1 mL of UV assay reaction mixture. The rate of NADPH formation was measured as described under Materials and Methods, and activation was determined by comparing the rate with that of a serum control (no activator present).

[b] Activation = rate with activated serum-rate with neat serum (no activator present).

Table 2
Serum Creatine Phosphokinase Activation by Small Particle Polymeric Thiols

| Polymeric thiol | μmol SH/g polymer | mM Polymeric SH tested in serum | Activation[a] |
|---|---|---|---|
| Glycophase 1350 Å/DADPA[b]/*N*-acetylhomocysteine | 3.0 | 0.6 | none |
| Affi-Gel 701/*N*-acetylhomocysteine | 3.5 (per mL) | 1.75 | none |
| Glycophase 1350 Å/PEI[c]/*N*-acetylhomocysteine | 15.0 | 0.6 | 1.7X |
| | | 1.2 | 2.8X |
| | | 2.4 | 3.6X |
| Glycophase 550 Å/PEI/*N*-acetylhomocysteine | 39.0 | 0.78 | 2.2X |
| | 3.12 | | 3.6X |
| Avicel TG-101/PEI/*N*-acetylhomocysteine | 18.5 | 2.2 | 2.1X |
| Avicel TG-101/PEI/*N*-acetylhomocysteine | 38.0 | 4.4 | 2.7X |
| Matrex 101/PEI/*N*-acetylhomocysteine | 38.0 | 5.0 | 4.2X |

[a] Activation = rate with activated serum/rate with neat serum (no activator present).
[b] DAPDA = diaminodipropylamine.
[c] PEI = polyethyleneimine (MW 4–6 × $10^4$).

modified agaroses were effective activators. The upper portion of Table 1 gives examples of polymers with the functional moiety modified by different sulfhydryls. Polymers prepared from *N*-acetylcysteine, cysteine·HCl, and *N*-acetylhomocysteine were efficient creatine phosphokinase activators. The inactivity of mercaptosuccinate and 3-mercaptopropionate polymers may reflect structural requirements for activation or may be related to the method of preparation.

The three polymers at the bottom of Table 1 were synthesized to study any correlation between the charge carried by the polymer and enzyme activation properties. For example, Polymers A carry a partial charge and are efficient activators. Polymer B contained the hydrazide linkage with no charge and produced marginal activation. It is not clear whether this difference in activating power is due to the differences in density of charged species within the polymers or actually to the difference in the levels of thiol substitution within the polymers, which could govern activation.

A direct kinetic comparison of AH Sepharose/*N*-acetylhomocysteine and thiolglucose as creatine phosphokinase activators is shown in Fig. 1.

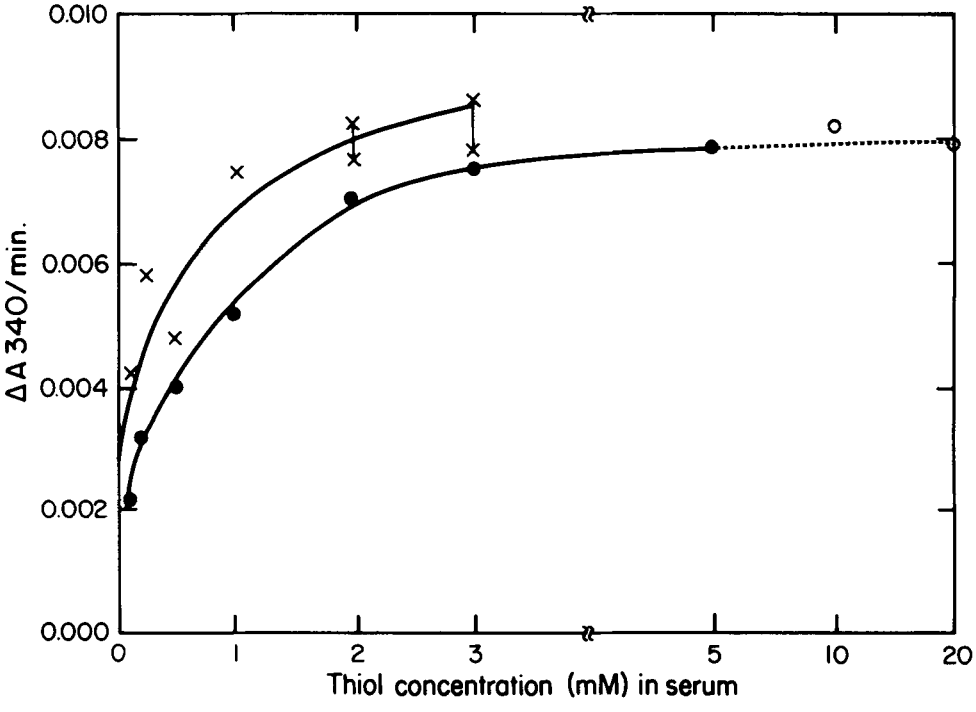



Fig. 1. Comparison of serum creatine phosphokinase activation by monomeric thioglucose and AH-Sepharose/*N*-acetylhomocysteine (Table 1). Serum was incubated with 0.1, 0.2, 0.5, 1, 2, 3, 5, 10, and 20 mM thioglucose and 0.1, 0.2, 0.5, 1, 2, and 3 mM SH (in polymeric activator) for 15 min on ice. Ten μL of the activated serum was then added into 1 mL of the UV assay reaction mixture, and the rate of NADPH formation at 340 nm was measured as described under Materials and Methods. Activation by thioglucose (●); activation by polymer (x).

As a basis for comparison, the monomeric activator thioglucose exhibits a 2.5X activation effect at 1-mM thiol concentration. Similar activation curves were observed with both monomeric and polymeric activators, suggesting efficient interaction of the polymeric thiol with the enzyme.

Table 2 summarizes activation studies with other polymeric thiols generally of smaller particle size than the agarose derivatives and representing carriers of different core composition (e.g., glass, acrylic, cellulose). Here again, several were found to be efficient activators of creatine phosphokinase. For example, Avicel/PEI/*N*-acetylhomocysteine at 4.4-mM thiol concentration gave a 2.7-fold activation of the enzyme, whereas a 2.8-fold activation of the same serum sample was achieved with 5 mM thioglucose.

In summary, many of the polymeric thiols activated creatine phosphokinase to a similar extent as the low-molecular-weight activator, thioglucose. Because the enzyme is activated via a disulfide reduction step, these polymers may show the same reductive capacity toward other protein disulfide linkages. The combination of a hydrophilic enzyme-compatible surface with a multitude of thiol groups may also make these and similar materials activators in other sulfhydryl-dependent enzyme systems.

Use of polymeric thiols can have a practical advantage over use of low molecular weight activators, particularly in systems where enzyme activation is followed by quantitation of enzymatic activity using protocols that are subject to interference by residual low molecular thiol. Alternatively, polymeric thiols may be recovered and reactivated for use. We are exploring the above applications.

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