# STIMULATION OF DNA POLYMERASE ACTIVITY BY THE COMBINATION OF p-HYDROXYMERCURIBENZOATE AND DITHIOTHREITOL

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SUMMARY: The combination in reaction mixtures of 1 mM dithiothreitol and 1 mM p-hydroxymercuribenzoate resulted in a 10-fold stimulation in the activity of a 2.5S mouse myeloma DNA polymerase. The stimulation, first detected using poly(rA)·oligo(dT) as template-primer, was also observed with several other template-primers. In addition, a comparable stimulation was observed in the poly(rA)-directed activity of the 3.4S DNA polymerase from mouse liver, murine leukemia virus DNA polymerase and E. coli DNA polymerase I, whereas no stimulation was observed with the intracisternal A-particle DNA polymerase or the 7.9S mouse myeloma DNA polymerase. The stimulatory effect of dithiothreitol:p-hydroxymercuribenzoate appeared to be related to the mercurial portion of p-hydroxymercuribenzoate and the specific properties of dithiothreitol because $HgCl_2$ substituted for p-hydroxymercuribenzoate whereas p-hydroxybenzoate did not, and because other chelating reagents and compounds containing monothiols, disulfides, or adjacent dithiols did not substitute for dithiothreitol whereas the isomer, dithioerythritol, did.

Previous studies on the sulfhydryl requirements of a 2.5S DNA polymerase from mouse myeloma demonstrated that enzyme activity was inhibited by the mercurial sulfhydryl-blocking reagent, p-hydroxymercuribenzoate (PHMB). As expected for inhibition through mercury-mercaptide bond formation, the activity of enzyme that had been preincubated with PHMB was restored by the sulfhydryl-reducing reagent dithiothreitol (DTT). Surprisingly, however, a 10-fold stimulation in enzyme activity was observed when both 1 mM PHMB and 1 mM DTT were added to the standard reaction mixture, without preincubation. Some properties of this stimulation of DNA polymerase activity were examined in the current study. Although the mechanism of the stimulation was not explained, the findings are presented because of their potential use in the detection and characterization of DNA polymerase activities.

Abbreviations: DTT, dithiothreitol; PHMB, p-hydroxymercuribenzoate; DTE, dithioerythritol; AMV, avian myeloblastosis virus; MuLV, murine leukemia virus.



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## METHODS AND MATERIALS

Enzyme Preparations: The mouse myeloma MOPC-104E 2.5S DNA polymerase (designated myeloma DNA polymerase II) was purified through the hydroxyapatite column chromatography step as described by Matsukage et al. (1). The enzyme, which was purified in the presence of DTT, was judged to be approximately 30% pure by polyacrylamide gel electrophoresis. The 3.4S mouse liver DNA polymerase (designated liver DNA polymerase II) was prepared from adult mouse liver exactly as described (1) for the purification of myeloma DNA polymerase II. *E. coli* DNA polymerase I fraction 7 was from General Biochemicals. The core component of avian myeloblastosis virus was prepared as described (2). Rauscher murine leukemia virus (Lot No. 146-4-4) was obtained from Electro-Nucleonics Laboratories, Bethesda, Maryland. Intracisternal A-particles were isolated from mouse myeloma MOPC-104E as described (3) and the highly purified 7.9S mouse myeloma DNA polymerase (designated myeloma DNA polymerase III) was prepared according to Matsukage et al. (4). Bovine pancreatic DNase I was from Worthington Biochemical Corp., and was used at a concentration of 0.5 mg/ml at 37° for 60 min.

Measurement of DNA Polymerase Activity: DNA polymerase activity was measured by determining the amount of incorporation of deoxynucleoside monophosphate into cold acid-insoluble material. Unless indicated otherwise, reactions contained the following components in a volume of 35 µl: 50 mM Tris·HCl buffer, pH 7.8, 0.5 mM $MnCl_2$, 70 mM KCl, 5 mM potassium phosphate buffer, 18% glycerol, 0.03% Nonidet P40, 0.1 mM dithiothreitol (Calbiochem. Lot No. 110147), 0.1 mM EDTA, 0.5 mM [$^3$H]dTTP (225-350 cpm/pmole), 40 µg/ml $(dT)_{12-18}$, termed $(dT)_{14}$ (Collaborative Res., Inc.), 200 µg/ml poly(rA) (Miles), 400 µg/ml bovine plasma albumin and 1 µg of myeloma DNA polymerase II protein. Unless indicated otherwise, reactions were mixed at 0° and incubated at 37° for 60 min. in silicon-treated soft glass tubes. Sulfhydryl reagents were added to reaction mixtures last. The final pH of the reaction mixture (7.8) was not changed more than ±0.1 units when reactions contained the various reagents tested including 1 mM PHMB-20 mM glycylglycine buffer. Reactions were stopped by freezing, and cold acid-insoluble material was collected and washed on a nitrocellulose filter using approximately 40 ml of 10% $CCl_3COOH$ at 4° (1). Each filter was then transferred to a glass scintillation vial and dissolved in 1 ml of methyl cellosolve at 24°. Ten ml of Triton X-100: toluene: Permafluor (Packard) (32:64:4) scintillation mixture was added, and after thorough mixing radioactivity was determined in a scintillation spectrometer at a counting efficiency of 15 to 17%. Results of enzyme assays, expressed as delta (Δ) pmoles [$^3$H] deoxynucleoside 5'-monophosphate incorporated per reaction indicate the enzyme dependent change in cold acid-insoluble radioactivity. The Δpmole value represents the difference between the amount of incorporation in the absence and in the presence of enzyme. Incorporations in reactions containing no enzyme were 0.5-1.5 pmoles [$^3$H]-deoxynucleoside 5'-monophosphate.

Rate-zonal Sedimentation Analysis: Sedimentation was performed using linear 10-30% glycerol gradients formed in a solution containing 250 mM KCl, 50 mM Tris·HCl buffer, pH 7.7, 0.1 mM EDTA and 1 mM dithiothreitol. Enzyme (50 µg protein) was layered over a 5.0 ml gradient and centrifuged in a SW 65 rotor at 60,000 rpm for 15 hrs. at 1°. The gradient was fractionated from below, and the refractive index and DNA polymerase activity were determined using 5 µl portions of fractions. $^{125}$I-labeled bovine plasma albumin (4.3S) and lysozyme (1.9S) were centrifuged in companion tubes as markers.

Reagents: Compounds were dissolved in $H_2O$ and stored frozen unless indicated otherwise, and were from the following sources: p-hydroxymercuribenzoate, sodium salt, Sigma, Lot No. 96-106 or 220-5250, p-hydroxybenzoic acid, Nutritional Biochem. Corp., and dithiodipropionic acid, Calbiochem, were

TABLE I

PROPERTIES OF THE POLY(rA)-DIRECTED ACTIVITY OF MOUSE MYELOMA DNA POLYMERASE II

| | Modification of Standard Reaction | Amount of [$^3$H]dTMP Incorporated Per Reaction | |
|---|---|---|---|
| | | Δpmoles | % |
| A. | | | |
| 1. | None | 10.2 | 100 |
| 2. | (+) 0.9 mM Dithiothreitol (DTT) | 8.3[a] | 81 |
| 3. | (+) 1 mM p-Hydroxymercuribenzoate (PHMB)[b] | 0.6 | 6 |
| 4. | (+) 0.9 mM DTT, 1 mM PHMB | 122.2 | 1200 |
| 5. | No. 1 minus $MnCl_2$, plus 4 mM magnesium acetate | 0.5 | 5 |
| 6. | No. 5, (+) 0.9 mM DTT, 1 mM PHMB | 0.9 | 9 |
| B. | | | |
| 1. | None, (+) 0.9 mM DTT, 1 mM PHMB | 122.2 | 100 |
| 2. | No. 1 (-) Enzyme | 0 | 0 |
| 3. | No. 1; incubation at 0° | 9.2 | 8 |
| 4. | No. 1; incubation time 15 min. | 52.6 | 43 |
| 5. | No. 1; incubation time 30 min. | 64.4 | 53 |
| 6. | No. 1 (-) $MnCl_2$ | 0.2 | <1 |
| 7. | No. 1 (+) 2 mM N-ethylmaleimide | 0.5 | <1 |
| 8. | No. 1 (+) DNase treatment after incubation | 1.3 | 1 |

[a] Similar incorporation was observed in reactions containing no DTT.

[b] Reactions containing PHMB also contained 20 mM glycylglycine buffer.

dissolved in 0.5M glycylglycine buffer, pH 7.6, at a concentration of 25 mM each. Iodoacetamide, Sigma, Lot No. 900-5250, and 2-mercaptoethanol, Eastman Kodak Co., were dissolved in $H_2O$ immediately prior to use; dithiothreitol, dithioerythritol, 2,2'-dithio-bis-(ethylamine) dihydrochloride, and glutathione, Calbiochem; N-ethylmaleimide, Sigma, Lot No. 61C-1850; cysteine hydrochloride, Eastman Kodak Co.; L-histidine monohydrochloride, Nutritional Biochem. Corp.; 1,10-phenanthroline, Allied Chemical, and ethylenediamine tetraacetate, disodium salt, Fisher, were dissolved in $H_2O$ and stored at 4°; 2,3-dimercaptopropanol, Calbiochem. was dissolved in 75% glycerol; L-cystine, Merck, was dissolved in 0.032 N NaOH; 4,6-dihydroxypyrimidine, 2,4-dithiopyrimidine and 2,8-dithio-6-oxypurine, Nutritional Biochem. Corp., were dissolved in 0.1 M glycylglycine buffer, pH 7.6; synthetic polynucleotide templates, Miles; oligonucleotide primers, Collaborative Res., Inc.; calf thymus DNA, Worthington Biochemical Corp., was activated as described (1).

## RESULTS AND DISCUSSION

Stimulation of DNA Polymerase Activity: As shown by the first experiment in Table I, activity of the 2.5S myeloma DNA polymerase was not altered by 1 mM

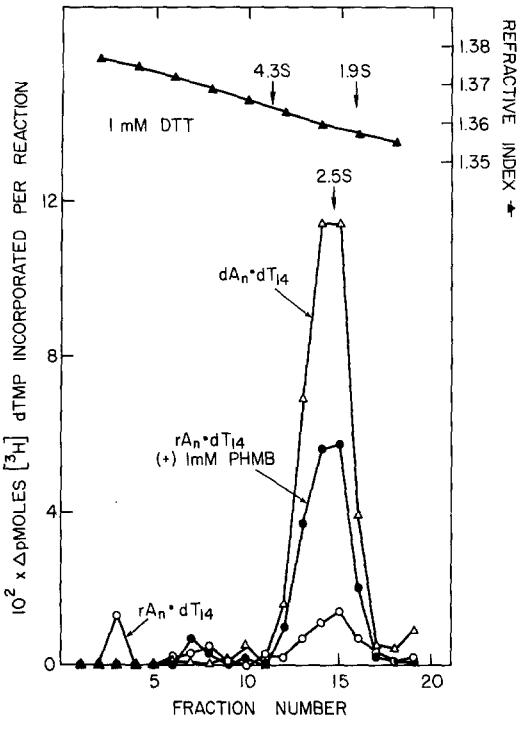


Fig. 1

Rate-zonal sedimentation of mouse myeloma DNA polymerase II. Experimental procedures are described in Methods and Materials, except that all reactions contained 1 mM DTT and the indicated template (200 µg/ml) and primer (40 µg/ml) combinations. Reactions shown by the solid circles also contained 1 mM PHMB.

DTT and was inhibited by 1 mM PHMB. However, when reactions contained both DTT and PHMB at a concentration of 1 mM each, a 12-fold stimulation of DNA polymerase activity was observed. Some properties of this stimulation were examined in the second experiment shown in Table I. Activity in reactions containing 1 mM PHMB:DTT was dependent upon enzyme and both incubation temperature and time. The stimulation was blocked by 2 mM N-ethylmaleimide and the cold acid-insoluble material produced in the stimulated reaction was rendered acid-soluble by incubation with DNase I. The sedimentation properties of the enzyme(s) responsible for the unstimulated and stimulated activities were investigated in the experiment shown in Fig. 1. Congruent peaks of poly(rA) and poly(dA)-directed DNA polymerase activity were observed sedimenting at 2.5S in reactions that contained 1 mM DTT alone.

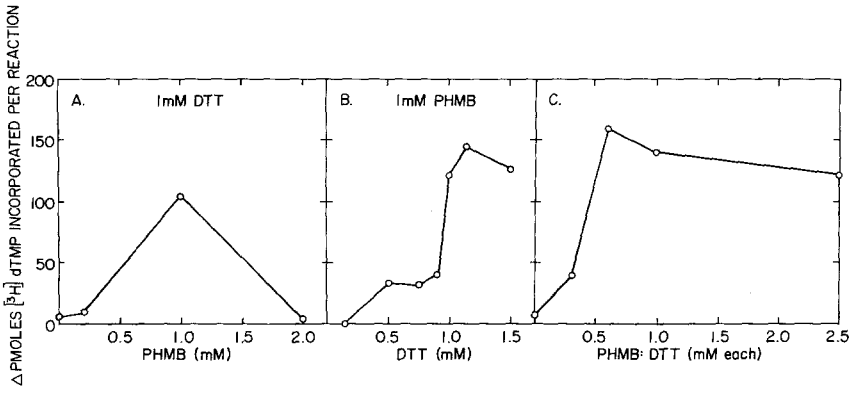


Fig.2

Effect of DTT and PHMB on the activity of mouse myeloma DNA polymerase II. Reactions were performed as described in Methods and Materials.

When the gradient fractions were tested using poly(rA)·$(dT)_{14}$ as template-primer in the presence of 1 mM PHMB and 1 mM DTT, a single peak of activity was observed at 2.5S. The amount of activity in this peak corresponded to a 5 to 6-fold stimulation of the poly(rA)-directed activity of the 2.5S enzyme. These results suggest that the stimulation in [$^3$H]dTMP incorporation represented an increase in the rate of DNA synthesis by the 2.5S myeloma DNA polymerase.

The relationship between enzyme activity and the concentrations of PHMB and DTT in reaction mixtures was investigated in the experiments shown in Fig. 2. In Panel A where all reactions contained 1 mM DTT, maximal enzyme activity was observed at a concentration of 1 mM PHMB, and in the experiment shown in Panel B where all reactions contained 1 mM PHMB, maximal activity was observed at slightly more than 1 mM DTT. When an equal amount of both DTT and PHMB were added to reaction mixtures maximal activity was observed in the range of 0.5 to 1 mM concentrations of both reagents and no inhibition was detected at higher concentrations.

Specificity of the Stimulation: A variety of compounds were tested for their capacity to substitute for DTT in producing the stimulation of myeloma DNA polymerase II activity (Table II).

TABLE II

THE EFFECT OF PHMB ON THE ACTIVITY OF MOUSE MYELOMA DNA POLYMERASE II IN THE PRESENCE OF VARIOUS REAGENTS

| Reagent Present (1 mM) | Modification[a] | |
|---|---|---|
| | (-) PHMB | (+) 1 mM PHMB |
| | Δ pmoles [$^3$H]dTMP Incorporated/Reaction | |
| A. | | |
| Dithiothreitol | 8.3 | 122.2 |
| Glutathione | 8.6 | 2.6 |
| 2-Mercaptoethanol | 11.7 | 5.1 |
| Cysteine | 5.5 | 1.9 |
| B. | | |
| Dithiothreitol | 3.0 | 50.2 |
| Histidine | 6.8 | 0.4 |
| 1,10-phenanthroline | 0.5 | 0.1 |
| EDTA | 0.4 | 0.5 |
| 4,6-Dihydroxypyrimidine | 3.7 | 0.3 |
| Dithiodipropionic acid | 4.5 | 0.5 |
| Cystine | 3.6 | 0.4 |
| 2,2'-Dithio-bis-(Ethylamine) | 2.6 | 0 |
| 2,4-Dithiopyrimidine | 4.4 | 0.3 |
| 2,8-Dithio-6-oxypurine | 0.6 | 0.5 |
| 2,3-Dimercaptopropanol | 4.6 | 1.2 |
| Dithioerythritol | 5.0 | 37.9 |

[a] Reactions were performed as described in Materials and Methods except that they contained the reagents indicated.

The sulfhydryl-reducing reagents, 2-mercaptoethanol, glutathione and cysteine did not substitute, nor did the disulfides and chelating reagents. Compounds containing adjacent SH groups also were ineffective; the isomer of DTT, dithioerythritol (DTE), was almost as effective as DTT.

In other experiments (data not shown), N-ethylmaleimide and iodoacetamide did not substitute for PHMB in producing the stimulation. Substitution of 1 mM p-hydroxybenzoic acid for PHMB also resulted in no stimulation, but the combination of 1 mM DTT and 1 mM $HgCl_2$ gave the same amount of stimulation as 1 mM DTT and 1 mM PHMB. Substitution of 1 mM concentrations of the metal ions, $Zn^{2+}$, $Fe^{2+}$, $Fe^{3+}$, $Cd^{2+}$, $Co^{2+}$, $Cu^{+}$, and $Cu^{2+}$, for PHMB resulted in inhibition of activity.

TABLE III

THE EFFECT OF DTT:PHMB ON THE ACTIVITY OF MOUSE MYELOMA DNA POLYMERASE II IN THE PRESENCE OF VARIOUS TEMPLATE-PRIMERS

| Template-Primer | Deoxynucleotide(s) | 1 mM DTT | 1 mM DTT: 1 mM PHMB |
|---|---|---|---|
| | | Δ pmoles [$^3$H]dXMP Incorporated/Reaction[a] | |
| None | [$^3$H]dTTP | <1 | 0 |
| None | [$^3$H]dATP | 0 | 0 |
| None | [$^3$H]dGTP | 0 | 0 |
| Poly(rA)·$(dT)_{14}$ | [$^3$H]dTTP | 8.8 | 99.1 |
| Poly(rA) | [$^3$H]dTTP | <1 | 0 |
| $(dT)_{14}$ | [$^3$H]dTTP | <1 | <1 |
| Poly(rU)·$(dA)_{14}$ | [$^3$H]dATP | 0 | 0 |
| Poly(rC)·$(dG)_{14}$ | [$^3$H]dGTP | 0 | 0 |
| Poly(dC)·$(dG)_{14}$ | [$^3$H]dGTP | 54.1 | 81.6 |
| Poly(dA)·$(dT)_{14}$ | [$^3$H]dTTP | 14.2 | 18.8 |
| Poly(dA)·Poly(dT) | [$^3$H]dTTP | <1 | 2.3 |
| Poly d(A-T) | [$^3$H]dATP, dTTP | <1 | 10.4 |
| Poly d(A-T)[b] | [$^3$H]dATP, dTTP | 2.2 | 13.3 |
| Poly(dA)·$(dT)_{14}$[b] | [$^3$H]dTTP | 28.8 | 37.6 |
| Activated Calf Thymus DNA[b] | [$^3$H]dTTP, dATP, dCTP, dGTP | <1 | <1 |

[a] Reactions were performed as described in Materials and Methods except they contained the components indicated at the following final concentrations: oligonucleotide primers, 40 μg/ml; templates, 200 μg/ml; the template-primers poly d(A-T) and DNA, 200 μg/ml; [$^3$H]dTTP, 0.5 mM; [$^3$H]dGTP, 0.25 mM; [$^3$H]dATP, dTTP, 0.25 mM each; deoxynucleotides with activated DNA, 0.5 mM each.

[b] Reactions contained 5 mM magnesium acetate instead of 0.5 mM $MnCl_2$.

The template specificity of the DTT:PHMB stimulation was investigated in the experiment shown in Table III. Enzyme activity was very low, but detectable in the presence of activated calf thymus DNA and no stimulation of this low activity was observed. However, activity in the presence of poly d(A-T) was stimulated more than 10-fold and 6-fold in the reactions containing manganese and magnesium, respectively. The poly(rA)·$(dT)_{14}$ dependent activity in the presence of manganese was stimulated 11-fold, and in cases where activity was observed with other template-primers it was stimulated slightly.

TABLE IV

THE EFFECT OF PHMB AND DTT ON THE POLY(rA)·$(dT)_{14}$-DIRECTED ACTIVITY OF VARIOUS DNA POLYMERASES

| Enzyme | Sulfhydryl Reagent | | | Fold Stimulation |
|---|---|---|---|---|
| | 1mM DTT | 1mM PHMB | 1mM DTT: 1mM PHMB | |
| | Δpmoles [$^3$H]dTMP Incorporated/Reaction[a] | | | |
| (2.5S) Mouse Myeloma DNA Polymerase II | 8.5 | 0.7 | 45.8 | 5.4 |
| (3.4S) Mouse Liver DNA Polymerase II | 1.0 | - | 15.8 | 15.8 |
| (7.9S) Mouse Myeloma DNA Polymerase III | 3.8 | 0 | 3.6 | 1 |
| E. coli DNA Pol I | 175 | 463 | 1340 | 7.6 |
| MuLV DNA Polymerase | 28.1 | 1.4 | 137 | 4.9 |
| AMV DNA Polymerase | 109 | 4.4 | 164 | 1.5 |
| A-Particle DNA Polymerase[b] | 24.5 | 0.4 | 23.9 | 1 |

[a] DNA polymerase reactions were performed as described in Materials and Methods except they contained no DTT unless indicated in the Table; reactions contained the following amounts of each enzyme protein: liver DNA polymerase II, 0.2μg; myeloma DNA polymerase II 1μg; myeloma DNA polymerase III, 0.75 ng; MuLV DNA polymerase, 700 ng; A-particle DNA polymerase, 800 ng; AMV DNA polymerase, 3.5μg; E. coli DNA polymerase I, 50 ng.

[b] 10 mM magnesium acetate, 120 mM KCl, poly(rA) and $(dT)_{14}$ at 143 μg/ml each.

The effect of DTT:PHMB on the activity of several other DNA polymerase preparations was investigated in the experiment shown in Table IV. In this study, the 2.5S myeloma DNA polymerase II was stimulated approximately 5-fold and the 3.4S mouse liver polymerase with identical chromatographic properties, termed liver DNA polymerase II, was stimulated 15-fold. Murine leukemia virus DNA polymerase was stimulated 8-fold, while the AMV DNA polymerase was stimulated only slightly. The mouse myeloma A-particle DNA polymerase and the 7.9S mouse myeloma DNA polymerase III were not stimulated.

The results demonstrate that the stimulation of DNA polymerase activity was observed with several DNA polymerases and with several template-primers in addition to poly(rA)·oligo(dT). The stimulation was specific for the combination of DTT or DTE and PHMB or $HgCl_2$ among the reagents tested. It is likely that the compound involved in the stimulation is a complex between DTT and mercury, and that mercury enhances the action of DTT (5) in the formation of monothiols on the enzyme. However, the mechanism of the observed stimulation was not determined, and it is not clear whether the mercurial:dithiol combination exerts a direct effect upon the DNA polymerase or alters a contaminant physically associated with the enzyme.

## REFERENCES

1. Matsukage, A., Bohn, E.W. and Wilson, S.H., Proc.Nat.Acad.Sci., USA, 71, 578 (1974).
2. Stromberg, K., Gantt, R. and Wilson, S.H., Biochim. Biophys. Acta., 304, 1 (1973).
3. Wilson, S.H., Bohn, E.W., Matsukage, A., Lueders, K.K. and Kuff, E.L., Biochemistry, 13, 1200 (1974).
4. Matsukage, A., Bohn, E.W. and Wilson, S.H., Submitted for Publication (1974).
5. Cleland, W.W., Biochemistry, 3, 480 (1964).