

# Studies on the Mechanism of Action of the Gluconate 6-Phosphate Dehydrogenase

THE PRESENCE OF A CYSTEINE RESIDUE IN THE ACTIVE CENTER\*

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## SUMMARY

The role of the sulfhydryl groups in the gluconate 6-phosphate dehydrogenase has been studied.

Nine cysteine residues have been detected by spectrophotometric titration with *p*-hydroxymercuribenzoate, but the reaction of only 3 of them is sufficient to inactivate the enzyme completely.

Chlorodinitrobenzene acts even more specifically. Incorporation of 1.6 dinitrobenzene residues leads to enzyme inactivation. The amino acid residues involved in dinitrophenylation have been identified as cysteine. Gluconate 6-phosphate completely protects the enzyme against inactivation by chlorodinitrobenzene.

Although the gluconate 6-phosphate dehydrogenase is of high significance in the oxidative shunt of hexose monophosphate, very few studies have been performed on its mechanism of action. Very recently we have shown that iodoacetate (1) inactivates the enzyme by carboxymethylation of 1.8 cysteine residues per mole of protein. In the present paper we report the study of two other sulfhydryl reagents, *p*-hydroxymercuribenzoate and chlorodinitrobenzene. The latter inactivates the enzyme through a specific reaction with a cysteine residue.

The strict relation between catalytic activity and modification of a cysteine residue suggests that this group is part of the active center of the enzyme.

## EXPERIMENTAL PROCEDURE

### Materials

D-Gluconate 6-phosphate dehydrogenase was prepared from *Candida utilis* according to the procedure of Pontremoli *et al.* (2) with minor modifications. D-Gluconate 6-phosphate, triphosphopyridine nucleotide,  $\epsilon$ -N-dinitrophenyllysine, and S-dinitrophenylcysteine were purchased from Sigma; *p*-hydroxymercuribenzoate sodium salt was purchased from Calbiochem, and chlorodinitrobenzene-<sup>14</sup>C (specific activity, 7.1 mC

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per mmole) from the Radiochemical Centre. All other materials were reagent grade.

### Methods

**Enzyme Assay**—The activity of D-gluconate 6-phosphate dehydrogenase was calculated from the initial rate of TPN reduction measured at 340  $m\mu$  in the test system described by Horecker and Smyrniotis (3). A unit of enzyme is defined as the quantity that would result in a change in absorbance of 1.0 per min.

**Protein Determination**—Protein was determined from the optical absorption at 280  $m\mu$ . A solution of 1 mg per ml of pure lyophilized enzyme has an optical density of 1.650 at 280  $m\mu$  (light path, 1 cm). The molar concentration of the enzyme was calculated for a molecular weight of 100,000 (1).

**Titration of Sulfhydryl Groups**—The spectrophotometric titration of free sulfhydryl groups in the D-gluconate 6-phosphate dehydrogenase was performed according to the procedure of Benesch and Benesch (4) based on the work of Boyer (5). The reaction mixture (1 ml) contained 0.51 mg of enzyme, 0.1 mM HMB,<sup>1</sup> and 0.01 M Tris-HCl buffer, pH 7.6. The temperature was 22°. The reaction was followed at 250  $m\mu$ . Reduced glutathione was used as standard.

**Reaction with Chlorodinitrobenzene-<sup>14</sup>C**—In a final volume of 0.5 ml, the reaction mixture contained 0.88 mg of gluconate 6-phosphate dehydrogenase, specific activity, 260 units per mg of protein; 0.5 M Tris-HCl buffer, pH 8.0; 0.01 M EDTA; and 50  $m\mu$ moles of chlorodinitrobenzene-<sup>14</sup>C, specific activity, 7.1 mC per mmole. The mixture was incubated, with stirring, in the dark at 22°, and aliquots were taken at intervals for the determination of enzyme activity.

**Radioactivity Measurements**—The DNP-protein was precipitated in 5% trichloroacetic acid, washed three times by centrifugation and suspension with 5% trichloroacetic acid, and finally dissolved in 0.25 ml of 1 M ammonium hydroxide. The small amount of undissolved material was discarded after centrifugation. Radioactivity measurements were made with dry samples on a low background Geiger-Müller counter.

**Protein Hydrolysis**—A 0.2-ml sample of the labeled DNP-protein (treated as above) was hydrolyzed in 5.7 N HCl at 106° for 16 hours in vials sealed under vacuum (6). The hydrolysate was taken to dryness under reduced pressure in a rotary evapo-

<sup>1</sup> The abbreviations used are: HMB, *p*-hydroxymercuribenzoate; DNP, 2,4-dinitrophenyl-

rator, dissolved and taken to dryness three times from water, and finally dissolved in 0.3 ml of water.

*Paper Chromatography of DNP-Amino Acids*—Monodimensional descending chromatography was performed on Whatman No. 3MM paper at 22° in the dark with the solvent system

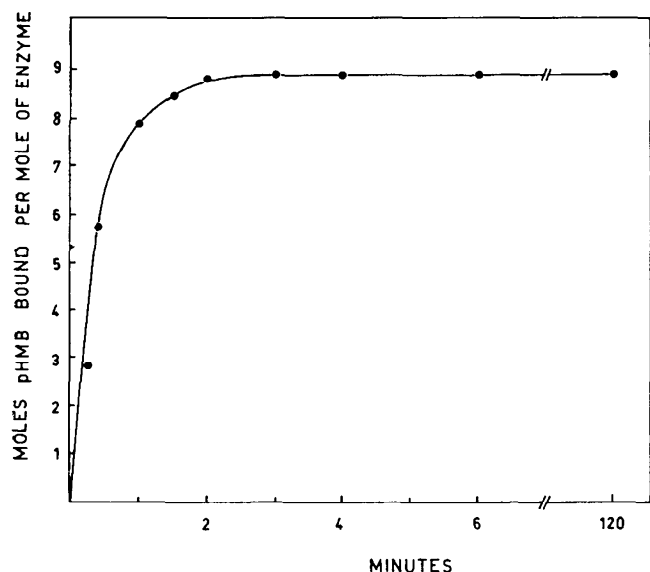


FIG. 1. Spectrophotometric titration of the sulfhydryl groups with *p*-hydroxymercuribenzoate (*pHMB*). The ratio of moles of *p*-hydroxymercuribenzoate bound per mole of enzyme has been calculated from the increment in optical density at 250  $m\mu$ . For details see "Methods."

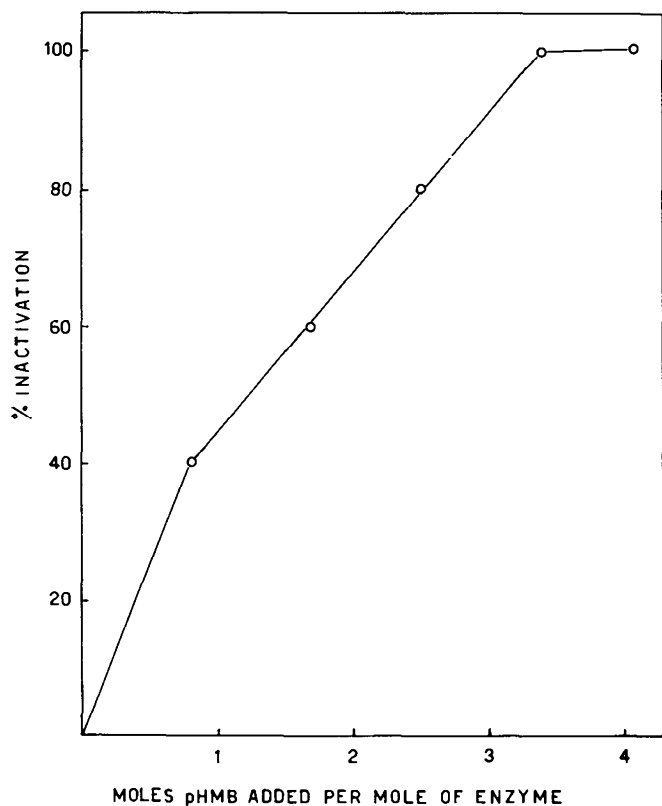


FIG. 2. Inactivation of gluconate 6-phosphate dehydrogenase as a function of the ratio of *p*-hydroxymercuribenzoate (*pHMB*) to enzyme. The 0.3-ml incubation mixture contained 0.066 M Tris-HCl buffer (pH 8.0), 0.012 mg of enzyme (0.12  $\mu$ mole), and 0.1 to 0.5  $\mu$ mole of *p*-hydroxymercuribenzoate. After 45 min of incubation at 0°, aliquots were taken for the determination of the enzymatic activity.

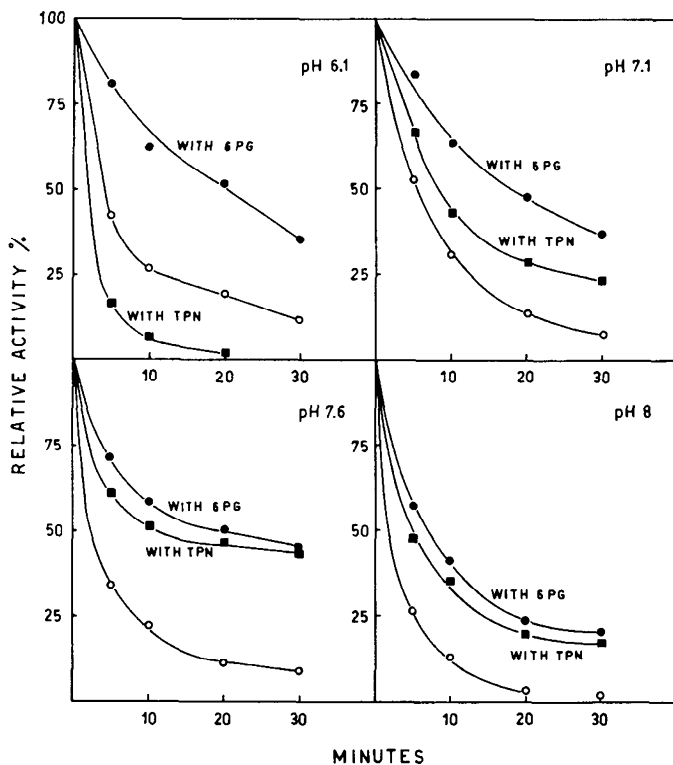


FIG. 3. Inactivation of gluconate 6-phosphate dehydrogenase by HMB and protection by substrate. The 0.3-ml incubation mixture contained 0.02 M phosphate buffer, pH as indicated; 0.03 mg of enzyme (0.3  $\mu$ mole);  $10^{-5}$  M *p*-hydroxymercuribenzoate; and, where indicated, either 1 mM gluconate 6-phosphate (6PG) or 0.1 mM TPN. Temperature was 0°. At intervals, aliquots were taken for determination of the enzymatic activity.

1-butanol-acetic acid-water (4:1:5). Spots were made with hydrolysate (about 1500 cpm).  $\epsilon$ -N-DNP-lysine and *S*-DNP-cysteine were used as internal standards. The colorless spot of *S*-DNP-cysteine was revealed by exposing the dry chromatogram to ammonia vapors. The areas of radioactivity were located by cutting the paper into pieces 1 cm wide and assaying them in a gas flow counter.

## RESULTS

*Number of Sulfhydryl Groups in Gluconate 6-Phosphate Dehydrogenase*—The number of sulfhydryl groups, determined by the spectrophotometric titration of the enzyme with *p*-hydroxymercuribenzoate, is 9. The same result is obtained also by titration with *p*-hydroxymercuribenzoate in 8 M urea or 1% sodium dodecyl sulfate. All the sulfhydryl groups are highly reactive; indeed, as shown in Fig. 1, with the enzyme concentration used the reaction is complete in 2 to 3 min.

*Effect of p-Hydroxymercuribenzoate on Enzymatic Activity and Substrate Protection*—The gluconate 6-phosphate dehydrogenase is inactivated by *p*-hydroxymercuribenzoate. At pH 8.0 a roughly linear correlation is established between inactivation and *p*-hydroxymercuribenzoate concentration. Complete inactivation is obtained when the number of moles of HMB added per mole of enzyme equals 3 (Fig. 2). Gluconate 6-phosphate and TPN, substrates of the enzyme, protect against inactivation (Fig. 3). The maximum protection is obtained at pH 7.6, which is the pH optimum for the enzymatic reaction. Addition of cysteine to the enzyme inactivated by *p*-hydroxymercuribenzoate results in complete recovery of the enzymatic activity.

*Effect of Chlorodinitrobenzene on Enzymatic Activity*—Since

*p*-hydroxymercuribenzoate appeared to be very reactive with the enzyme, and since the substrates did not afford good protection, we turned to chlorodinitrobenzene. We have found this reagent to be less reactive than *p*-hydroxymercuribenzoate and fluorodinitrobenzene. Indeed, the time required for the complete inactivation of the enzyme is longer, and the results are more consistently reproducible.

When the enzyme is treated with chlorodinitrobenzene at pH 8.0, 70% inactivation is obtained after 60 min of incubation (Fig. 4); while a control sample without chlorodinitrobenzene retains the enzyme activity completely.

Complete protection against inactivation for at least 40 min is obtained with 1 mM gluconate 6-phosphate, while 0.3 mM TPN does not protect. Inorganic phosphate, 0.15 M, affords only partial protection. This effect can probably be explained by the fact that inorganic phosphate competes with gluconate 6-phosphate for the enzyme (Fig. 5) and can thus partially replace the substrate.

**Relationship of Dinitrobenzene Labeling and Loss of Enzymatic Activity**—In order to establish the relationship between dinitrobenzene labeling and loss of enzymatic activity, the enzyme was incubated with <sup>14</sup>C-labeled chlorodinitrobenzene. After 40 min of incubation the residual activity of the enzyme was determined, and the labeled dinitrophenylated protein was isolated as described under "Methods." As shown in Table I, complete inactivation of gluconate 6-phosphate dehydrogenase is obtained when 1.6 moles of dinitrobenzene are bound per mole of enzyme. Gluconate 6-phosphate not only protects the en-

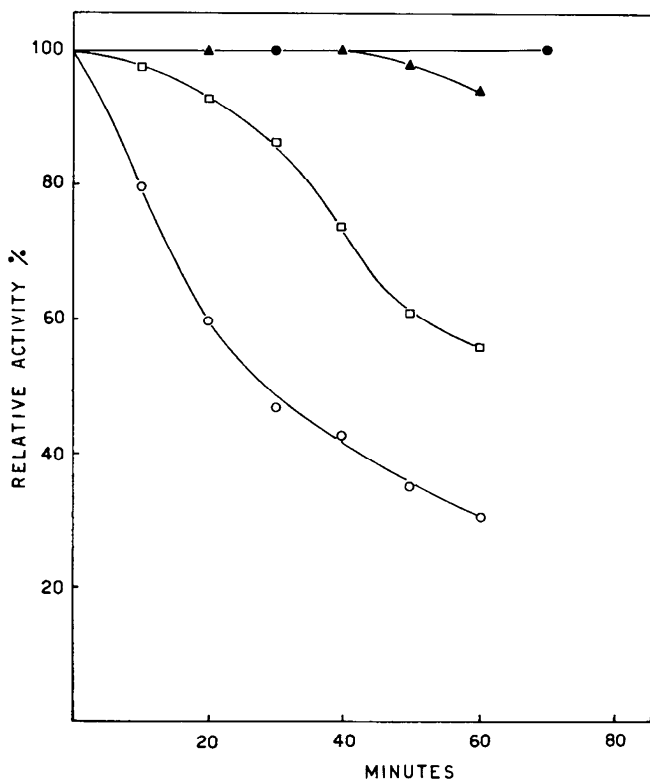


FIG. 4. Inactivation of gluconate 6-phosphate dehydrogenase by chlorodinitrobenzene and protection by substrate. For details see "Methods." ●—●, control sample; ○—○, chlorodinitrobenzene added, or chlorodinitrobenzene and 0.3 mM TPN added; □—□, 0.15 M orthophosphate and chlorodinitrobenzene added; ▲—▲, 1 mM gluconate 6-phosphate and chlorodinitrobenzene added.

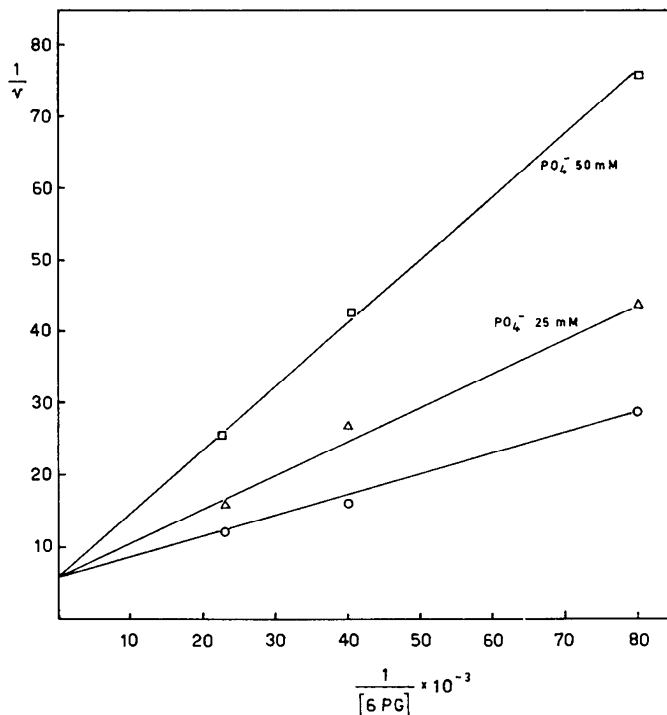


FIG. 5. Phosphate inhibition of gluconate 6-phosphate dehydrogenase as a function of the concentration of gluconate 6-phosphate (6PG). The 1-ml incubation mixtures contained 0.98  $\mu$ g of gluconate 6-phosphate dehydrogenase, specific activity, 260 units per mg of protein; 0.06 M glycylglycine buffer, pH 7.5; 0.1 mM TPN; gluconate 6-phosphate, and phosphate as indicated. The lowest curve was obtained in absence of inorganic orthophosphate.

TABLE I

Incorporation of labeled dinitrobenzene and loss of enzymatic activity

Dinitrophenylation of the enzyme was performed as described under "Methods." The time of incubation was 40 min; the temperature was 22°.

Addition	Observed inactivation		Protein			<sup>14</sup> C-Chlorodinitrobenzene fixed		
	Chlorodinitrobenzene present (A)	Controls (B)	Total <sup>a</sup>	Active <sup>b</sup>	Chlorodinitrobenzene-inactivated <sup>c</sup>	cpm	$\mu$ moles	$eq^d$
None.....	67	0	8.1	2.6	5.5	47,000	9	1.6
Gluconate 6-phosphate, 1 mM.....	0	0	8.1	8.1	0.0	16,000	3.1	

<sup>a</sup> Based on a molecular weight of 100,000.

<sup>b</sup> Calculation: (100% - A)  $\times$  total protein.

<sup>c</sup> Calculation: (A - B)  $\times$  total protein.

<sup>d</sup> Millimicromoles of chlorodinitrobenzene fixed per  $\mu$ mole of protein inactivated by chlorodinitrobenzene.

zyme against inactivation by chlorodinitrobenzene but also prevents the incorporation of the reagent in the protein.

**Identification of Amino Acid Residues Affected by Dinitrophenylation**—The identification of the amino acid residues affected by dinitrophenylation has been performed on the acid

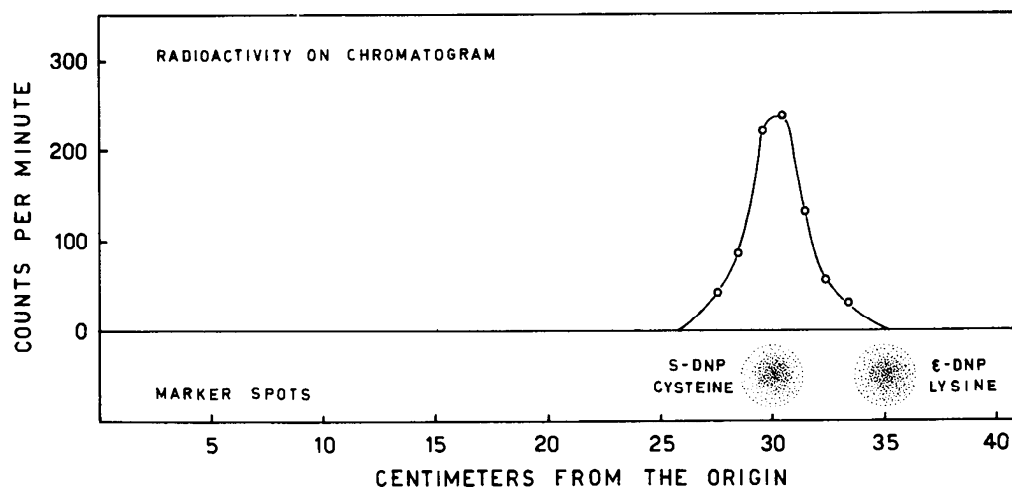


Fig. 6. Chromatographic analysis of the DNP-protein hydrolysate. For details see "Methods"

hydrolysate of the dinitrophenylated protein. The recovery of radioactive material after the hydrolysis was 70%. An aliquot of the hydrolysate was subjected to paper chromatography as described under "Methods." *S*-DNP-cysteine and  $\epsilon$ -*N*-DNP-lysine were used as internal standards. After chromatography, the radioactive material was detected in a single peak corresponding to the spot of the *S*-DNP-cysteine (Fig. 6).

#### DISCUSSION

Several facts point out the relation in the gluconate 6-phosphate dehydrogenase between free sulfhydryl groups and enzymatic activity. *p*-Hydroxymercuribenzoate, iodoacetate (1), and chlorodinitrobenzene inactivate the enzyme. In all cases cysteine residues are involved. The lowest ratio of moles of reagent bound per mole of enzyme inactivated is obtained with chlorodinitrobenzene. As shown in Table I, 1.6 moles of reagent are incorporated per mole of enzyme inactivated. In the experiment with gluconate 6-phosphate, in which no loss of activity is detected, a fraction of 1 mole of reagent is still incorporated. This suggests that part of the 1.6 eq found in the other part of the experiment may involve nonessential groups and allows the reduction of the number of essential cysteine residues

to 1. The modification of this cysteine residue either by carboxymethylation or by dinitrophenylation leads to complete loss of the catalytic activity.

Gluconate 6-phosphate affords protection against both the inactivation and the incorporation of chlorodinitrobenzene or iodoacetate. TPN protects only against *p*-hydroxymercuribenzoate and only at a pH higher than 7. With iodoacetate (1) or with chlorodinitrobenzene, no protection by TPN is obtained.

A peculiar result is the increase, in the presence of TPN at pH 6.1, of the rate of inactivation of the enzyme by *p*-hydroxymercuribenzoate. No explanation, at the moment, can be given for this observation.

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