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Mechanism of Inactivation of Rabbit Muscle Glyceraldehyde 3-Phosphate Dehydrogenase by Ethacrynic Acid

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SUMMARY

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The reaction of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) with rabbit muscle glyceraldehyde 3-phosphate dehydrogenase [D-glyceraldehyde 3-phosphate:NAD^+ oxidoreductase (phosphorylating), EC 1.2.1.12] consists of an initial burst of reaction over several minutes, corresponding to 2 thiols/subunit, followed by a further slow reaction of the 2 remaining groups. The initial fast release of 2 thionitrobenzoate anions per subunit is shown to be due to reaction of two sulphydryl groups with DTNB. One of these reacts very fast, with consequent inactivation of the enzyme, and is therefore assumed to be the active site thiol, Cys-149. A mechanism involving reaction of DTNB with Cys-149, followed by formation of an intramolecular disulphide bridge between Cys-149 and a second thiol, is shown to be unlikely. Incubation of the enzyme with ethacrynic acid abolishes the initial fast reaction with DTNB of 2 groups/subunit, and 2 ethacrynic acid residues/subunit remain attached to the enzyme after thorough dialysis. Ethacrynic acid treatment results in inactivation of the enzyme, which is not reversed by dilution or by addition of 2-mercaptoethanol. Furthermore, the reactivity of the 2 sulphydryl groups blocked by ethacrynic acid is not restored by denaturation or by exhaustive dialysis. One thiol per subunit reacts rapidly with ethacrynic acid, and one more slowly. The rate of inactivation of the enzyme correlates with the rate of reaction of the fast group, which is assumed to be the active site thiol. Increasing the extent of saturation of the enzyme with NAD^+ results in protection against inactivation by ethacrynic acid. The data suggest that ethacrynic acid reacts with 2 thiols/enzyme subunit and that inactivation of the enzyme by the drug results from modification of the active site thiol.

INTRODUCTION

Ethacrynic acid acts at several sites within the cell. Inhibitory effects have been demonstrated on mitochondrial oxidative phosphorylation (1–3), on membrane Na^+- and K^+-activated ATPase (4, 5), and on the glycolytic pathway (1, 6, 7). None of the effects, however have been directly correlated with the diuretic action of the drug. In addition to its diuretic action, ethacrynic acid has been shown to cause vasodilation (8), and Ogilvie has suggested (9) that this is due to interference by the drug with the ability of vascular smooth muscle to recover from anoxic work.

Ethacrynic acid was shown by Jones

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and Landon (10) to inhibit glycolysis in rabbit kidney slices. Gordon and de Hartog (6) extended this observation to Ehrlich ascites tumour cells and were able to place the site of inhibition between the fructose 1,6-diphosphate and 3-phosphoglycerate levels. In a later study (7) they demonstrated that incubation of glyceraldehyde 3-phosphate dehydrogenase [D-glyceraldehyde 3-phosphate:NAD^+ oxidoreductase (phosphorylating), EC 1.2.1.12] with ethacrynic acid resulted in complete inactivation of this enzyme. Because of the known reactivity of ethacrynic acid with sulphydryl compounds, Gordon and de Hartog (7) suggested that the inactivation of glyceraldehyde 3-phosphate dehydrogenase was due to reaction of the drug with the active site thiol of this enzyme.

Rabbit muscle glyceraldehyde 3-phosphate dehydrogenase is a tetramer composed of 4 identical subunits, each containing 4 cysteine residues (11). The active site thiol (Cys-149) reacts rapidly with a number of sulphydryl reagents (12, 13). The other 3 sulphydryls are relatively nonreactive, although Cys-153 has been reported to participate in formation of a disulphide bridge with Cys-149 (14).

The present work indicates that ethacrynic acid inactivates glyceraldehyde 3-phosphate dehydrogenase by reacting with 2 thiols, 1 of which is probably Cys-149. The disappearance of DTNB[2]-reactive sulphydryls was used to follow modification of the enzyme by ethacrynic acid. As a prerequisite to this method, the reaction of DTNB with the rabbit muscle enzyme has been studied in some detail.

MATERIALS AND METHODS

Triethanolamine HCl and iodoacetic acid were obtained from Sigma Chemical Company. NAD^+ (free acid), NADH (disodium salt), ATP (disodium salt), glycerate 3-phosphate (tricyclohexylammonium salt), and phosphoglycerate kinase were obtained from C. F. Boehringer and Sons. Sephadex G-25 (coarse grade) was a Pharmacia product. L-Cysteine, 2-mercaptoethanol, DTNB, ethylenediaminetetraacetic acid, and sodium dodecyl sulphate were supplied by British Drug Houses, Ltd. Ethacrynic acid was a gift of Merck Sharp & Dohme.

Glyceraldehyde 3-phosphate dehydrogenase was prepared from rabbit skeletal muscle by the procedure of Amelunxen and Carr (15), except that KCN was not used. The enzyme was recrystallized six times. Before use the crystals were centrifuged and dissolved in 0.1 M triethanolamine buffer (pH 7.6) containing 1 mM EDTA, and ammonium sulphate was removed by gel filtration on a column of Sephadex G-25 (coarse grade). The A_{280}:A_{260} ratio of this enzyme (holoenzyme) was 1.09. Removal of NAD^+ was accomplished by mixing the holoenzyme with a 10-fold excess (by weight) of acid-washed activated charcoal. After stirring for 1 hr at 0°, the charcoal was removed by filtration with gentle suction. The A_{280}:A_{260} ratio of this enzyme (apoenzyme was in the range 1.75–1.95, which represents 0.3 mole or less of NAD^+ bound per mole of enzyme (16)). The enzyme concentration was measured using the published extinction coefficients of $E_{280}^{0.1\%} = 1.002$ for the holoenzyme and $E_{280}^{1.0\%} = 0.89$ for the apoenzyme (17).

The NAD^+ content of the holoenzyme was determined enzymatically after dialysis of 2 ml of enzyme (22.5 mg/ml) against two changes of 2 litres of 0.05 M triethanolamine buffer, pH 7.6, containing 1 mM EDTA. The enzyme was then incubated with 2 mg of pepsin at pH 2 and 25° for 2 hr. After centrifugation NAD^+ was determined by conversion to NADH in a solution containing 2 ml of 0.5 M glycine buffer, pH 9.0, with 0.4 M hydrazine and 10 mM lactate, 0.02 ml of lactate dehydrogenase (2 mg/ml), and 0.4 ml of digested glyceraldehyde 3-phosphate dehydrogenase. The accuracy of the method was checked by the use of NAD^+ standards and by addition of NAD^+ to the enzyme preparation prior to assay.

Glyceraldehyde 3-phosphate dehydrogenase activity was measured at 25° in a solution containing 0.1 M triethanolamine buffer (pH 7.6), 0.5 mM EDTA, 6 mM $MgSO_4$, 1 mM ATP, 0.25 mM NADH, 3 mM glycerate 3-phosphate, and phosphoglycerate

[2] The abbreviation used is: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

kinase (3 μg/ml). After the phosphoglycerate kinase reaction had reached equilibrium, glyceraldehyde 3-phosphate dehydrogenase was added to a final concentration of about 0.5 μg/ml and the decrease in A_{340} was followed. The specific activity of the holoenzyme was 80 μmoles/min/mg.

The following procedure was used to study the reaction of enzyme with DTNB. DTNB (0.1 ml, usually 5 mM in triethanolamine buffer) was added to 2 ml of a solution containing 1–2 μM glyceraldehyde 3-phosphate dehydrogenase. The reaction was followed by the change in absorbance at 412 nm, and the reaction course was analysed by the procedure of Freedman and Radda (18). Under pseudo-first-order conditions the end point of the reaction can be derived using the expression

$$\Delta E_\infty = \frac{\Delta E_b^2 - \Delta E_a \cdot \Delta E_c}{2\,\Delta E_b - (\Delta E_a + \Delta E_c)}$$

where ΔE_a, ΔE_b, and ΔE_c are the changes in absorption after time intervals a, b, and c such that $b - a = c - b$. These time intervals should be of the same order as the half-time of the reaction. The stoichiometry of the reaction can then be calculated using an extinction coefficient of 13,600 M^{-1} cm^{-1} for the thionitrobenzoate anion (19). For a single reaction under pseudo-first-order conditions a plot of $\log_e [\Delta E_\infty/(\Delta E_\infty - \Delta E_t)]$ against time results in a straight line whose slope gives the pseudo-first-order rate constant. If the reaction consists of more than one exponential, a curved plot will result. The pseudo-first-order rate constant for the most slowly reacting set is given by the slope of the linear segment, and extrapolation of this linear region to the ordinate allows calculation of the ΔE_∞ value of the fast reacting set. The contribution of the slow set to the change in absorption at each time t is then given by

$$\Delta E_{t\,\text{slow}} = \Delta E_{\infty\,\text{slow}}(1 - e^{-tk\ \text{slow}})$$

The contribution of the fast set to the change in absorption at each time t is then calculated by subtraction of the slow set contribution from the observed change in absorption. The pseudo-first-order rate constant for the fast set can then be derived from a plot of

$$\log_e \left(\frac{\Delta E_{\infty\text{fast}}}{\Delta E_{\infty\text{fast}} - \Delta E_{t\text{fast}}}\right) \text{against time.}$$

Enzyme activity measurements, time courses of reactions with DTNB, and other optical density measurements were performed on a Unicam SP 1800 ultraviolet spectrophotometer fitted with a Unicam SP 1805 programme controller.

RESULTS

Reaction of DTNB with glyceraldehyde 3-phosphate dehydrogenase. Figure 1 shows the time course of reaction of DTNB with the holoenzyme in the presence and absence of 1 mM NAD^+. The complete reaction corresponds to modification of 4 thiols/enzyme subunit (16/tetramer). In the absence of added NAD^+ there is an initial burst of reaction, which corresponds to 2 sulphydryl groups/subunit, and this is followed by the slow reaction of the 2 remaining thiols.

Analysis of the reaction with DTNB shows that it can be described in terms of 2 slowly reacting thiols, 1 reacting at an intermediate rate and 1 reacting very fast. Analyses of reactions of DTNB with holo-

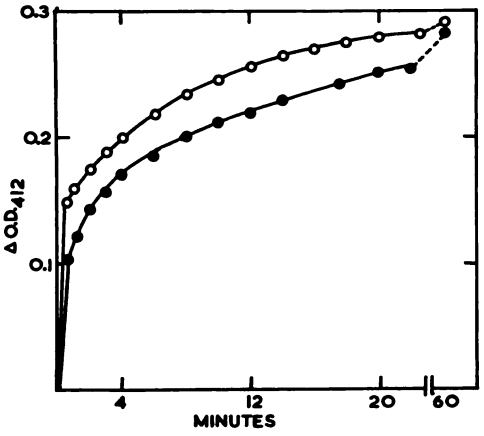


FIG. 1. *Reaction of DTNB with glyceraldehyde 3-phosphate dehydrogenase*

The reactions were carried out at 25° and pH 7.6 in 0.1 M triethanolamine buffer containing 1 mM EDTA. The DTNB concentration was 0.24 mM. O——O, holoenzyme (1.4 μM); ●——●, holoenzyme (1.39 μM) + 1 mM NAD^+.

TABLE 1

Reaction of DTNB with glyceraldehyde 3-phosphate dehydrogenase

The conditions were as described in Fig. 1, and the reactions were analysed as described under MATERIALS AND METHODS. K_{slow} and $K_{intermediate}$ are pseudo-first-order rate constants (DTNB concentration was 0.24 mM).

Conditions	Total groups/ subunit	Slow groups/ subunit	K_{slow}	Intermediate groups/ subunit	$K_{intermediate}$	Fast groups/ subunit
			min^{-1}		min^{-1}	
Holoenzyme	3.93	2.06	0.127	0.74	5.0	1.08
Apoenzyme	3.84	2.05	0.141	0.67	6.0	1.12
Holoenzyme + 1 mM NAD^+	3.74	1.8	0.06	0.78	0.54	1.16

enzyme, apoenzyme, and holoenzyme in the presence of 1 mM NAD^+ are given in Table 1. There is no significant difference between apo- and holoenzymes, but in the presence of saturating NAD^+ (1 mM) the rate of the slow set is reduced by 50% and the rate of the intermediate group is reduced by a factor of 10. The reaction of the fast group is too rapid to be followed by these techniques. The enzyme is totally inactivated within 10 sec of adding DTNB.

Effect of varying DTNB concentration. Wassarman and Major (20), working with lobster muscle glyceraldehyde 3-phosphate dehydrogenase, found that at low concentrations of DTNB the number of sulphydryl groups reacting, as indicated by the number of thionitrobenzoate anions released, was greater than could be explained by the amount of DTNB added. They suggested that reaction of DTNB with the active site thiol (Cys-148 in the case of the lobster muscle enzyme) was followed by formation of a disulphide bridge between Cys-148 and Cys-152, with the consequent release of a second thionitrobenzoate anion. Formation of the disulphide bridge was considered to cause a conformational change in the enzyme with exposure of the previously buried and nonreactive thiols.

A similar lack of stoichiometry at low DTNB concentrations has been observed with the rabbit muscle enzyme. At molar ratios of DTNB to glyceraldehyde 3-phosphate dehydrogenase of less than 1:1, all the added DTNB reacts within 10 sec. At molar ratios above 1:1, 1 thiol/subunit reacts within 10 sec, and reaction of this thiol results in complete loss of enzyme activity. At molar ratios of up to 2:1, essentially all the reacted thionitrobenzoate anion is slowly released over a period of 30 min. The release of the thionitrobenzoate anion is not accompanied by reappearance of free sulphydryls, as is shown by the following experiment. Glyceraldehyde 3-phosphate dehydrogenase and DTNB were mixed at a molar ratio of 2 DTNB/enzyme subunit and allowed to react until 3.4 thionitrobenzoate anions/subunit had been released. The enzyme was then denatured by addition of 1% sodium dodecyl sulphate and a large excess of DTNB was added. Reaction of only 0.4 additional group/subunit was observed; i.e., 3.4 groups/subunit remained nonreactive.

The release of 1 thionitrobenzoate anion/subunit, which occurs at an intermediate rate at high DTNB concentrations (Table 1), could be due to the formation of a disulphide bridge with consequent release of 1 thionitrobenzoate anion/subunit from the fast reacting thiol, or to the reaction of DTNB with 1 of the 3 remaining sulphydryls. The former case represents an intramolecular first-order process, the rate of which should be independent of DTNB concentration. The rate of release of the second thionitrobenzoate anion is increased with increasing concentrations of DTNB, and therefore probably represents reaction of DTNB with a second thiol.

The slow reaction of 2 groups/subunit (Table 1) is first-order even at a low DTNB

concentration, and the rate is independent of the DTNB concentration.

Effect of prior treatment with iodoacetic acid. Holoenzyme was incubated at 0° for 1 hr with a 10-fold molar excess of iodoacetate, and the unreacted iodoacetate was removed by gel filtration on a column of Sephadex G-25. Under these conditions the active site thiol (Cys-149) is specifically carboxymethylated (21). DTNB reacts very slowly with the carboxymethylated enzyme, one group being modified over a period of 2 hr. Addition of 1 mM NAD^+ further reduces the rate of reaction of this group to a negligible level.

Reaction of ethacrynic Acid with glyceraldehyde 3-phosphate dehydrogenase. Figure 2 shows the reaction of DTNB with the enzyme before and after a 30-min incubation with ethacrynic acid. Preliminary incubation with ethacrynic acid completely abolishes reaction of the two fast groups, so that only 2 of the 4 thiols/subunit react with DTNB. These 2 thiols react as a single set, with a rate constant similar to that observed for the slow set in the absence of ethacrynic acid.

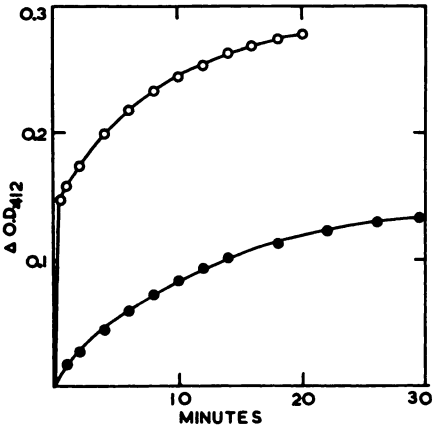


FIG. 2. *Reaction of DTNB with glyceraldehyde 3-phosphate dehydrogenase after incubation with ethacrynic acid*

Enzyme (1.4 μM) was incubated for 60 min at 25° and pH 7.6 with 325 μM ethacrynic acid. DTNB was then added to a concentration of 0.24 mM, and the reaction course was followed. No reaction of DTNB with ethacrynic acid was observed. ○——○, before incubation with ethacrynic acid; ●——●, after incubation with ethacrynic acid.

TABLE 2

Reaction of DTNB with glyceraldehyde 3-phosphate dehydrogenase after incubation with ethacrynic acid

Enzyme (2 μM) was incubated with 0.325 mM ethacrynic acid for 60 min at 25° and pH 7.6. DTNB (240 μM) was then added, and the increase in A_{412} was observed. The reactions were analysed as described under MATERIALS AND METHODS. No change in A_{412} was observed when DTNB was added to ethacrynic acid in the absence of enzyme.

Conditions	Total groups/ subunit	Slow groups/ subunit	K_{slow}	Fast groups/ subunit
			min^{-1}	
Holoenzyme	1.83	1.83	0.078	0
Apoenzyme	2.07	2.07	0.075	0
Holoenzyme + 1 mM NAD^+	3.05	1.81	0.1	1.24

Analyses of the reaction of glyceraldehyde 3-phosphate dehydrogenase with DTNB after incubation for 1 hr with ethacrynic acid are presented in Table 2. In the case of both the holo- and the apoenzyme, there is a slow reaction of 2 groups/subunit and the initial burst of reaction is no longer present.

The number of thiols that have reacted with ethacrynic acid can be determined by extrapolation to zero time of the linear segment between 1 and 2 min of the reaction of the enzyme with DTNB. There is an initial rapid reaction of the enzyme with ethacrynic acid, followed by a slow increase in the number of groups modified, to a maximum of 1.9/subunit. The semilogarithmic plot (Fig. 3) shows that this reaction is biphasic, with 0.8 group/subunit reacting rapidly and 1.1 groups reacting more slowly. The pseudo-first-order rate constant for the slow phase is 0.075 min^{-1} (ethacrynic acid concentration, 0.325 mM). Removal of NAD^+ from the holoenzyme results in an increase in the number of fast reacting groups to 1.1/subunit, whereas the rate of the slow phase is not greatly changed (0.05 min^{-1}).

The rate of reaction of glyceraldehyde 3-phosphate dehydrogenase with ethacrynic acid is decreased by addition of 1 mM NAD^+ (saturating under these condi-

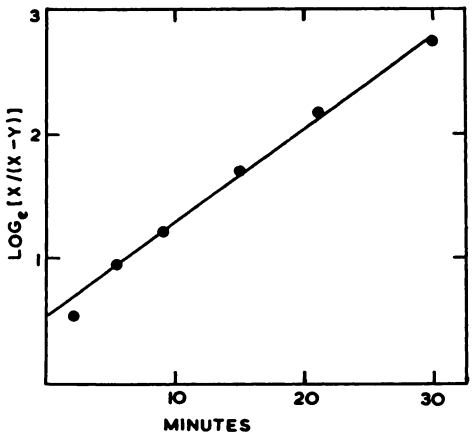


FIG. 3. *Semilogarithmic plot of reaction of glyceraldehyde 3-phosphate dehydrogenase with ethacrynic acid*

X represents the total number of sulphydryl groups which react with ethacrynic acid, and *Y* is the number of thiols reacted at various times. Holoenzyme (5.5 μM subunits) was incubated with 0.325 mM ethacrynic acid at 25° and pH 7.6 in 0.1 M triethanolamine buffer containing 1 mM EDTA. The extent of reaction with ethacrynic acid was determined as described in the text.

tions). Incubation with ethacrynic acid for 20 min in the presence of 1 mM NAD^+ results in modification of 0.2 group/subunit, whereas the comparable figure in the absence of NAD^+ is 1.6 groups/subunit.

The irreversible nature of the modification of glyceraldehyde 3-phosphate dehydrogenase by ethacrynic acid has been shown in several ways.

1. The enzyme was incubated with ethacrynic acid for 1 hr and then denatured by addition of 1% sodium dodecyl sulphate. The sulphydryl content was then determined by immediate addition of DTNB. Even after denaturation 2 of the 4 thiols remained blocked by ethacrynic acid.

2. Glyceraldehyde 3-phosphate dehydrogenase was incubated with ethacrynic acid for 1 hr and then dialysed for 24 hr at 4° against two changes of triethanolamine-EDTA buffer, representing a 1:10,000 dilution of the drug. The enzyme was then denatured by addition of 1% sodium dodecyl sulphate and the sulphydryl content was determined using DTNB. Two groups per subunit remained nonreactive.

3. Glyceraldehyde 3-phosphate dehydrogenase (0.8 mg/ml) was incubated for 45 min at 25° with ethacrynic acid (325 μM). Ethacrynic acid-treated enzyme (2 ml) and 2 ml of untreated enzyme were then dialysed overnight against the same 2 litres of triethanolamine-EDTA buffer at 4°. The absorbance of the treated enzyme at 280 nm was greater than that of the untreated preparation by an amount corresponding to the presence of 2 ethacrynic acid residues/subunit.

Reaction of glyceraldehyde 3-phosphate dehydrogenase with ethacrynic acid results in inactivation of the enzyme (Fig. 4). The time course of inactivation is biphasic to an extent which depends on the percentage of saturation with NAD^+. Inactivation of the apoenzyme proceeds at a single fast rate for nearly 90% of the total reaction (Fig. 4). Addition of NAD^+ to 50% saturation causes the inactivation to become markedly biphasic, with the slow phase constituting about 50% of the total reaction, and the presence of saturating NAD^+ affords essentially complete protection against inactivation. The correlation between percentage of saturation with NAD^+ and the proportion of slow phase inactivation is shown in Table 3. The correlation is excellent except in the case of the holoenzyme. This anomaly is considered further below. The inactivation of glyceraldehyde 3-phosphate dehydrogenase by ethacrynic acid is not reversed by addition of 1 mM 2-mercaptoethanol, 1 mM NAD^+, or 10 mM P_i.

DISCUSSION

The reaction of DTNB with lobster muscle glyceraldehyde 3-phosphate dehydrogenase has been studied by Wassarman and Major (20), who concluded that reaction of DTNB with the active site cysteine (Cys-148) was followed by formation of an intramolecular disulphide bond between Cys-148 and Cys-152. The reaction of DTNB with thiols is followed by the yellow colour of the thionitrobenzoate anion released into solution (19). The spectrophotometric assay therefore does not distinguish between reaction of 2 mole-

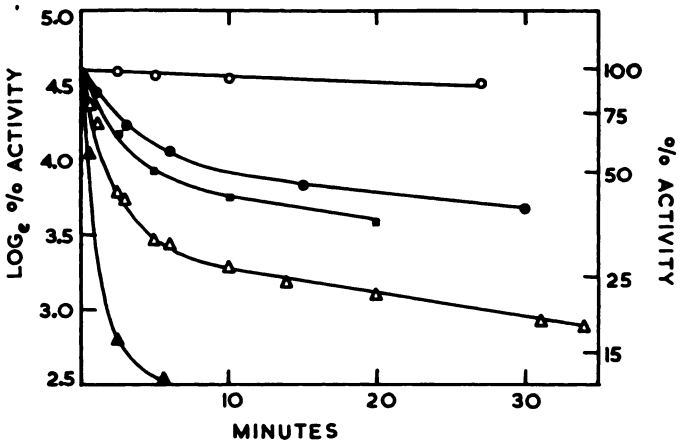


FIG. 4. *Inactivation of glyceraldehyde 3-phosphate dehydrogenase by ethacrynic acid*
Enzyme (0.5 μM) was incubated with 0.1 mM ethacrynic acid at 25° and pH 7.6. Aliquots were removed at the times shown and assayed as described in the text. ▲——▲, apoenzyme; △——△, holoenzyme; ■——■, apoenzyme + 2.8 μM NAD^+; ●——●, apoenzyme + 5.8 μM NAD^+; ○——○, apoenzyme + 1 mM NAD^+.

cules of DTNB with 2 sulphydryl groups and reaction of 1 molecule of DTNB with 1 sulphydryl group followed by release of the reacted thionitrobenzoate anion. It was important to distinguish between these two possibilities, as it was proposed in the present study to use DTNB to follow the reaction of ethacrynic acid with the enzyme.

Rabbit muscle glyceraldehyde 3-phosphate dehydrogenase has 4 cysteine residues/polypeptide chain, all of which apparently react with DTNB; i.e., 4 thionitrobenzoate anions are released per enzyme subunit. The reaction course consists of an initial burst of reaction over 1–2 min, corresponding to 2 groups/subunit, followed by a further slow reaction of the remaining 2 groups/subunit.

Slow reaction of 2 groups/subunit. This is first-order even at low DTNB concentrations, and the rate is independent of DTNB concentration. This reaction may represent a rate-limiting unfolding of the protein with exposure of previously buried thiols to reaction with DTNB. The data do not, however, allow differentiation between this and other possible schemes.

Fast reaction of 2 groups/subunit. DTNB reacts rapidly (within 10 sec) with 1 thiol/subunit even when added at a concentration corresponding to 1 mole of DTNB per mole of glyceraldehyde 3-phosphate dehydrogenase subunit. Reaction of this thiol results in complete inactivation of the enzyme. The rapid reaction of 1 thiol/subunit with DTNB is abolished by carboxymethylation of Cys-149. It is therefore likely that the immediate reaction with DTNB repre-

TABLE 3
Correlation between NAD^+ saturation and proportion of total ethacrynic acid inactivation constituted by slow phase

Enzyme	NAD^+ bound	Saturation	Slow phase
	moles/mole enzyme	%	*% inactivation*
Apoenzyme	0.3	7.5	15
Holoenzyme	3.5[a]	80	34
Apoenzyme + 2.8 μM NAD^+	2.1[b]	53	48
Apoenzyme + 5.6 μM NAD^+	2.2[b]	55	56
Apoenzyme + 1 mM NAD^+	4	100	100

[a] Determined enzymatically as described under MATERIALS AND METHODS.

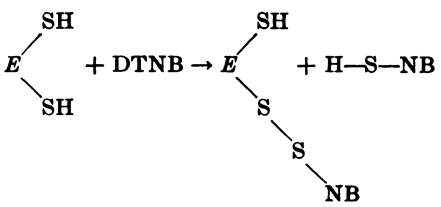
[b] The amount of NAD^+ bound was calculated as described by Price and Radda (22), using values of K_3 = 20.5 μM and K_4 = 318 μM, where K_3 and K_4 are the dissociation constants for binding of the third and fourth NAD^+ molecules to the enzyme.

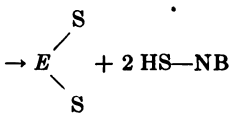
sents modification of the active site thiol, Cys-149.

The slower release of a second thionitrobenzoate anion per subunit could be due to reaction of a second thiol with DTNB. An alternative possibility is that it represents formation of a disulphide bond between Cys-149 and another thiol, with release of the thionitrobenzoate anion from Cys-149. In this case there would be an apparent stoichiometry of 2 groups/subunit although only 1 group had reacted with DTNB.

If the release of the second thionitrobenzoate anion is due to disulphide formation between Cys-149 and another thiol, it should occur at the same rate whether or not there is an excess of DTNB present. At a 1:1 ratio of DTNB to enzyme subunit, there is an immediate reaction of 1 group/subunit followed by a slow release (over 30 min) of the reacted anion. At higher DTNB to enzyme ratios the second anion is released increasingly rapidly, so that at a 20:1 ratio 2 anions/subunit are released within 1 min. It is therefore likely that release of the second thionitrobenzoate anion represents reaction of DTNB with a second thiol.

The stoichiometry observed with excess glyceraldehyde 3-phosphate dehydrogenase is likely to be due to formation of intra- or intermolecular disulphide bridges. DTNB was added at a 2:1 ratio with enzyme subunits (i.e., 1 DTNB/2 enzyme thiols). and the reaction was allowed to proceed until 3.4 thionitrobenzoate anions had been released per enzyme subunit. The enzyme was denatured and allowed to react with an excess of DTNB; 3.4 groups/subunit remained nonreactive. This means that reaction with DTNB and subsequent release of the thionitrobenzoate anion results in blockage of 2 thiols. A model which could explain these results is as follows:





where E represents glyceraldehyde 3-phosphate dehydrogenase and —S—NB represents thionitrobenzoate.

When DTNB is allowed to react with an *excess* of cysteine or glutathione, 2 thionitrobenzoate anions are released per molecule of DTNB added. When DTNB is in excess, however, only 1 anion is released.[3] It is therefore likely that the model proposed above represents the sequence of events only when the enzyme is present in excess. These considerations suggest that when DTNB is present in excess, the rapid release of 2 thionitrobenzoate anions represents reaction of 2 molecules of DTNB with 2 thiols. One of these is probably the active site thiol, Cys-149.

Ethacrynic acid was developed in the course of a search for non-organomercurial inhibitors of sulphydryl-dependent systems. The molecule contains an α,β-unsaturated ketone structure that reacts readily with sulphydryl groups (23).

Incubation of glyceraldehyde 3-phosphate dehydrogenase with ethacrynic acid abolishes the fast reaction with DTNB of 2 thiols/subunit, and 2 ethacrynic acid residues remain attached to the enzyme after thorough dialysis. The reactivity with DTNB of the 2 thiols blocked by ethacrynic acid is not restored by denaturation of the enzyme with sodium dodecyl sulphate or by exhaustive dialysis. Incubation with ethacrynic acid results in inactivation of the enzyme, which is not reversed by dilution into the assay solution or by addition of 2-mercaptoethanol. It is concluded that ethacrynic acid reacts irreversibly with 2 thiols/enzyme subunit, 1 of which is probably Cys-149. It is assumed that the second ethacrynic acid-reactive thiol is that which reacts at an intermediate rate with DTNB.

The time course of reaction of ethacrynic acid with glyceraldehyde 3-phosphate dehydrogenase has been followed by the rate of disappearance of sulphydryl groups re-

[3] D. J. Birkett, unpublished observations.

acting with DTNB. The time course is shown to be biphasic, with, in the case of the apoenzyme, approximately 1 group/subunit reacting rapidly and 1 group reacting at a slower rate. The rate of reaction of the slower group is much less than the rate of inactivation of the apoenzyme by ethacrynic acid (0.05 min^{-1} compared to 2.86 min^{-1} at 0.325 mM drug). This supports the conclusion that the thiol which reacts rapidly with ethacrynic acid is the active site thiol, Cys-149.

When NAD^+ is added to the apoenzyme, the inactivation by ethacrynic acid becomes markedly biphasic. The extent of the slow phase inactivation correlates well with the percentage of saturation with NAD^+ (Fig. 4 and Table 3). In the presence of saturating NAD^+ both inactivation and modification of sulphydryls (Tables 2 and 3) proceed at a very slow rate. These data indicate that binding of NAD^+ protects the active site thiol against modification by ethacrynic acid.

The effect of ethacrynic acid on the holoenzyme is anomalous (Table 3). The A_{280}: A_{260} ratio of 1.09 suggests that 3–4 moles of NAD^+ are bound per enzyme subunit, i.e., that the enzyme is essentially saturated with NAD^+. Yet several aspects of the behaviour of the enzyme suggest that the NAD^+ saturation is about 30%. (a) Ethacrynic acid reacts rapidly with the holoenzyme, about 0.7 group/subunit being modified at a fast rate and 1.2 reacting more slowly. Addition of 1 mM NAD^+ causes a decrease in the rate of reaction to a very low level. (b) Ethacrynic acid inactivation of the holoenzyme is rapid and biphasic, the slow phase constituting about 30% of the total reaction. Addition of 1 mM NAD^+ affords essentially complete protection.

Neither of these results would be expected if the holoenzyme was 90% saturated with NAD^+. Block *et al.* (24) have shown that crystalline preparations of glyceraldehyde 3-phosphate dehydrogenase may contain bound adenosine diphosphate ribose, a degradation product of NAD^+, and it seemed that this might be the explanation for the anomalous behaviour of the holoenzyme. However, enzymatic analysis for the NAD^+ content of the holoenzyme after exhaustive dialysis at pH 7.6 (representing a $1:10^6$ dilution) showed that 3.5 moles of NAD^+ were bound per mole of enzyme. This figure was surprising in view of the published values for K_3 and K_4, the dissociation constants for the third and fourth molecules of NAD^+ to bind. Price and Radda (22), working under the same conditions of pH and temperature as in this work, found values for K_3 and K_4 of 20.5 and 318 μM, respectively. It would be expected, therefore, that dialysis would remove the third and fourth NAD^+ molecules, resulting in an NAD^+ content of 2 moles/mole of enzyme. These considerations suggest that removal of NAD^+ by charcoal treatment, followed by addition of 4 moles of NAD^+ per mole of enzyme, does not reconstitute the holoenzyme in the initial form. This problem has not been further investigated in the present work.

The results presented in this paper provide a mechanism at the molecular level for the inhibition of glycolysis by the drug. Ethacrynic acid reacts rapidly with the active site sulphydryl of glyceraldehyde 3-phosphate dehydrogenase, causing inactivation of the enzyme.

Extrapolation of effects *in vitro* to the mode of action of the drug *in vivo* is difficult, as relatively high concentrations are needed to observe inhibition of these systems *in vitro*. However, Daniel *et al.* (1) have shown that ethacrynic acid is concentrated 3–10-fold in rat uterine tissue and, furthermore, the effective concentration of the drug in the various intracellular compartments is not known. The inactivation of glyceraldehyde 3-phosphate dehydrogenase by ethacrynic acid is irreversible, so that in this case equilibrium conditions do not apply and relatively low extracellular concentrations of ethacrynic acid may be effective. The view that the drug reacts with thiols *in vivo* is supported by studies showing a decrease in the content of protein-bound sulphydryls in renal cortex (25, 26), and studies are currently being undertaken in this laboratory to determine whether

ethacrynic acid inactivates glyceraldehyde 3-phosphate dehydrogenase *in vivo*.

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