



## Dithranol, Glucose-6-phosphate Dehydrogenase Inhibition and Active Oxygen Species

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Dedicated to Professor Dr. Dr. Ernst Mutschler on the occasion of his 60th birthday

### Summary

Inhibition of glucose-6-phosphate dehydrogenase (G6-PDH) by dithranol (anthralin, CAS 480-22-8) has been studied in the presence of catalase, superoxide dismutase (SOD) and various scavengers of active oxygen species. Most scavengers were found to be either inhibitors of G6-PDH by themselves or simply without effect. The combined addition of catalase and SOD as well as the heat-denatured enzymes and the oxygen radical scavengers  $\alpha$ -tocopherol and salicylic acid markedly reduced the inhibitory effect of dithranol. The direct exposure of G6-PDH to active oxygen species led to different results. When liberated from a water-soluble naphthalene endoperoxide, singlet oxygen was without effect whereas photosensitization with methylene blue resulted in a total loss of enzyme activity. Experiments under anaerobic conditions revealed that this inhibition was accomplished by the triplet state of the sensitizer. Superoxide anion radical was highly effective at concentrations corresponding to the amount of that produced by a 10  $\mu$ mol/l dithranol solution. In contrast, hydroxyl, alkylperoxyl and alkoxy radicals were all less efficient.  $H_2O_2$  and alkylhydroperoxides did not alter the enzyme activity. The results suggest that  $\cdot O_2^-$  is the potent species towards G6-PDH, if dithranol acts through formation of active oxygen species.

### Zusammenfassung

Dithranol, Glucose-6-phosphat-Dehydrogenase-Hemmung und aktive Sauerstoffspezies

**Key words:** Anthralin, *in vitro* studies · Antipsoriatic drugs · CAS 480-22-8 · Dithranol · Free radicals · Glucose-6-phosphate dehydrogenase, inhibition

### 1. Introduction

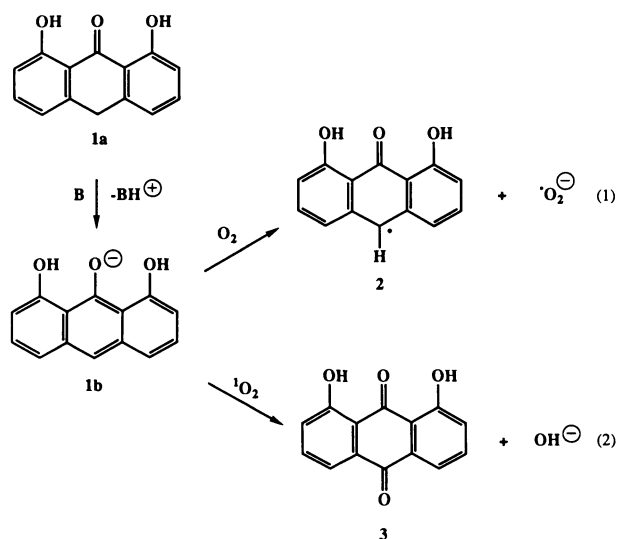
Dithranol (anthralin, **1a**; CAS 480-22-8) has been used as an effective topical antipsoriatic drug for over 70 years

Glucose-6-phosphat-Dehydrogenase (G6-PDH)-Hemmung durch Dithranol (Anthralin, CAS 480-22-8) wurde in Gegenwart von Catalase, Superoxid-Dismutase (SOD) und verschiedenen Fängern für aktive Sauerstoffspezies untersucht. Die meisten Fänger waren entweder selbst Hemmstoffe der G6-PDH oder wirkungslos. Sowohl die kombinierte Zugabe von Catalase und SOD als auch die denaturierten Enzyme, ebenso die Sauerstoffradikalfänger  $\alpha$ -Tocopherol und Salicylsäure zeigten eine deutliche Reduzierung der durch Dithranol verursachten Hemmeffekte. Die direkte Einwirkung von aktiven Sauerstoffspezies auf G6-PDH führte zu unterschiedlichen Resultaten. Freisetzung von Singulett-Sauerstoff aus einem wasserlöslichen Endoperoxid war ohne Effekt, hingegen führte die Photosensibilisierung mit Methylenblau zum vollständigen Verlust der Enzymaktivität. Experimente unter anaeroben Bedingungen zeigten jedoch, daß diese Hemmung durch den Triplett-Zustand des Sensibilisators verursacht wurde. Superoxid-Anion-Radikal erwies sich in Konzentrationen, die von einer 10  $\mu$ mol/l Dithranol-Lösung produziert werden, als hochwirksam. Hydroxyl-, Alkylperoxyl- und Alkoxy-Radikale waren hingegen weniger wirksam.  $H_2O_2$  und Alkylhydroperoxide führten zu keiner Beeinträchtigung der Enzymaktivität. Die Resultate deuten darauf hin, daß  $\cdot O_2^-$  die potente Spezies der G6-PDH-Hemmung durch Dithranol ist, wenn diese über die aktiven Sauerstoffspezies erfolgt.

[1]. The mode of action of the drug at the molecular level is still not known in detail, but it may act, at least in part, on mainly four cellular targets: a) interaction with DNA [2–6], b) alteration of mitochondrial functions [7–10], c) inhibition of the 5- and 12-lipoxygenase pathways of arachidonic acid metabolism [11–13], and d) inhibitory action on cytosolic enzymes of the glycolytic pathway, such as glucose-6-phosphate dehydrogenase (G6-PDH) [14–18]. This key enzyme of the hexose monophosphate shunt is elevated in psoriatic skin [19, 20]. In view of the

instability of dithranol in weakly basic aqueous solution [18], it is unlikely that the molecule itself is responsible for the biological properties. Consequently, the inhibitory effects might be related to decomposition products formed during the autoxidation of dithranol [18]. Since the only two known oxidation products, the dimer bianthrone and the anthraquinone danthron (**3**), are only moderate inhibitors of G6-PDH, it has been suggested that other breakdown products must be the most active species towards G6-PDH [18].

The free radical mechanism of dithranol oxidation starts with deprotonation followed by electron abstraction to give 1,8-dihydroxy-9-anthrone-10-yl-radical (**2**) [21, 22]. Because of its stability and inertness towards oxygen this radical was discounted as an important intermediate [23]. In the presence of oxygen, the univalent reduction of oxygen by dithranol anion results in the formation of superoxide anion ( $\text{O}_2^-$ ) [24, 25] (Scheme 1 (1)), dismutation of this species generates hydrogen peroxide [24], and by the iron-catalyzed Haber-Weiss-reaction the far more reactive hydroxyl radical ( $\text{OH}^\bullet$ ) is produced [26]. Photochemically, electronically excited molecular oxygen (singlet oxygen,  $^1\text{O}_2$ ) is produced by the dithranol anion (**1b**) [27, 28], which in turn is oxidized to danthron (**3**, Scheme 1 (2)) in a self-sensitized process most probably via an endoperoxide [27].



Scheme 1

Although this production of active oxygen species *in vitro* by dithranol is well documented, it has not yet been established whether this property is relevant to the mechanism of action of anthralin on biological targets. Dithranol interacts irreversibly with G6-PDH [18]. Irreversible inhibition may be derived from an undetermined redox pathway involving oxygen radicals generated by dithranol, resulting in an inactive enzyme through destruction of specific amino acids, particularly histidine and tryptophan [29]. Studies not related to dithranol showed that the membrane-bound SH enzyme glyceraldehyde-3-phosphate dehydrogenase was inhibited by oxidation of the SH groups by active oxygen species [30].

The objective of the present study was to assess the role of active oxygen species in the inhibitory action on G6-PDH by dithranol. This inhibition is utilized for a first screening for antipsoriatic activity to date [31, 32]. Disregarding the question whether G6-PDH inhibition is a significant mechanism for the mode of action or not we find this test a useful tool in our efforts to obtain more insight into the nature of the inhibitory species. If dith-

ranol acts through active oxygen formation, addition of catalase, superoxide dismutase (SOD), and various oxygen radical scavengers to the reaction system should cancel or diminish the rate of inactivation of G6-PDH by dithranol. Another approach which would allow us to understand the influence of oxygen derivatives on this reaction system is to test the effects of active oxygen species — generated independently of dithranol — on G6-PDH activity.

### Abbreviations

BAS — bovine serum albumin  
 DABCO — 1,4-diazabicyclo[2.2.2] octane  
 DTPA — diethylenetriaminepentaacetic acid  
 EDTA — ethylenediaminetetraacetic acid  
 G6-P — glucose-6-phosphate disodium salt  
 G6-PDH — glucose-6-phosphate dehydrogenase (EC. 1.1.1.49)  
 HSA — human serum albumin  
 NADP<sup>+</sup> — nicotinamide adenine dinucleotide phosphate disodium salt  
 NBT — nitro blue tetrazolium  
 NDPO<sub>2</sub> — endoperoxide of 3,3'-(1,4-naphthylene)dipropionate  
 NDGA — nordihydroguaiaretic acid  
 SOD — superoxide dismutase (EC. 1.15.1.1)  
 XO — xanthine oxidase (EC. 1.1.3.22)

## 2. Materials and methods

### 2.1. Chemicals

Dithranol (anthralin, 1,8-dihydroxy-9(10H)-anthracenone) was prepared by reduction of danthron [33] and purified by column chromatography ( $\text{SiO}_2/\text{CH}_2\text{Cl}_2$ ); NDPO<sub>2</sub> was prepared according to the method of Aubry [34]; BSA,  $\beta$ -carotene, catalase from bovine liver (E.C. 1.11.1.6; 20000 U/mg protein), chelating resin (sodium form), deferoxamine mesylate, 2,2'-dipyridyl, DTPA, G6-P, glutathione, HSA, NADP<sup>+</sup>, NDGA, propyl gallate, pyrogallol, SOD from bovine erythrocytes (2800 U/mg protein), xanthine, XO (Sigma, Munich, FRG), cumene hydroperoxide, EDTA,  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ ,  $\text{FeSO}_4 \cdot \text{H}_2\text{O}$ , hydrogen peroxide (30%), mannitol, methylene blue, NBT, sodium citrate,  $\alpha$ -tocopherol (E. Merck, Darmstadt, FRG),  $\beta$ -alanine, ascorbic acid, L-cysteine, DABCO, glycine, rose bengal, salicylic acid, L-serine, sodium benzoate, thiourea (Aldrich, Steinheim, FRG). G6-PDH (350 U/mg protein; Boehringer Mannheim GmbH, Mannheim, FRG), tert-butyl hydroperoxide (Janssen, Nettekald, FRG).

### 2.2. G6-PDH assay

Incubation experiments were performed in a Ringer buffer composed of 140 mmol/l NaCl, 2.7 mmol/l KCl, 5 mmol/l  $\text{NaHCO}_3$ , 1.8 mmol/l  $\text{CaCl}_2$  and 1.1 mmol/l  $\text{MgCl}_2$  at pH 7.5. The commercial enzyme suspension (350 U/mg) was diluted 1:2000 in the buffer and kept in an ice bath (at time zero the control activity was 70 U/l). Dithranol stock solutions (4.4 mmol/l in acetone) were kept in the dark under  $\text{N}_2$ . In typical runs G6-PDH activity was determined in four different incubation sets (final volume — 5 ml) consisting of (a) 4.48 ml of Ringer buffer, 0.5 ml of enzyme solution and 0.02 ml acetone, (b) 4.48 ml of Ringer buffer, 0.5 ml of enzyme solution and 0.02 ml of dithranol stock solution, (c) 3.98 ml of Ringer buffer, 0.5 ml of enzyme solution, 0.5 ml of oxygen scavenger solution and 0.02 ml acetone, and (d) 3.98 ml of Ringer buffer, 0.5 ml of enzyme solution, 0.5 ml of oxygen scavenger solution and 0.02 ml of dithranol stock solution. Test solutions were incubated for 1 h at 37 °C in a shaking thermostat bath in the dark. After incubation, 0.5 ml of the test solution was pipetted in a 3.0 ml cell, 2.4 ml of Ringer buffer and 0.05 ml of a 0.03 mmol/l NADP<sup>+</sup> solution were added, mixed and kept for 5 min at 25 °C. After addition of 0.05 ml of a 0.04 mmol/l G6-P solution the rate of increase in absorbance at 339 nm was measured over a period of 5 min on a Uvikon 810 spectrophotometer (Kontron Instruments, Eching, FRG).

### 2.3. Catalase assay

Catalase activity was measured following the decomposition of  $\text{H}_2\text{O}_2$  at 240 nm [35]. Reaction mixtures contained 1.98 ml of phosphate buffer, 1 ml of 0.03 mmol/l  $\text{H}_2\text{O}_2$  and 0.02 ml of enzyme dilution (2 U/mg protein). Incubation experiments for the determination of catalase inhibition by dithranol were performed for 30 min at 37 °C in a shaking thermostat bath under light protection. Control: acetone.

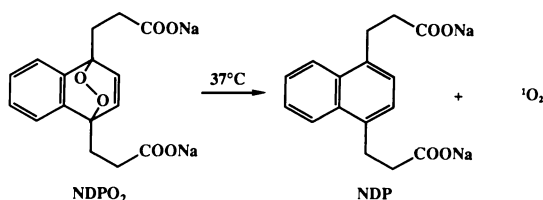
## 2.4. SOD assay

SOD activity was determined by measuring the inhibition of pyrogallol autoxidation, monitored spectrophotometrically at 420 nm [36]. Incubation experiments for the determination of SOD inhibition by dithranol were performed for 60 min at 37 °C in a shaking thermostat bath in the dark. Control: acetone.

## 2.5. Active oxygen species generating systems

All incubation experiments for G6-PDH inhibition by active oxygen species were performed aerobically in a phosphate buffer composed of 50 mmol/l  $K_2HPO_4 \cdot 3H_2O$  and 5 mmol/l  $KH_2PO_4$  at pH 7.8 under shaking. Incubation mixtures contained 4.47 or 4.48 ml of phosphate buffer, 0.5 ml of enzyme solution and 0.02 or 0.03 ml of an active oxygen generating system. Incubation time was 30 min.

$^1O_2$  was produced either by means of a dye-sensitized photochemical method or with a chemical source. In the case of photosensitized oxidations the incubation mixtures were irradiated with a cooled halogen lamp (Osram Halostar, 100 W) in the presence of rose bengal or methylene blue ( $10^{-5}$  mmol/l), respectively. A 1 % solution of  $K_2Cr_2O_7$  in  $H_2O$  was used as a cutoff filter (550 nm). Controls: Ar/in the dark. As a chemical source the decomposition of the water-soluble NDPO<sub>2</sub> [34, 37] (Scheme 2) was used.



Scheme 2

The decomposition of the endoperoxide was examined at 37 °C in the phosphate buffer described above. The disappearance of NDPO<sub>2</sub> (10 μmol/l in the incubation mixture of 5 ml) was monitored by HPLC (Kontron 420, column: Nucleosil-100 RP 18; methanol/water/acetic acid (77:23:0.1); UV detection at 232 nm, Kontron Uvikon 735 LC). The first order rate constant was  $4.2 \pm 0.2 \cdot 10^{-4} s^{-1}$  which corresponds to a half-life of 28 min.

In order to produce a flux of  $^1O_2$  a system of XO and xanthine was used [38, 39]. Generation of  $^1O_2$  was ascertained spectrophotometrically by monitoring the reduction of NBT at 560 nm [40]. The amounts of XO (0.02 U/ml) and xanthine (30 μmol/l) used were chosen on the basis of an  $IC_{50}$  of dithranol for G6-PDH of about 10 μmol/l. Thus, the system was adjusted in such a manner that the reduction rate of NBT corresponded to 55 % [24] of that caused by a 10 μmol/l dithranol solution (45 % of NBT reduction are due to direct electron transfer from dithranol to NBT, for details on  $^1O_2$  production during dithranol autoxidation see [24]). XO was added last to initiate the reaction. The phosphate buffer solution was passed through a column of chelating resin to remove traces of iron [41]. DTPA [42, 43] (0.1 mmol/l) or deferoxamine mesylate [44] (0.1 mmol/l) was added to prevent  $^1OH$  formation.

$^1OH$  was generated from the xanthine/XO system either by addition of  $Fe^{3+}$ -EDTA [42, 45] (iron-catalyzed Haber-Weiss reaction) or  $Fe^{2+}$ -DTPA [46] (superoxide driven Fenton reaction). The final concentrations in the reaction mixtures were 0.1 mmol/l for  $FeCl_3 \cdot 6H_2O$  or  $FeSO_4 \cdot H_2O$ , respectively. Controls were performed with the ferrous salt,  $H_2O_2$ , xanthine, and the chelators alone. Additionally, hydroxyl radicals were produced in a Fenton reaction with hydrogen peroxide and  $Fe^{2+}$ -DTPA [46].  $H_2O_2$  was added last in three portions (final concentration 0.18 mmol/l) to initiate the reaction.

Alkoxyl radicals were generated by the catalytic decomposition of tert-butyl hydroperoxide [47] and cumene hydroperoxide by  $Fe^{2+}$ -dipyridyl [48], whereas  $Fe^{3+}$  as a catalyst was employed to predominantly generate alkylperoxyl radicals [49, 50]. Incubation experiments contained 0.1 mmol/l of the alkylhydroperoxide, 0.1 mmol/l ferrous dipyridyl or iron DTPA, respectively.

## 3. Results and discussion

### 3.1. Effects of catalase and SOD on the system G6-PDH/dithranol

Since dithranol was reported to lower the activities of both the enzymes [51], we examined the activities of catalase and SOD as a function of dithranol concentration. The  $IC_{50}$  of dithranol for catalase was found to be 5 μmol/l (Fig. 1). With a dithranol concentration of  $1.7 \cdot 10^{-5}$  mol/l catalase activity decreased to about 70 % of initial activity after 30 min of incubation (Fig. 2). Although this inhibition of catalase by dithranol has to be considered, there still remains sufficient activity for studying the effect of catalase on G6-PDH inhibition by dithranol under these conditions.

Contrary to catalase the maximal degree of inhibition of SOD that could be achieved was 35 % (Fig. 3), even at higher concentrations of dithranol.

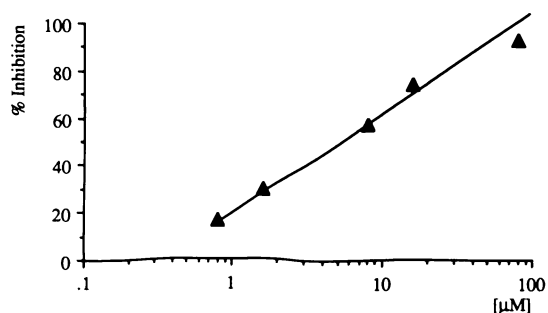


Fig. 1: Inhibition of catalase as a function of dithranol concentration (abscissa). Incubation mixtures contained catalase (5 μg/ml) and indicated concentrations of dithranol in a final volume of 1 ml phosphate buffer for 30 min at 37 °C in a shaking water bath. Results are the average of 3 experiments (SD < 5 %).

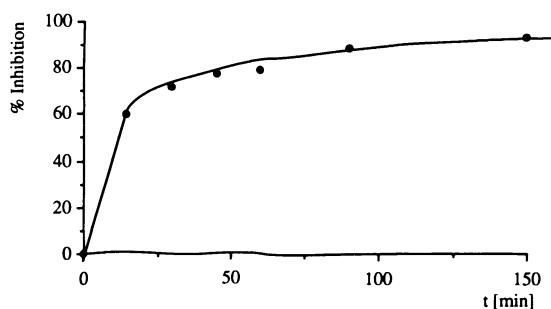


Fig. 2: Time course of inhibition of catalase by dithranol. Incubation mixtures contained catalase (5 μg/ml) and  $1.7 \cdot 10^{-5}$  mol/l dithranol in a final volume of 1 ml phosphate buffer for 30 min at 37 °C. Results are the average of 3 experiments (SD < 5 %).

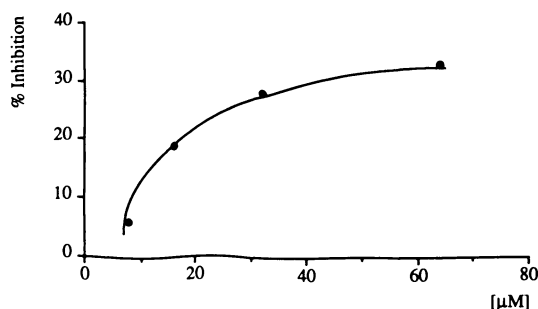


Fig. 3: Inhibition of SOD as a function of dithranol concentration (abscissa). Incubation mixtures contained SOD (10 μg/ml) and indicated concentrations of dithranol in a final volume of 2 ml phosphate buffer for 1 h at 37 °C in a shaking water bath. Results are the average of 3 experiments (SD < 5 %).

Data from Table 1 reveal that neither catalase nor SOD show significant changes in dithranol efficacy towards G6-PDH when added to the incubation sets. On the other hand, combined treatment with catalase and SOD at high concentrations markedly lowered inhibitory action on G6-PDH by dithranol. The combined addition of the enzymes to the system removes both H<sub>2</sub>O<sub>2</sub> (catalase) and ·O<sub>2</sub><sup>-</sup> (SOD), and — by interaction of these two species — ·OH as a secondary reaction product, as previously shown for dithranol [26]. However, the denatured enzymes were effective, too. Moreover, addition of the proteins BSA or HSA led to a total loss of inhibitory action. Similar although weaker effects were obtained with amino acids.

These results allow two possible explanations for the decrease of G6-PDH inhibition by dithranol. Firstly, they suggest an interaction of dithranol with proteins or amino acids. Observations from other studies that dithranol is partially bound or inactivated by serum proteins [17, 52–54] are in agreement with this interpretation. Secondly, it is known that even catalase and SOD pro-

Table 1: Effects of catalase, SOD, proteins and amino acids on G6-PDH inhibition by dithranol.

Scavenger added	% Inhibition
None	68 ± 4 <sup>a)</sup>
Catalase (2 U/ml)	66 ± 2
Catalase (300 U/ml)	52 ± 4
Superoxide dismutase (28 U/ml)	65 ± 6
Superoxide dismutase (175 U/ml)	55 ± 2
Catalase (300 U/ml) + superoxide dismutase (175 U/ml)	14 ± 1
Heated catalase (300 U/ml) + heated superoxide dismutase (175 U/ml)	17 ± 3
BSA (30 µg/ml)	0
HSA (30 µg/ml)	0
β-Alanine (1.6 × 10 <sup>-4</sup> mol/l)	60 ± 3
L-Cysteine (1.6 × 10 <sup>-4</sup> mol/l)	31 ± 4
Glutathione (1.6 × 10 <sup>-4</sup> mol/l)	44 ± 5
Glycine (1.6 × 10 <sup>-3</sup> mol/l)	50 ± 1
L-Serine (1.6 × 10 <sup>-4</sup> mol/l)	38 ± 5

Incubation mixtures contained 7 mU/ml G6-PDH, 1.7 × 10<sup>-5</sup> mol/l dithranol and indicated concentrations of scavengers at 37 °C in Ringer buffer (30 min). % Inhibition is expressed as mean ± SD; n ≥ 3. All scavengers added did not impair the enzyme activity (controls without dithranol). <sup>a)</sup> n = 22.

teins (e.g. the inactive enzymes) react with active oxygen derivatives [55]. The same is true for other proteins including heat-denatured SOD and catalase [55]. Amino acids are highly reactive with ·OH radicals [56]. Thus, scavenging of active oxygen species produced by dithranol may be responsible for the observed decline of G6-PDH inhibition in the presence of catalase, SOD and proteins.

### 3.2. Effects of singlet oxygen quenchers and oxygen radical scavengers on the system G6-PDH/dithranol

With the exception of the singlet oxygen quenchers β-carotene [57] and ascorbic acid [58], scavengers of oxygen radicals, such as mannitol, sodium benzoate, sodium citrate, salicylic acid, and thiourea did not influence G6-PDH activity of controls (no dithranol) to an appreciable amount (Table 2). Higher concentrations of scavengers did inhibit G6-PDH activity. In addition, the free radical scavengers NDGA, propyl gallate and pyrogallol are stronger inactivators of G6-PDH than dithranol. Pertinent experiments revealed that addition of only small amounts of these scavengers resulted in a total loss of enzyme activity. Of all tested compounds only α-tocopherol, although an inhibitor by itself, and salicylic acid did weaken the inhibitory effect of dithranol.

Table 2: Effects of singlet oxygen quenchers and oxygen radical scavengers on G6-PDH inhibition by dithranol.

Scavenger added	% Inhibition without dithranol	% Inhibition
None	0	68 ± 4 <sup>a)</sup>
Ascorbic acid (1.6 × 10 <sup>-4</sup> mol/l)	28 ± 1	84 ± 4
β-Carotene (8 × 10 <sup>-6</sup> mol/l)	44 ± 6	81 ± 5
DABCO (1.6 × 10 <sup>-3</sup> mol/l)	0	93 ± 1
Mannitol (1.6 × 10 <sup>-3</sup> mol/l)	0	78 ± 3
NDGA (1.6 × 10 <sup>-5</sup> mol/l)	100	100
Propyl gallate (1.6 × 10 <sup>-6</sup> mol/l)	100	100
Pyrogallol (1.6 × 10 <sup>-6</sup> mol/l)	100	100
Salicylic acid (1.6 × 10 <sup>-4</sup> mol/l)	0	56 ± 1
Sodium benzoate (1.6 × 10 <sup>-3</sup> mol/l)	0	76 ± 2
Sodium citrate (1.6 × 10 <sup>-4</sup> mol/l)	0	72 ± 3
Thiourea (1.6 × 10 <sup>-3</sup> mol/l)	0	84 ± 2
α-Tocopherol (3.2 × 10 <sup>-5</sup> mol/l)	29 ± 6	50 ± 6

Incubation mixtures contained 7 mU/ml G6-PDH, 1.7 × 10<sup>-5</sup> mol/l dithranol and indicated concentrations of scavengers at 37 °C in Ringer buffer (30 min). % Inhibition is expressed as mean ± SD; n ≥ 3. <sup>a)</sup> n = 22.

Our results demonstrate that most of the oxygen radical scavengers were G6-PDH inhibitors by themselves at concentrations necessary for the inactivation of oxygen free radicals produced by dithranol. This indicates that experiments with the system G6-PDH/dithranol/scavenger are not much of an appropriate tool in search of the active intermediate towards G6-PDH.

### 3.3. Effects of active oxygen species on G6-PDH activity

If the toxic effects of dithranol on G6-PDH occurred by oxygen free radicals or singlet oxygen, a direct exposure of the enzyme to these species should lead to loss of activity. Since dithranol inhibited this enzyme at an IC<sub>50</sub> value of about 10 µmol/l (Fig. 4), the effects of active oxygen species on G6-PDH activity were tested at concentrations corresponding approximately to the amount of active oxygen species generated by a 10 µmol/l dithranol solution.

#### 3.3.1. Singlet oxygen

<sup>1</sup>O<sub>2</sub> causes damage to a variety of biological targets [59]. Destruction of key active-site amino acids leads to inactivation of many enzymes. Indeed, studies on G6-PDH photooxidation by rose bengal resulted in a specific destruction of histidine residues [60]. In this study we made use of two different <sup>1</sup>O<sub>2</sub>-sources. Photosensitization with rose bengal leading to a total loss of activity (Table 3, entry 1) was consistent with a previous report [60]. However, pertinent experiments in the dark revealed that even ground-state rose bengal is an inhibitor of G6-PDH. Contrary, the <sup>1</sup>O<sub>2</sub>-sensitizer methylene blue did not inhibit the enzyme in the dark. Although photosensitiza-

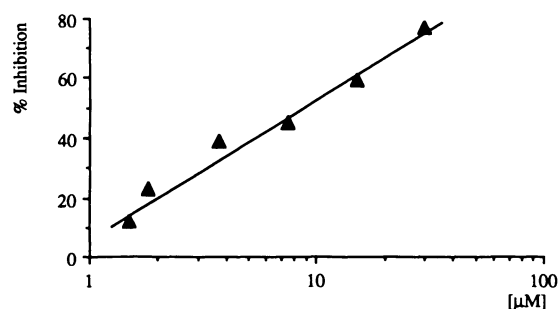


Fig. 4: Inhibition of G6-PDH as a function of dithranol concentration (abscissa). Incubation mixtures contained 7 mU/ml G6-PDH and indicated concentrations of dithranol in a final volume of 5 ml phosphate buffer at 37 °C in a shaking water bath (30 min). Results are the average of three experiments (SD < 5 %).

Table 3: Effects of active oxygen species on G6-PDH.

Entry	Active oxygen species-generating system	% Inhibition
1	$^1\text{O}_2$ rose bengal (10 $\mu\text{mol/l}$ )/ $\text{O}_2$ /100 W	100
2	rose bengal (10 $\mu\text{mol/l}$ )/dark <sup>a)</sup>	100
3	methylene blue (10 $\mu\text{mol/l}$ )/ $\text{O}_2$ /100 W	100
4	methylene blue (10 $\mu\text{mol/l}$ )/Ar/100 W <sup>a)</sup>	100
5	methylene blue (10 $\mu\text{mol/l}$ )/dark <sup>a)</sup>	0
6	NDPO <sub>2</sub> (10 $\mu\text{mol/l}$ )	0
7	$^{\cdot}\text{O}_2^-$ xanthine (30 $\mu\text{mol/l}$ )/XO (0.02 U/ml)/DTPA (100 $\mu\text{mol/l}$ )	60 $\pm$ 3
8	xanthine (30 $\mu\text{mol/l}$ )/XO (0.02 U/ml)/EDTA (100 $\mu\text{mol/l}$ )	62 $\pm$ 5
9	xanthine (30 $\mu\text{mol/l}$ )/XO (0.02 U/ml)/deferoxamine (100 $\mu\text{mol/l}$ )	59 $\pm$ 4
10	$^{\cdot}\text{O}_2^-/\text{OH}$ xanthine (30 $\mu\text{mol/l}$ )/XO (0.02 U/ml)/DTPA (100 $\mu\text{mol/l}$ )/Fe <sup>2+</sup> (100 $\mu\text{mol/l}$ )	59 $\pm$ 4
11	xanthine (30 $\mu\text{mol/l}$ )/XO (0.02 U/ml)/EDTA (100 $\mu\text{mol/l}$ )/Fe <sup>3+</sup> (100 $\mu\text{mol/l}$ )	59 $\pm$ 4
12	$^{\cdot}\text{OH}$ FeSO <sub>4</sub> (100 $\mu\text{mol/l}$ )/H <sub>2</sub> O <sub>2</sub> (180 $\mu\text{mol/l}$ )/DTPA (100 $\mu\text{mol/l}$ )	16 $\pm$ 1
13	FeSO <sub>4</sub> (100 $\mu\text{mol/l}$ ) <sup>a)</sup>	17 $\pm$ 1
14	FeSO <sub>4</sub> (100 $\mu\text{mol/l}$ )/DTPA (100 $\mu\text{mol/l}$ ) <sup>a)</sup>	2 $\pm$ 0
15	H <sub>2</sub> O <sub>2</sub> H <sub>2</sub> O <sub>2</sub> (100 $\mu\text{mol/l}$ )	0
16	ROOH tert-butyl hydroperoxide (100 $\mu\text{mol/l}$ )	0
17	tert-butyl hydroperoxide (100 $\mu\text{mol/l}$ )	0
18	RO $^{\cdot}$ tert-butyl hydroperoxide (100 $\mu\text{mol/l}$ )/dipyridyl-Fe <sup>2+</sup> (100 $\mu\text{mol/l}$ )	14 $\pm$ 1
19	ROO $^{\cdot}$ cumene hydroperoxide (100 $\mu\text{mol/l}$ )/dipyridyl-Fe <sup>2+</sup> (100 $\mu\text{mol/l}$ )	12 $\pm$ 1
20	ROO $^{\cdot}$ cumene hydroperoxide (100 $\mu\text{mol/l}$ )/DTPA (100 $\mu\text{mol/l}$ )/Fe <sup>3+</sup> (100 $\mu\text{mol/l}$ )	17 $\pm$ 1

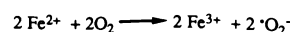
Incubation was performed for 30 min at 37 °C in phosphate buffer. Incubation mixtures contained 7 mU/ml G6-PDH and indicated concentrations of active oxygen generating systems (% inhibition is expressed as mean  $\pm$  SD; n  $\geq$  3). Controls with xanthine, XO or the chelators alone did not impair the enzyme activity. <sup>a)</sup> Control.

tion with methylene blue completely inactivated G6-PDH, experiments performed under anaerobic conditions (argon) led to similar results demonstrating that enzyme inactivation was caused by the triplet state of methylene blue, i.e. in a  $^1\text{O}_2$ -independent fashion. Since photosensitizers may also interact directly with the substrate resulting in hydrogen atom or electron transfer to produce radicals [59], reactions that are not  $^1\text{O}_2$  mediated are likely. On the other hand, preparation of  $^1\text{O}_2$  from naphthalene endoperoxides (Table 3, entry 6) [61] seems to be a clean source, since no side reactions have yet been reported.  $^1\text{O}_2$  generated by the thermal decomposition of the water-soluble NDPO<sub>2</sub> was completely ineffective. This result clearly rules out any effectiveness of  $^1\text{O}_2$  towards G6-PDH.

### 3.3.2. Hydrogen and alkyl peroxides, hydroxyl, alkoxy and alkyperoxy radicals

Beside electronically excited molecular oxygen, reactive species include H<sub>2</sub>O<sub>2</sub> and  $^{\cdot}\text{O}_2^-$ . Furthermore, there are stronger oxidants derived from these oxygen intermediates, such as  $^{\cdot}\text{OH}$  radicals [42, 45, 62]. Results from our work do not point to deleterious effects of H<sub>2</sub>O<sub>2</sub>,  $^{\cdot}\text{OH}$ , ROOH, RO $^{\cdot}$  and ROO $^{\cdot}$  towards G6-PDH (Table 3, entries 12–20). The enzyme activity was only slightly inhibited by  $^{\cdot}\text{OH}$  generated by the Fenton reaction (Table 3, entry 12). Surprisingly, Fe<sup>2+</sup> alone showed the same effectiveness. This observation can be explained by oxidation of Fe<sup>2+</sup> to Fe<sup>3+</sup> and  $^{\cdot}\text{O}_2^-$ , dismutation of the latter would yield H<sub>2</sub>O<sub>2</sub>, which in turn is oxidized by Fe<sup>2+</sup> to finally generate  $^{\cdot}\text{OH}$  (Schemes 3–5) [63].

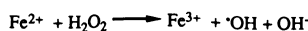
Addition of the chelator DTPA almost completely cancelled this inhibition, since ferrous DTPA is relatively stable to oxidation [63]. The effects of alkyperoxy and



Scheme 3



Scheme 4



Scheme 5

alkoxy radicals on G6-PDH were similar to those of  $^{\cdot}\text{OH}$ . Incubation with H<sub>2</sub>O<sub>2</sub> and alkylhydroperoxides did not produce any alteration of the enzyme activity.

### 3.3.3. Superoxide anion radical

$^{\cdot}\text{O}_2^-$  (Table 3, entries 7–9), was the most potent inactivating oxygen derivative towards G6-PDH at concentrations which caused the same reduction rate of NBT as did 55 % of a 10  $\mu\text{mol/l}$  diethanol solution (NBT reduction by diethanol could be inhibited by SOD to this extent [24], indicating 55 % of the reduction to be caused by  $^{\cdot}\text{O}_2^-$ ). G6-PDH activity was decreased to 60 %. Similar effects were achieved by addition of Fe<sup>2+</sup>-DTPA or Fe<sup>3+</sup>-EDTA (Table 3, entries 10, 11) to the xanthine/XO-system in order to produce  $^{\cdot}\text{OH}$  as a secondary product. In this case it cannot be differentiated between the effects of  $^{\cdot}\text{OH}$  and  $^{\cdot}\text{O}_2^-$ , but because  $^{\cdot}\text{OH}$  generated by the Fenton reaction (Table 3, entry 12) was only a modest inhibitor, these findings rather suggest that in this system  $^{\cdot}\text{O}_2^-$  is the inactivating species exerting the deleterious effects independently of its iron-catalyzed interaction with H<sub>2</sub>O<sub>2</sub> [64].

Our experiments with oxygen derivatives, produced independently of the drug, demonstrate that G6-PDH is inert to H<sub>2</sub>O<sub>2</sub> and  $^1\text{O}_2$ , but markedly susceptible to  $^{\cdot}\text{O}_2^-$ . Hydroxyl radical leads to a modest decrease of G6-PDH activity. In conclusion, if diethanol acts through formation of active oxygen species,  $^{\cdot}\text{O}_2^-$  is the potent species towards G6-PDH.

## 4. References

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