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Formation of Active Oxygen Species by Dithranol, III[1]

Dithranol, Active Oxygen Species and Lipid Peroxidation in vivo

Klaus Müller[a], Wolfgang Wiegrebe[a)*)**)] and Maged Younes[b]

[a] Institute of Pharmacy, University, P.O. Box 397, D-8400 Regensburg
[b] Institute of Toxicology, Medical University, Ratzeburger Allee 160, D-2400 Lübeck
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At pH 7.8 the dithranol anion **1a** is oxidized in the dark to yield the bianthrone **3** besides dithranol brown, whilst O_2 is reduced to $O_2^{\cdot-}$ and H_2O_2. $O_2^{\cdot-}$ was detected by the reduction of nitroblue-tetrazolium (NBT, **4**). The formation of the formazan **5** was inhibited by superoxide dismutase (SOD). Exclusion of O_2 and addition of SOD reveal a direct electron transfer from **1a** to **4** and to cytochrome c which was not affected in the presence of SOD. – *In vivo* experiments indicate a low rate of lipid peroxidation which was determined on the basis of ethane exhalation.

Bildung aktiver Sauerstoffspezies durch Dithranol, 3. Mittl.[1]: Dithranol, aktive Sauerstoffspezies und Lipidperoxidation in vivo

Dithranolanion **(1a)** wird bei pH 7.8 unter Lichtausschluß zum Bianthron **3** und zu Dithranolbraun oxidiert, während O_2 zu $O_2^{\cdot-}$ und H_2O_2 reduziert wird. $O_2^{\cdot-}$ wurde durch Reduktion von Nitrotetrazoliumblau (NBT, **4**) nachgewiesen, die Bildung des Formazans **5** wurde durch Superoxid Dismutase (SOD) gehemmt. O_2-Ausschluß und Zugabe von SOD weisen einen direkten Elektronentransfer von **1a** auf **4** bzw. Cytochrom c nach, der in Gegenwart von SOD nicht beeinträchtigt wurde. – *In vivo*-Experimente weisen auf eine geringfügige Lipidperoxidation hin, die über die Ethanausatmung gemessen wurde.

According to *Gollnick*[2] photooxygenations are classified as type I- and type II-processes. Type I processes are characterized as reactions of the sensitizer in the excited state either with ground state oxygen ($^3O_2$) or with the reactant *via* electron transfer, whilst type II reactions produce oxygen in an excited state ($^1O_2$)[3]. We have shown that the antipsoriatic compound dithranol **(1)** produces $^1O_2$, the main product of this type II process being chrysazin **(2)**[1,4]. In the dark, however, only the bianthrone **3** and the so called dithranol brown arise. In both cases, **1**-anion is attacked by O_2. *Mustakallio* et al.[5] have found a radical by ESR-spectroscopy, which was identified as 1,8-dihydroxy-9-anthrone-10-yl-radical **(1b)** by *Davies*[6]. One electron oxidation of **1a** should lead, therefore, to superoxide-radical-anion $O_2^{\cdot-}$ by electron transfer from **1a** to $^3O_2$ (scheme 1).

**) Dedicated to Prof. Dr. Dr. h.c. K. E. Schulte, Münster, on the occasion of his 75th anniversary.

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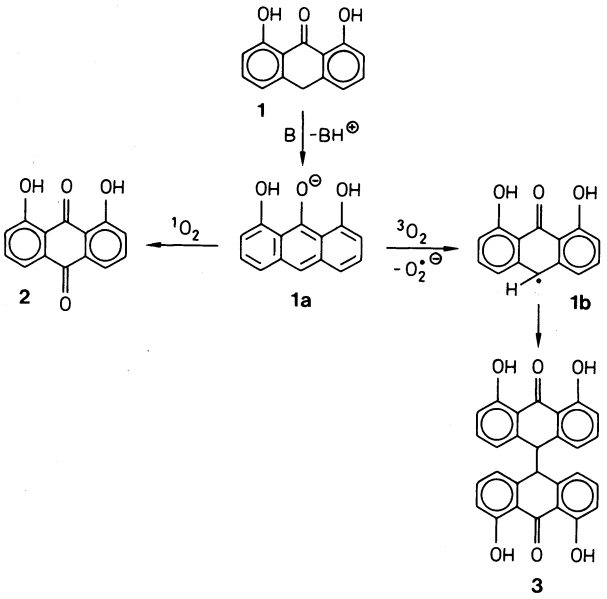
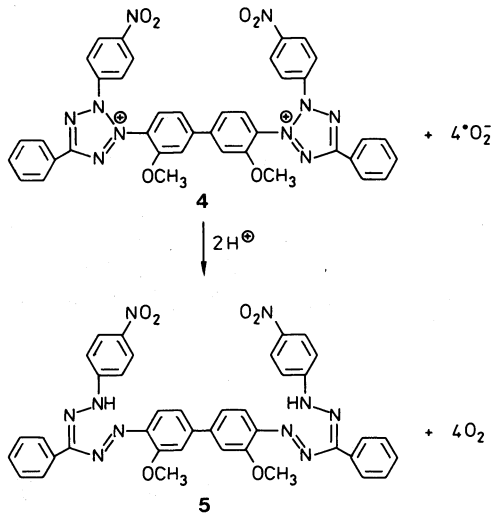


Amoung various tests for $O_2^{\cdot -}$[7], NBT **(4)**[8,9] turned out to be useful for our experiments because its reduced form **5** (λ max = 560 nm[10]) does not interfere with the absorption of **1a** in aqueous buffer[11] (pH = 7.8: λ max = 384 nm).



Results

After a steep rise at the beginning, **1** reduces **4** to the corresponding diformazan **5** steadily (fig. 1). Therfore, the reduction rate was determined at 560 nm after this initial

phase. As it is shown in fig. 2, the rate of formation of **5** is a linear function of the concentration of **1**.

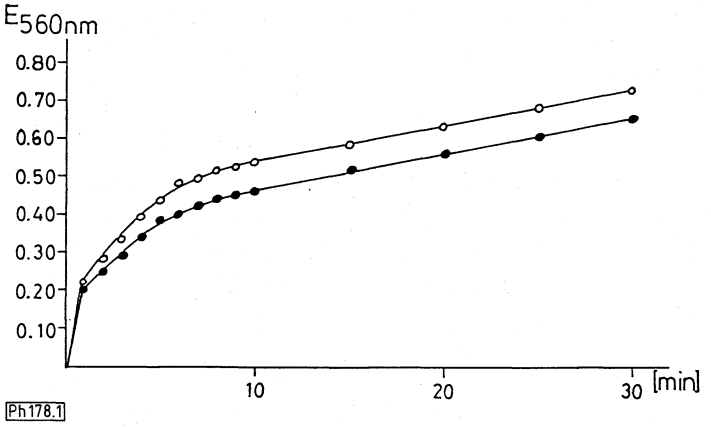


Fig. 1: Time course of the reduction of NBT in the presence of dithranol ● O_2; ○ Ar

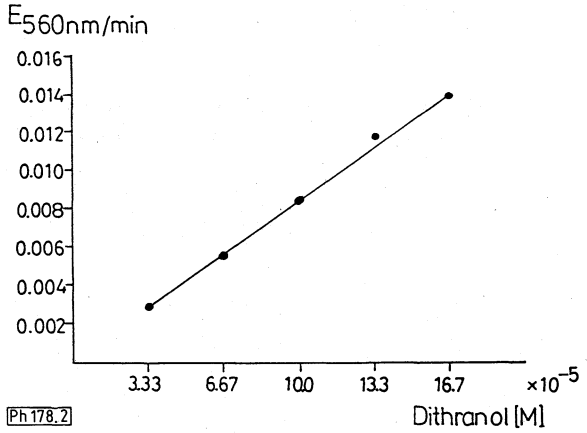


Fig. 2: Rate of reduction of NBT as a function of dithranol-concentration

Control experiments under N_2 and Ar, however, show that **4** is reduced not only by $O_2^{\cdot-}$, but also by a direct electron transfer from **1a** to **4** under anaerobic conditions. As to specify the identification of $O_2^{\cdot-}$ the experiments were repeated in the presence of SOD. This enzyme accelerates the conversion of $O_2^{\cdot-}$ (eq. 4, see below) at pH 7.4 by a factor 10^4 as compared to the spontaneous dismutation[12]. – We standardized the concentration of **1** so that the increase of absorption at 560 nm became 0.012/min (fig. 2). Fig. 3 shows that the reduction of **4** to **5** is lowered down by SOD to 55 %, indicating that this reduction is provoked by $O_2^{\cdot-}$ to this extent. This inhibition does not depend on whether the enzyme is added before or during the reaction (fig. 4).

Further control experiments were run by adding SOD to NBT and **1** under N_2 and testing the influence of desactived SOD (100°, 5 min) on the system **1**/NBT/O_2: in both cases the curves correspond to fig. 1, indicating that there are no inhibitory effects ei-

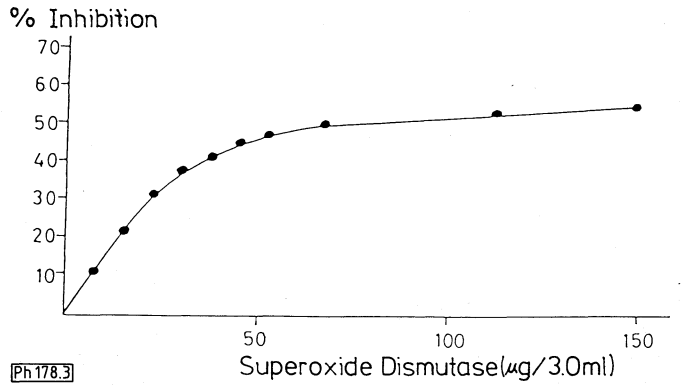


Fig. 3: Inhibition of NBT reduction by SOD in the system dithranol/O_2

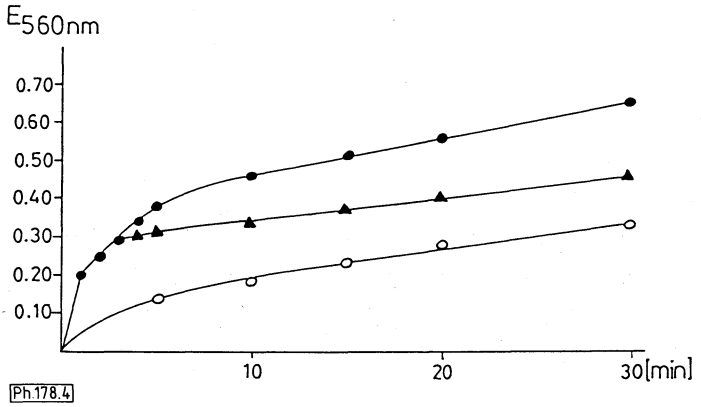


Fig. 4: Reduction of NBT in the presence of dithranol ● without SOD; ▲ SOD (75 µg) was added after 3 min; ○ SOD (75 µg) was added before the reaction

ther by SOD under anaerobic conditions or by the desactivated enzyme. Therefore, an interference of traces of oxygen is excluded which could influence the formation of **5** catalytically *via* the recyclic system of equations 2 and 3 with **1a** as the ultimate electron source:

$$4\ \mathbf{1a} + \mathrm{NBT} \longrightarrow 4\ \mathbf{1b} + \mathbf{5} \qquad \text{(eq. 1)}$$

$$\mathbf{1a} + O_2 \longrightarrow \mathbf{1b} + O_2^{\bullet -} \qquad \text{(eq. 2)}$$

$$4\ O_2^{\bullet -} + \mathrm{NBT} + 2H^{\oplus} \longrightarrow 5 + 4\ O_2 \qquad \text{(eq. 3)}$$

$$O_2^{\bullet -} + O_2^{\bullet -} + 2H^{\oplus} \longrightarrow O_2 + H_2O_2 \qquad \text{(eq. 4)}$$

Under anaerobic conditions NBT is reduced only according to eq. 1. Obviously (fig. 1) this direct electron transfer is favoured over the $O_2^{\bullet -}$ mediated formation of **5.**

As an additional test for $O_2^{\bullet -}$ we used the reduction of cytochrome c[13]; this reaction is very much faster than the reduction of NBT: after 15 min the reduction was complete and it was scarcely influenced by SOD. This result corresponds to those of other groups, who – contrary to NBT – did not observe an inhibition of cytochrome c

reduction by SOD, testing the $O_2^{\cdot -}$-producing systems phenazine methosulfate/NADH[14, 15] or flavine nucleotid/NADH[16], respectively.

The type II product chrysazin **(2)** does not reduce NBT, **3** shows a very weak effect.

Because $O_2^{\cdot -}$ dismutates rapidly, the formation of H_2O_2 in the system **1**/O_2 was conceivable, at least as a secondary product. *Unna*[17] has already found this active oxygen species (KI/starch; benzidine-reaction) when he examined the hypothesis that **1** (Cignolin) practises its efficacy *via* H_2O_2. We identified H_2O_2 by a modified Fe(III)-thiocyanate test (λ max = 480 nm)[18].

The formation of active oxygen species and of the dithranol radical **1b**[5, 6] points towards lipid peroxidation by **1**. **1b** is known to catalyze the UV-induced peroxidation of epidermis lipids[19]. $^1O_2$ converts polyunsaturated fatty acids (PUFAHs) to lipidperoxides *via* a non-radical mechanism[20], in a model experiment we have shown that hydroperoxides of unsaturated fatty acids are formed by **1a**, acting as a photosensitizer[4]. On the contrary, H_2O_2 does not provoke lipid peroxidation in biomembranes[21] and $O_2^{\cdot -}$ does not react with PUFAHs[22]; according to *Haber-Weiß*[23] or by a modified *Haber-Weiß*-reaction[24–26], however, the very reactive $^{\cdot}OH$ is formed so that H_2O_2 and $O_2^{\cdot -}$ can lead to lipid peroxidation indirectly. – The formation of alkanes, mainly ethane and pentane, is the most reliable index for lipid peroxidation *in vivo* and *in vitro*[27]. Ethane is formed from ω-3-unsaturated fatty acids[28]; the quantity of ethane corresponds to the degree of peroxidation, the determination of exhaled alkanes is generally used for quantifying the lipid peroxidation *in vivo*[29–32]. We used the device developed by *Siegers* et al.[30] for CCl_4-exposition (Table 1):

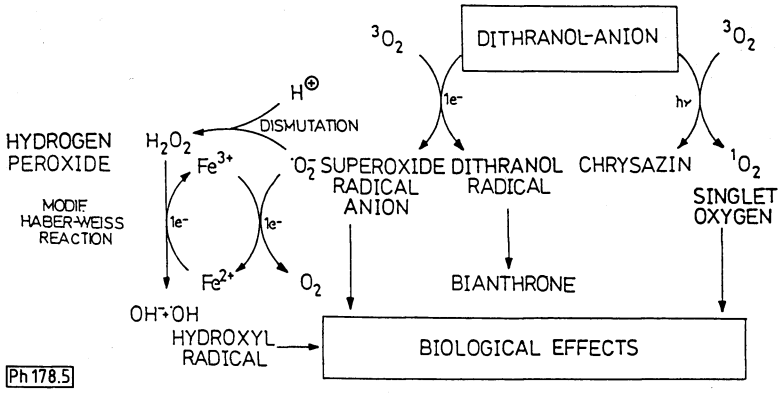
Table 1: *Ethane exhaled* (nmol/kg body weight) *after cutaneous application of 10 ml/kg body weight. Results are expressed as means and their standard errors of three experiments.*

t [min]	Dithranol, 2 % in Olive oil	Olive oil (controls)
30	7.1 ± 0.6	2.9 ± 1.0
60	8.2 ± 0.6	3.6 ± 0.6
90	9.3 ± 0.6	5.8 ± 1.0
120	10.2 ± 0.5	5.2 ± 1.0

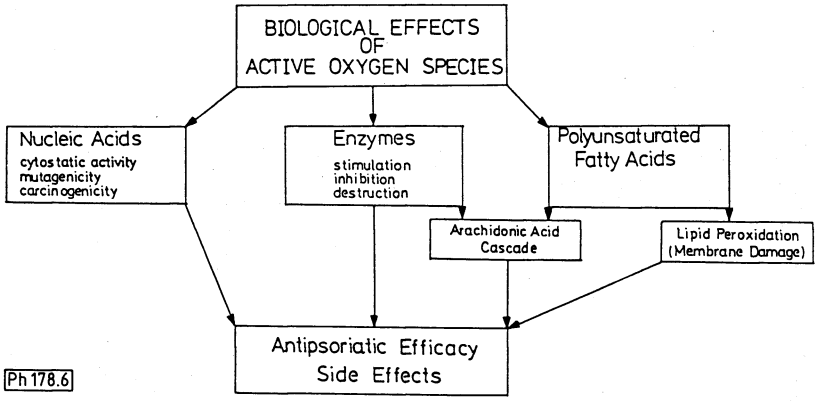
After cutaneous application of **1** in olive-oil the exhaled ethane is twice as high as in the control animals, nevertheless the values are far below those of substances well known for their efficacy in lipid peroxidation (under identical conditions CCl_4 leads to 39.6 ± 4.1 nmol C_2H_6 after 2 h[32]).

Discussion

Fig. 5 gives a comprehensive view of **1**-dependent oxygen activation. Because $^{\cdot}OH$ can result from $^1O_2$, $O_2^{\cdot -}$ and H_2O_2 *via* secondary reactions[23–26], a lot of biological effects are conceivable[33].



The importance of active oxygen species for antipsoriatic efficacy is significantly demonstrated: $^1O_2$ and $O_2^{\cdot -}$ are generated by psoralenes[34]; coal tar which is still used as an antipsoriatic remedy contains photosensitizers[3]. Considering the targets of the active oxygen species mentioned above, the various antipsoriatic effects of **1**[35, 36] can be summarized as in fig. 6.



Side effects by lipid peroxidation are unlikely in the modern "minutes-therapy"[37], when **1** is applied in high concentrations only for 20 min; lipid peroxidation can not be excluded *a priori* for the combined regimen **1** + UV-light (*Ingram*), because an increased formation of $^1O_2$ is conceivable (**1a** is a photosensitizer[4]). Moreover, lipid peroxidation is stimulated by UV light[18, 38, 39] and by visible light in the presence of photosensitizers[40].

Experimental Part

UV-spectroscopy: Shimadzu 210; Uvikon 810 (Kontron) with Uvikon recorder 21 and thermostatized cell holder; pathlenght 10 mm, vol. 3.0 ml, temp. 25°. – *Gas chromatography*: Fractovap 4200, Carlo Erba. –

Chemicals: Nitrobluetetrazolium (NBT, **4**): Merck; Superoxide dismutase (EC 1.15.1.1.) type I from bovine blood: Sigma; cytochrome c type III from horse heart: Sigma; 1.8-dihydroxy-9.10-anthraquinone (chrysazin, **2**): Synochem; 1.8-dihydroxy-9-anthrone (dithranol, **1**) and 1.1′.8.8′-tetrahydroxy-10.10′-bisanthrone: lit.[41], purification by column chromatography (SiO_2/CH_2Cl_2).

Stock solutions:

1: 4.52 mg (0.02 mmol) were dissolved in 10.00 ml EtOH and kept under N_2 in the dark. – NBT: 40.9 mg (0.05 mmol) in 10.00 ml EtOH, N_2, dark. – Buffer: 11.41 g $K_2HPO_4 \cdot 3H_2O$ (0.05 mol) and 0.68 g KH_2PO_4 (0.005 mol) in ca. 800 ml bidist. water were adjusted to pH 7.8 with 0.1 N-NaOH and filled up to 1000.0 ml with bidist. water. – Superoxide dismutase: 3.75 mg enzyme (2800 units/mg protein) in 5.00 ml buffer. – Cytochrome c: 18.6 mg (1.5 μmol) in 5.00 ml EtOH/buffer (1:1-vol.).

Identification of $O_2^{\cdot -}$

NBT-conc.: $1.67 \cdot 10^{-4}$ M (100 μl stock solution). The reduction product **5** is nearly insoluble in water; at this conc. precipitation of **5** is avoided[10]. **1**-conc.: $3.33 \cdot 10^{-5}$ M – $1.67 \cdot 10^{-4}$ M (50–250 μl stock solution). The mixture of NBT and **1** was made up to 3.00 ml with buffer and saturated with O_2, N_2 or Ar, respectively. In the case of SOD experiments, 7.5–225 μg (10–300 μl stock solution) were added before filling up. The velocity of the formation of **5** was determined at 560 nm. – The cytochrome c reduction was followed at 550 nm with a cytochrome conc. $2 \cdot 10^{-5}$ (200 μl stock solution).

Identification of H_2O_2

22.6 mg (0.1 mmol) **1** in 10 ml acetone saturated with O_2, and 2 drops saturated $Ba(OH)_2$ were shaken at r.t. for 2 h. After addition of 5 drops 5 N-H_2SO_4 the mixture was filtered, 0.2 ml 10 mM-$(NH_4)_2Fe(SO_4)_2$ and 0.1 ml 2.5 M-KSCN were added to 3.0 ml filtrate. The mixture became red, λ max = 480 nm.

In vivo test of lipid peroxidation

Male NMRI mice were shaved on the back. Approximately 0.3–0.4 ml of 200 mg **1** in 10 ml olive-oil (~ 200 mg/kg) were applied over an area of 2 × 2 cm. Immediately after application 4 mice were transferred to a gas-tight closed system[30] and the exhaled ethane was measured: from 3 parallel experiments 7 ml gas samples were taken *via* a rubber septum in 30 min intervals using a gas-tight syringe. The gas samples were injected into a sample loop of the gas chromatograph. – column: Porapack Q (80/100 mesh), 3 m, temp. 90°. – detector: FID, temp. 170°. – N_2-flow rate: 60 ml/min. – H_2-flow rate: 20 ml/min. – Flow rate of synthetic air: 200 ml/min. Standardization by 0.25 ppm ethane in N_2. – Retention time for C_2H_6 under these conditions: 75 sec[27, 32].

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