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HYDROXYL RADICAL FORMATION BY DITHRANOL

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Abstract—Ethene formation from 2-keto-thiomethyl-butyric acid (KMBA) was used to measure hydroxyl radical generation from the antipsoriatic drug dithranol in phosphate buffer pH 7.8. Because the singlet oxygen ($^1O_2$) sensitizer, rose bengale, showed enlarged production of ethene when irradiated in the presence of KMBA, experiments were performed in the dark in order to avoid $^1O_2$ production by dithranol. KMBA was converted to ethene by dithranol under aerobic conditions, whereas ethene formation was negligible in the absence of oxygen. Addition of catalytic amounts of $FeCl_3$ resulted in increased ethene formation, indicating the catalysis of a superoxide-driven Fenton-reaction. $O_2^{\cdot}$- and $^{\cdot}OH$-scavengers such as rutin, catechin, dimethyl sulfoxide, mannitol, ethanol, sodium salicylate and propyl gallate as well as catalase and superoxide dismutase inhibited ethene formation.

Although dithranol (1,8-dihydroxy-9-anthrone, **1a** in Fig. 1) has been used for the treatment of psoriasis for seventy years, the molecular mechanism by which it is effective against this skin disease has yet to be elucidated. In our studies [1, 2] we have shown the formation of active oxygen species by dithranol (Fig. 1). In the presence of light the trihydroxyanthracene anion (**1b** in Fig. 1) is a singlet oxygen ($^1O_2$) sensitizer [1], whereas in the dark the electron transfer from the anion to molecular oxygen leads to the formation of superoxide radical anion ($O_2^{\cdot}$) [2]. These reactive oxygen species and the dithranol free radical (**2** in Fig. 1) formed under physiological conditions [3] may play an important role in the mode of action and the induction of side effects of this drug. Moreover, bacterial reduction of the laxative agent danthron (1,8-dihydroxyanthraquinone, **3** in Fig. 1) in the colon leads to dithranol which is considered to be the active principle [4]. Because of genotoxic and carcinogenic effects observed in laboratory animals danthron was drawn off the market. These effects could be caused by active oxygen species derived from dithranol or by enzyme-catalyzed redox cycling [5] of danthron, which can be biologically reduced to an autoxidizable semiquinone radical yielding $O_2^{\cdot}$ [6].

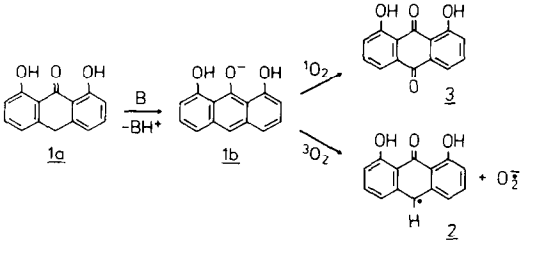

Fig. 1. Scheme of dithranol autoxidation.

However, the mechanism by which $O_2^{\cdot}$ might be deleterious to the cell remains unclear. Because $O_2^{\cdot}$ itself is poorly reactive in aqueous solution [7], it has been suggested that the hydroxyl radical ($^{\cdot}OH$) derived from $O_2^{\cdot}$ is the real toxic species [8–12]. There are several assays for the detection of $^{\cdot}OH$ [13]. For example, spin trapping by 5,5-dimethyl-1-pyrroline-1-oxide (DMPO) leads to DMPO-OH that can be observed by ESR-spectroscopy [13]. Unfortunately, nitrones form adducts with both $^{\cdot}OH$ and $O_2^{\cdot}$. The adduct formed with $O_2^{\cdot}$ (DMPO-OOH) decomposes into DMPO-OH [14], so that in the presence of $O_2^{\cdot}$ it is not possible to assign the ESR-spectrum unequivocally to $^{\cdot}OH$. DMPO-OOH has been detected recently with dithranol [15], indicating the formation of $O_2^{\cdot}$. The gas chromatographic measurement of ethene formation from thioether aldehydes and ketones, such as methional [16, 17] or 2-keto-4-thiomethyl-butyric acid (KMBA) [18], is a commonly used assay to determine $^{\cdot}OH$. Because other oxy radicals are also capable of producing ethene from methional [19], we also examined the influence of a number of $^{\cdot}OH$-scavengers on ethene formation from KMBA.

MATERIALS AND METHODS

Chemicals. 1,8-Dihydroxy-9-anthrone (dithranol, anthralin) was prepared by reduction of danthron (Janssen, Nettetal, F.R.G.) [20] and purified by column chromatography (SiO_2/CH_2Cl_2). Mannitol, propyl gallate, rutin, catechin, 2-keto-thiomethyl-butyric acid (KMBA) and superoxide dismutase

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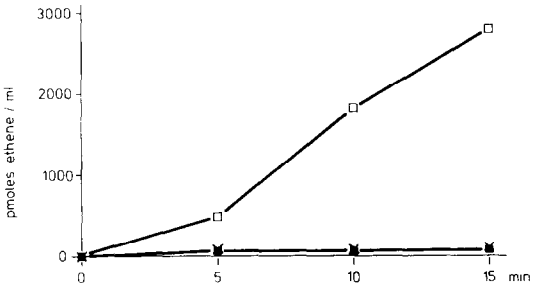

Fig. 2. Time course of ethene formation from KMBA by rose bengale (RB, 10 μM) after aerobic incubation: □, with RB/with light; ■, with RB/without light; ×, without RB/with light.

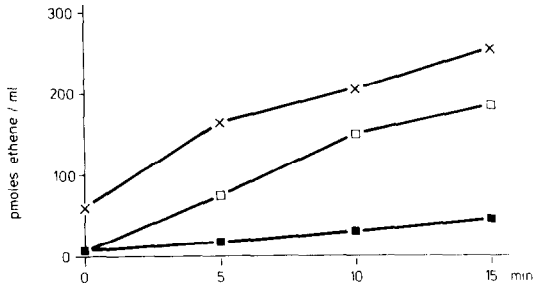

Fig. 3. Time course of ethene formation from KMBA by dithranol (100 μM) incubated in the absence of light: □, aerobic system; ■, anaerobic system (helium); ×, positive control without dithranol: H_2O_2 (70 μM) + $FeSO_4$ (2.5 μM in 0.1 mM EDTA).

from bovine erythrocytes (EC 1.15.1.1) were obtained from Sigma (München, F.R.G.). Sodium salicylate, $FeCl_3 \cdot 6H_2O$, ethanol and all other chemicals were from Merck (Darmstadt, F.R.G.); beef liver catalase (EC 1.11.1.6) was from Boehringer (Mannheim, F.R.G.), and rose bengale from Aldrich (Steinheim, F.R.G.).

Ethene formation. Experiments were carried out in 25 ml Erlenmeyer flasks (total volume 40 ml) equipped with a screw cap and a silicon septum as already described [21]. The standard assay mixture (2 ml) consisted of dithranol, 2 mM KMBA and 66 mM Soerensen phosphate buffer pH 7.8 and was aerobically incubated at 37° under shaking. Control assays were performed under both aerobic and anaerobic (helium) conditions. In some experiments $FeCl_3$-EDTA or scavengers were added as indicated. In the case of photosensitized oxidations the incubation mixture was irradiated with a Philips Photocrescenta lamp (150 W). Otherwise the reaction vessels were wrapped up with aluminium foils. The reaction was initiated by injection of KMBA via the septum. Gas samples removed from the head space of the flask were analyzed for ethene by gas chromatography as previously described [21, 22]. All experiments were run at least in triplicate. Ethene formation from KMBA only (max. 1 pmol/ml/min) has been subtracted.

RESULTS

In order to determine whether $^1O_2$ reacts directly with KMBA to form ethene a control experiment was carried out with the $^1O_2$-sensitizer rose bengale. Figure 2 shows that in the presence of light KMBA is converted to ethene by rose bengale to a great extent, indicating that KMBA is reactive with $^1O_2$, whereas in the dark no increased amounts of ethene arise from this system. Because the dithranol anion has about 25% of the efficiency of rose bengale as $^1O_2$ sensitizer [23], the following experiments were performed under light protection. The reaction of ferrous sulfate/EDTA with H_2O_2 (Fenton's reagent) was used as a standard system which generates $^{\cdot}OH$ [24]. Figure 3 illustrates that with 100 μM dithranol ethene formation from KMBA increases almost linearly with time. It also reveals that in the absence of oxygen (under helium) the generation of ethene is negligible. As demonstrated in Fig. 4, the rate of ethene formation is a linear function of the dithranol concentration (20–200 μM). Higher concentrations of dithranol do not yield high amounts of ethene. This may be related to the poor aqueous solubility of dithranol.

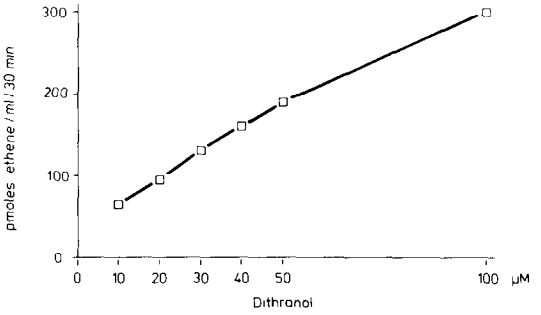

Fig. 4. Rate of ethene formation as a function of the dithranol concentration incubated in the absence of light.

Table 1. Effects of inhibitors and stimulators of ethene formation from KMBA after 30 min incubation of dithranol in the absence of light; mean values ± SD (N ≥ 3)

	% Ethene
Control (100 μM Dithranol)	100
+ Rutin (5 μM)	35 ± 4
+ Catechin (5 μM)	47 ± 10
+ Dimethyl-sulfoxide (5 mM)	66 ± 3
+ Mannitol (5 mM)	82 ± 3
+ Ethanol (5 mM)	87 ± 2
+ Sodium salicylate (5 mM)	49 ± 3
+ Propyl gallate (0.05 mM)	9 ± 2
Catalase (300 U/ml)	6 ± 2
Superoxide dismutase (175 U/ml)	86 ± 4
Catalase (300 U/ml) + Superoxide dismutase (175 U/ml)	0
$FeCl_3$ (0.5 μM)	169 ± 11

Addition of catalytic amounts of Fe^{3+}-EDTA are sufficient for the enhancement of the ethene for-

mation (Table 1). This effect was not increased with higher Fe^{3+}-concentrations (data not shown). Likewise irradiation of the dithranol containing reaction mixture leads to enhanced ethene production due to the sensitizing properties of dithranol (data not shown).

Scavengers such as catechin, rutin, dimethyl sulfoxide, mannitol, ethanol, sodium salicylate, propyl gallate, and the enzymes catalase and superoxide dismutase (SOD) were all able to inhibit ethene formation to different extents (Table 1).

DISCUSSION

Because KMBA is susceptible to direct oxidation by $^1O_2$, care must be taken with compounds that act as $^1O_2$ sensitizers. As all experiments were performed in the dark, this active oxygen species cannot be the cause of ethene production observed during dithranol autoxidation. Furthermore, neither $O_2^{\cdot}$ nor H_2O_2 reacts directly with KMBA [25] to cause liberation of ethene.

On the other hand, hydroxyl radical-trapping agents (mannitol, ethanol, dimethyl sulfoxide, sodium salicylate) inhibited ethene formation. Their relatively weak inhibitory potency is in agreement with results of previous studies [25]; but it has also to be considered that because of their competition with a relatively high concentration of KMBA (2 mM) high concentrations of scavengers (5 mM) are needed. Only propyl gallate was an effective inhibitor by the addition of small amounts (0.05 mM), just as recently reported in other systems [26]. That the superoxide scavengers rutin and catechin [27] inhibit generation of ethene indicates participation of $O_2^{\cdot}$ as precursor of $^{\cdot}OH$. The slight inhibition by SOD agrees with this suggestion. On the other hand, ethene formation is almost completely inhibited by catalase indicating that H_2O_2 must also be involved. As control experiments with H_2O_2 alone do not result in enhanced ethene production (data not given) H_2O_2 is presumably active by interaction with $O_2^{\cdot}$ leading to $^{\cdot}OH$ as secondary reaction product. This is further supported by the strong inhibitory effect of catalase plus SOD.

These results are compatible with hydroxyl radical formation via the iron-catalyzed Haber–Weiss-reaction [28]

$$Fe^{3+} + O_2^{\cdot} \rightarrow Fe^{2+} + O_2 \quad (1)$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + {}^{\cdot}OH \quad (2)$$

That ethene is produced without added iron ions can be explained by trace metal ions present as contaminants which we were unable to remove from all reagents including the dithranol preparation used. This mechanism is further supported by the increased ethene production observed after addition of catalytic amounts of $FeCl_3$. Metal ion chelators were not used in order to prove this further because they are not highly specific.

In view of the relative chemical inertness of $O_2^{\cdot}$ [29], the therapeutic efficacy and the skin-irritating properties of dithranol may therefore depend on the efficiency of its conversion to $^{\cdot}OH$. Thus, the discovery of dithranol-induced $^{\cdot}OH$ formation could lead to a better understanding of the inflammatory process provoked by dithranol. Whether this side effect can be diminished or prevented in therapy by scavengers (e.g. rutin) is dependent on their possible interference with the therapeutic effect. Although $^{\cdot}OH$ is highly reactive, the biological effects after topical application of dithranol would be restricted to the skin. Indeed, systemic toxicity of dithranol after percutaneous absorption of metabolites is negligible in man [30].

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