Diesel Soot – Catalyzed Production of Reactive Oxygen Species: Cooperative Effects with Bisulfite

S. C. Hippeli and E. F. Elstner

Institut für Botanik und Mikrobiologie, Biochemisches Labor, Technische Universität München, Arcisstraße 21, D-8000 München 2, Bundesrepublik Deutschland

Z. Naturforsch. **44c**, 514–523 (1989); received December 1, 1988

Dedicated to Professor Achim Trebst on the occasion of his 60th birthday

Diesel Soot Particles, Reactive Oxygen Species, Chemiluminescence, Sulfite, Air Pollutants

The fragmentation of KMB (4-(methylthio)-2-oxobutyric acid) as an indicator reaction for the production of strong oxidants is catalyzed by diesel soot particles in the light. This reaction operates also in the dark and can be strongly stimulated by the addition of sulfite. The cooperative activity of diesel soot particles (DP) and sulfite also bleaches the carotenoid crocin. This molecule represents a model for both the groups of plant pigments as well as polyene structures embedded as flexible backbones in cellular membranes. Enhanced chemiluminescence generated by linolenic acid in the presence of either *tert*-butylhydroperoxide or hydrogenperoxide is detected in the presence of both DP and HSO_3^-. Luminescence originates from singlet oxygen or chemically excited molecules such as triplet ketones and is thus a valuable indicator for peroxidation of lipids involved in membrane damage. All reactions were inhibited by SOD (superoxide dismutase) whereas catalase had no effect.

Introduction

Several well known compounds such as NO_x and SO_2 play an outstanding role in air pollution. Diesel exhaust is another airborne pollutant with growing importance.

From the environmental standpoint, diesel engines have low hydrocarbon and carbon monoxide emissions. NO_x levels are comparable to those of gasoline-driven cars equipped with catalysts. Of special importance are the particulate emissions, however, which are 30 to 70 times higher than those from catalyst equipped spark ignition engines, arising to approximately 1 g per kilometer [1]. The soot particles are small in size (less than 0.5 μm), easily respirable, and have carbon cores with a very large surface area onto which a variety of organic compounds is adsorbed. 15–65% of the mass consists of extractable organic material such as polycyclic aromats, nitro aromats and quinones. Number and structures of these compounds depend on the type of engine and the mode of its operation and may thus be extremly different [1–3].

Several investigators demonstrated that organic extracts from DP are mutagenic in both bacterial and mammalian cell systems. Especially products from the reaction between diesel fuel and NO_2 seem to be highly cytotoxic and mutagenic [4, 5].

Chronic effects and tumor induction in the respiratory tracts of animals were observed after inhalation of unfiltered diesel engine emissions [6].

A heavy load of soot particles was found in long term dieselexposed lungs of animals, initiating tissue reactions including inflammations and fibrotic lesions. The massive accumulation of soot correlates very well with the deterioration of the alveolar lung clearence mechanism [7].

Mammalian cell mutagenesis bioassays capable of detecting gene mutations, DNA damage and chromosomal aberrations have confirmed the mutagenic activity of diesel exhaust [8].

The carcinogenic activity of polycyclic hydrocarbons obtained in diesel emission has been correlated with their ability to form free radicals [9].

Nachtman [10] demonstrated, that 1-nitropyrene (the main nitro-PAH in diesel exhaust) generates reactive oxygen radicals such as superoxide radical anions in the presence of rat lung microsomes.

Much is known on the other hand about SO_2 as one main air pollutant and its role in generating "acid rain".

Abbreviations: KMB, 4-(methylthio)-2-oxobutyric acid; DP, diesel soot particles; SOD, superoxide dismutase; LA, linolenic acid; *t*-BuOOH, tert-butyl hydroperoxide.

This work was supported by Bayerisches Staatsministerium für Landesentwicklung und Umweltfragen.

Reprint requests to Prof. Dr. E. F. Elstner.

Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/89/0500–0514 $ 01.30/0

During the nasal passages and in the lung, SO_2 is rapidly hydrated forming sulfite or bisulfite depending on the pH of the relevant media. At physiological pH, bisulfite, HSO_3^-, predominates. It was shown, that bisulfite incubated with prostaglandine synthase and arachidonic acid, 15-hydroperoxyarachidonic acid or H_2O_2 results in the formation of the reactive sulfur trioxide anion radical ($SO_3^{\cdot -}$). This free radical was detected with electron spin resonance technique and spin trapping [11]. Prostaglandine synthase is widespread in biological systems including the lungs.

In green plants chlorophyll destruction has been described as a cooxidative process during the reaction between linolenic acid hydroperoxide and bisulfite [12]. The authors suggested, that alkoxy radicals formed as metabolites might be responsible for the destruction of chlorophyll through free radical mechanisms.

Diesel soot particles on the other hand are able to catalyze ethylene release from KMB [13], which is a sensitive indicator for the production of strong oxidants such as the $OH^{\cdot}$ radical, singlet oxygen, transitionmetal-oxygen or peroxide complexes of the Fenton type [14].

In the present communication we report that

a. ethylene release from KMB mediated by diesel soot particles is stimulated synergistically by sulfite. This cooperative activity between soot particles and sulfite is further shown to

b. bleach crocin, a water soluble model substance representing carotenoids and polyene structures, and

c. initiate chemiluminescence of linolenic acid or in the presence of preformed peroxides such as *tert*-butylhydroperoxide or H_2O_2.

Materials and Methods

Materials

All chemicals were of the highest grade of purity available (Merck). KMB, SOD, linolenic acid and H_2O_2 (30%) were obtained from Sigma; Catalase was from Boehringer and *t*-BuOOH (70%) from Aldrich-Chemie. Crocin was isolated from commercially available saffron as earlier described [15]. Diesel soot particles were carefully removed with a small brush from the exhaust pipe of a fire brigade motor car (Magirus, Fire brigade Lohr/Main). All experiments with DP were done with the same batch of soot, in order to warrant standardized conditions for the experiments.

KMB-Fragmentation: The formation of ethylene from KMB was determined by gas chromatography as described previously [16].

The reaction mixture contained in 2 ml

phosphate buffer		0.1	M/pH 7.8
KMB		5	µmol
DP		400	µg
HSO_3^-		0.5	mM
SOD		100	U
catalase		100	U
H_2O	up to	2	ml

The reactions were done at 37 °C in the light (30 klux) or at the same temperature in the dark. Standard deviations represent $n = 6$. Crocin bleaching was followed at 440 nm in a UVIKON-810 spectrophotometer (Kontron, Eching). Crocin destruction was also determined by registrating the absorption spectra between 350 and 550 nm.

The reaction mixtures were either illuminated for 30 min (30 klux) or incubated in the dark in a water bath at 37 °C ($n = 6$).

The reaction mixture containd in 2 ml

phosphate buffer		0.1	M/pH 7.8
crocin		8.18	µM
DP		400	µg
HSO_3^-		0.5	mM
SOD		100	U
H_2O	up to	2	ml

Chemiluminescence was measured at 37 °C in a Berthold Biolumat LB 9500 T.

The reaction mixtures contained in 2 ml

phosphate buffer		0.1	M/pH 6.8 in reactions with *t*-BuOOH and H_2O_2 pH 6.3 in reactions with linolenic acid
linolenic acid, *t*-BuOOH or H_2O_2		11.3	µM
DP		1–400	µg
HSO_3^-		0.5	mM
SOD		100	U
catalase		100	U
H_2O	up to	2	ml

0.1 g linolenic acid was dissolved in 40 ml borate buffer, 0.2 M, pH 9.0, containing 0.1 ml Tween 20 and 0.26 ml 1 N NaOH.

Light emissions arising from the reactions were integrated over 60 seconds. The reactions were started by automatic injection of LA-, *t*-BuOOH- or H_2O_2 solutions *via* a Dispensor LB 95-C-300.

The data shown are differences between the values with and without LA, *t*-BuOOH or H_2O_2. Standard deviations were calculated for $n = 8$.

Results

A. KMB-Fragmentation

As shown in Fig. 1 the release of ethylene from KMB both in the light and in the dark is strongly stimulated by addition of both DP and sulfite. In the light DP or sulfite release 5 and 7.3 nmol ethylene, respectively. In combination the amount of ethylene is increased by a factor of nearly 6 as compared to the sum of ethylene produced by the individual reactions. In the dark DP alone is not active in the release of ethylene from KMB. Sulfite alone causes the formation of 1.5 nmol ethylene whereas both compounds together enhance the KMB-fragmentation 10-fold yielding 16 nmol ethylene.

Both light and dark reactions of the combined system are terminated after approximately 30 min (Fig. 2).

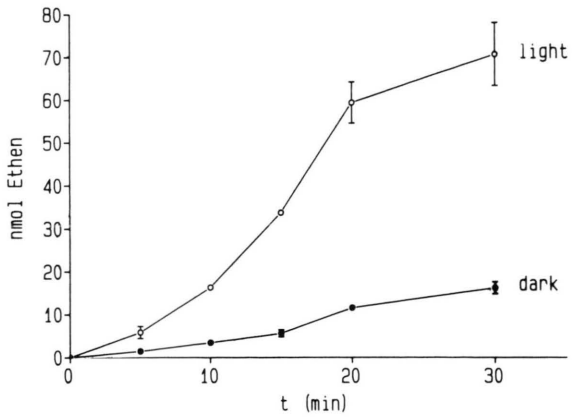


Fig. 2. Formation of ethylene from KMB under the influence of DP/sulfite. Reaction mixtures as described for Fig. 1. Standard deviations for $n = 6$.

The effects of antioxidative enzymes are shown in Fig. 3. SOD inhibits ethylene formation in the light as well as in the dark yielding 65% and 95% inhibition, respectively, after 30 min whereas catalase has no effect.

B. Crocin-bleaching

Crocin destruction by illuminated DP-suspensions is shown in Fig. 4. After 30 min crocin without additions undergoes negligible degradation. In the presence of DP the decrease in absorbance followed at 440 nm is about 13%; in the presence of sulfite the

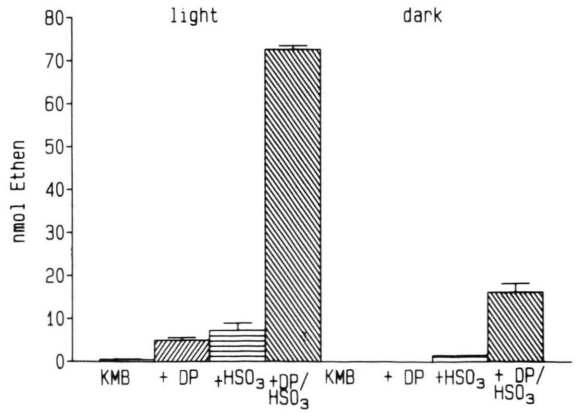


Fig. 1. Ethylene release from KMB by DP and sulfite in the light and in the dark. Reaction mixtures contained in a total volume of 2 ml: 2.5 mM KMB, 400 µg DP, 0.5 mM sulfite, 0.1 M phosphate buffer (pH 7.8).

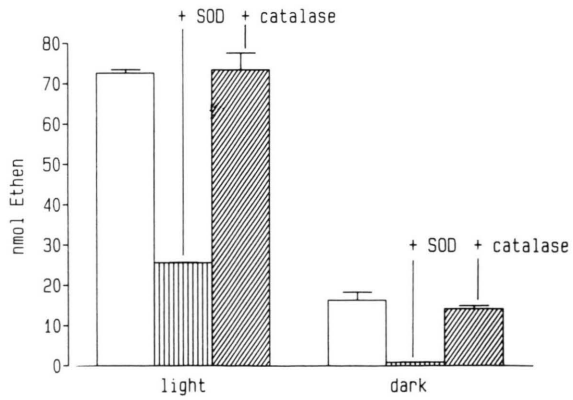
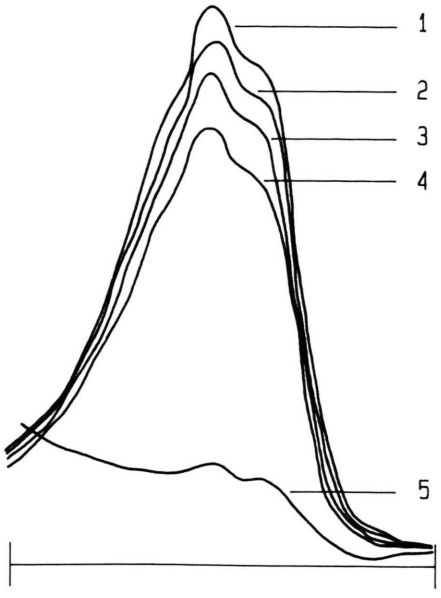


Fig. 3. Influence of SOD and catalase on KMB-fragmentation by DP/sulfite. The reaction mixtures (total volume of 2 ml) contained: 2.5 mM KMB, 400 µg DP, 0.5 mM sulfite, 100 U SOD, 100 U catalase, 0.1 M phosphate buffer (pH 7.8).



1: Crocin, 0 min, E 440nm: 0.978
2: Crocin, 30 min, E 440 nm: 0.929
3: Crocin + DP, 30 min, E 440 nm: 0.853
4: Crocin + sulfite, 30 min, E 440 nm: 0.750
5: Crocin + DP/sulfite, 30 min, E 440 nm: 0.145

Fig. 4. Comparison of the spectra of crocin treated by DP, sulfite and DP/sulfite. The reaction mixture (total volume of 2 ml) was as follows: 8.18 μM crocin, 400 μg DP, 0.5 mM sulfite, 0.1 M phosphate buffer (pH 7.8).

decay is about 23% in 30 min. Total destruction of the carotenoid is observed within 30 min in the presence of both DP and sulfite.

The time kinetics of the light and dark reactions are shown in Fig. 5 and 6. In the presence of both DP and sulfite crocin is bleached in the light by 88% and in the dark by 72%. The single substances degrade crocin by about 25% where the effects in the light are more pronounced. SOD inhibits crocin bleaching by DP/sulfite in the light as well as in the dark reaction yielding 46% inhibition in the light and 86% in the dark.

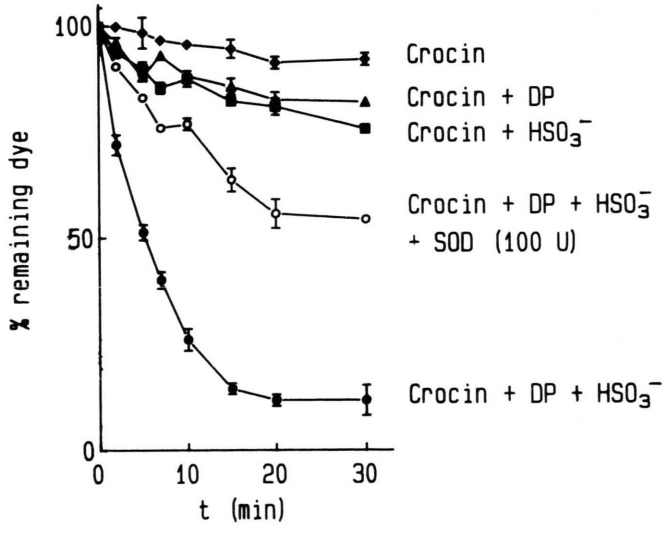


Fig. 5. Crocin bleaching in the light and the effect of SOD. The reaction mixture (total volume of 2 ml) was as described for Fig. 4. 100 U SOD were added. Standard deviations for $n = 6$.

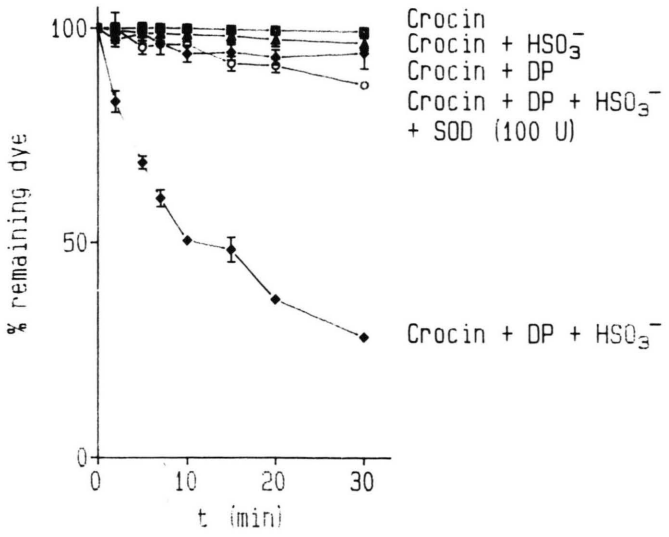


Fig. 6. Crocin bleaching in the dark. Reaction mixtures and standard deviations are identical to those in Fig. 5.

C. Chemiluminescence generated by reactions between DP/sulfite and LA, t-BuOOH or H_2O_2

Lipid peroxidation proceeds *via* a free radical mechanism generating a series of reactive intermediates including alkoxyl radicals, peroxyl radicals and hydroperoxides. Treatment of linolenic acid with DP and sulfite causes light emission with 4.222 "counts" during the first minute. The degree of light emission depends on the age of the LA solution (and thus probably on the stage of its autoxidation) and on the pH. The strongest light emissions were observed at acid pH and aged LA solutions (data not shown). The pH finally chosen for this reaction was 6.8, the pH optimum for the reactions with *t*-BuOOH or H_2O_2. Fig. 7 shows, that pure LA or LA with added DP caused no light emission, LA plus sulfite to some degree. Like in the other test systems (KMB-fragmentation and crocin bleaching) catalase has no effect on light emission whereas SOD inhibits by about 40%. Conducting the same experiment with *t*-BuOOH or H_2O_2 in place of LA, we found almost identical results. The light emission was reduced during the reactions between sulfite and *t*-BuOOH or H_2O_2 in comparison to the light production of the complete system, were 11,000 counts were recorded with *t*-BuOOH, and 1500 counts with H_2O_2. SOD inhibited the light emission in the *t*-BuOOH-system

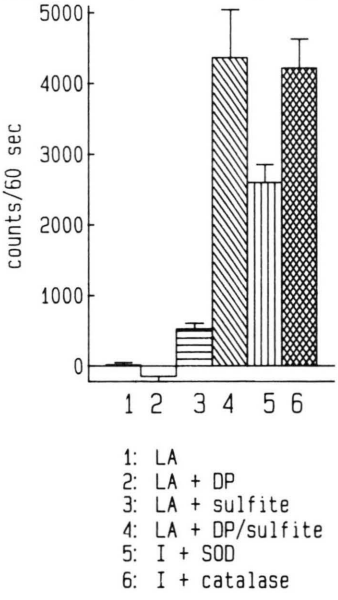


Fig. 7. Chemiluminescence by LA treated with DP and sulfite. Experimental conditions were as described in Materials and Methods. Reaction mixtures contained in a total volume of 2 ml: 11.3 μM LA, 400 μg DP, 0.5 mM sulfite, 100 U SOD, 100 U catalase, 0.1 M phosphate buffer (pH 6.3). (1), LA; (2), LA with DP; (3), LA with sulfite; (4), LA with DP/sulfite (= I); (5), I with SOD; (6), I with catalase. Standard deviations for $n = 8$.

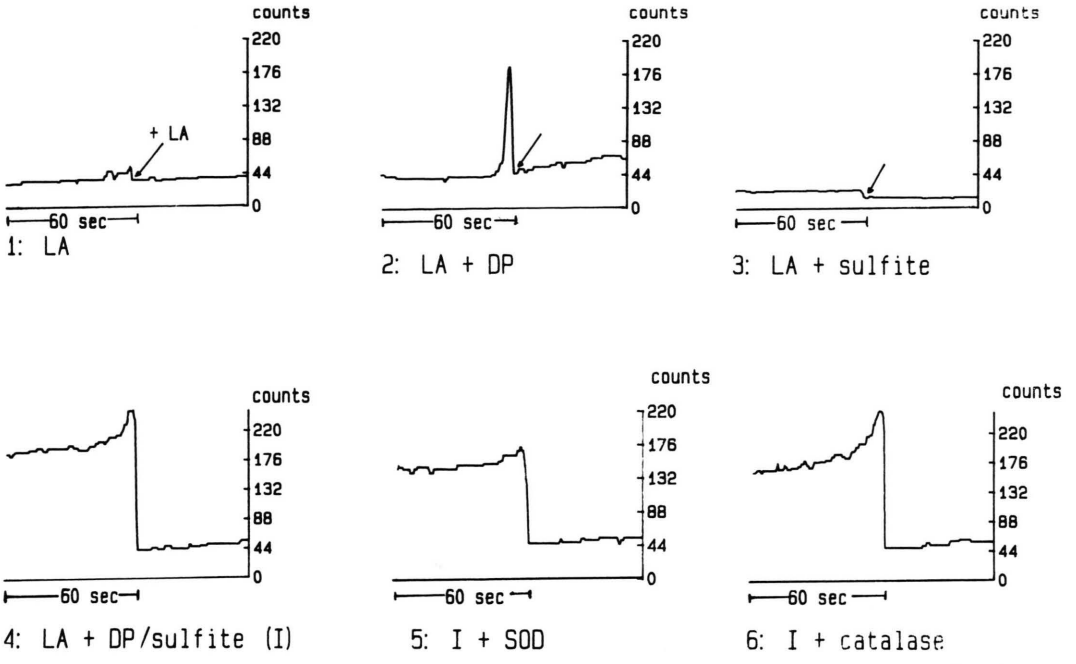


Fig. 8. Kinetics of the reactions between LA and DP and sulfite. The reaction mixture was as described in Fig. 7. Numbers 1–6 were in accordance with those in Fig. 7. Experimental conditions were as indicated in Materials and Methods.

by 30%, in the H_2O_2-system by 97% whereas catalase had no effect in the first system. The decrease of chemiluminescence in the H_2O_2-system in the presence of catalase is trivially a result of the enzymatic destruction of the added H_2O_2. The light emission of the reaction between *t*-BuOOH and DP/sulfite depends on the DP-concentration. Fig. 11 and 12 show that already 5 µg DP significantly catalyze the light emission. At a concentration of 200 µg/2 ml DP a saturation of the reaction is observed.

Fig. 8, 10 and 14 show the reaction kinetics of the three chemiluminescence systems. The numbers in the individual kinetic graphs are in accordance with the numbers in the corresponding figures (Fig. 7, 9, 13) representing the integrated areas underlying these curves. Chemiluminescence increases rapidly after adding LA, *t*-BuOOH or H_2O_2 to the reaction mixtures containing DP and sulfite (exp.-No. 4) and decreases slowly. The inhibiting effect of SOD is shown as a lower peak of light emission (No. 5) whereas the kinetics in the presence of catalase (No. 6) are identical with those of the complete system (No. 4).

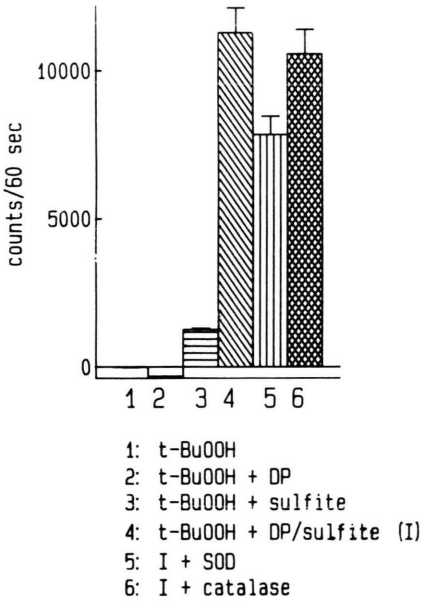


Fig. 9. Chemiluminescence by *t*-BuOOH treated with DP and sulfite. Reaction mixture was as described for Fig. 7. LA was replaced by *t*-BuOOH (11.3 µM). 0.1 M phosphate buffer (pH 6.8) was used.

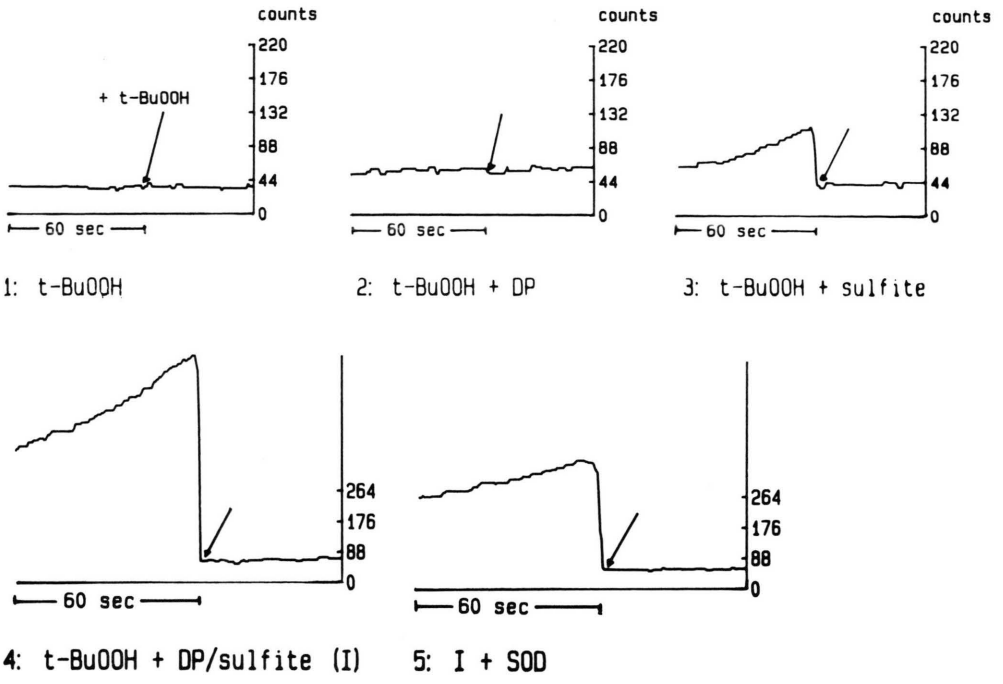


Fig. 10. Kinetics of chemiluminescence by *t*-BuOOH. Reaction mixtures and combinations (No 1–6) were as described for Fig. 7; *t*-BuOOH instead of LA.

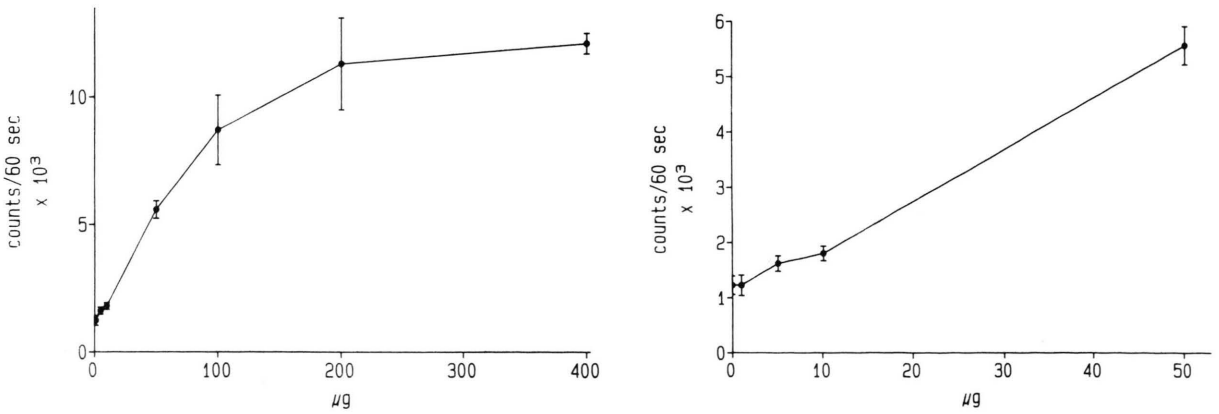


Fig. 11 and 12. Light emissions in the system *t*-BuOOH/DP/sulfite in dependence on DP concentrations. The reaction mixtures (total volume of 2 ml) contained: 11.3 µM *t*-BuOOH, 0.5 mM sulfite, 0–400 µg DP, 0.1 M phosphate buffer (pH 6.8). Standard deviations for $n = 6$.

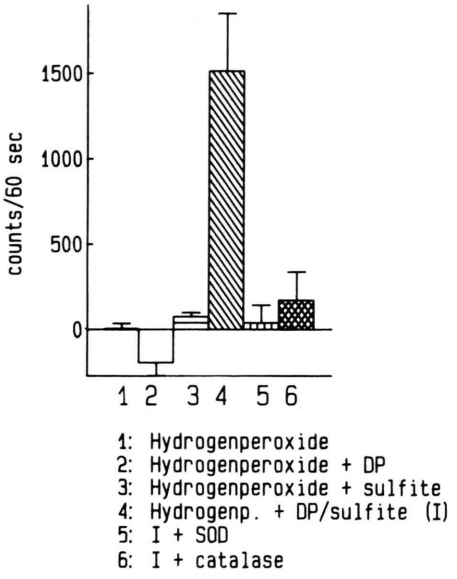


Fig. 13. Chemiluminescence of the combination H_2O_2/DP/sulfite. The reaction mixture was as indicated for Fig. 7. In place of LA, H_2O_2 (11.3 μM) was used; 0.1 M phosphate buffer (pH 6.8); standard deviations for $n = 8$.

Discussion

In three different test systems the combinations of DP and sulfite yield synergistic effects of molecular destructions, which were inhibited by SOD whereas catalase had no influence. KMB-Fragmentation and crocin bleaching also proceed as light reactions were the single substances as well as the combinations were stimulatory. It is remarkable, that the light reactions were only partially inhibited by SOD whereas the dark reactions were inhibited by nearly 100%. This suggests, that light and dark reactions are due to different mechanisms where superoxid radical anion is involved and H_2O_2 seems to be of no importance.

In all systems sulfite supports the degradations probably by acting as electron donor. Peiser and Yang [12] reported that, during HSO_3^--driven lipid peroxide degradation, $HSO_3^{\cdot -}$ radicals, alkoxy radicals and O_2^- radicals were generated as follows:

$$HSO_3^- + LOOH \xrightarrow{k1} LO^{\cdot} + HSO_3^{\cdot} + OH^-$$

$$HSO_3^{\cdot} + O_2 + H_2O \xrightarrow{k2} SO_4^{2-} + O_2^{\cdot -} + 3H^+$$

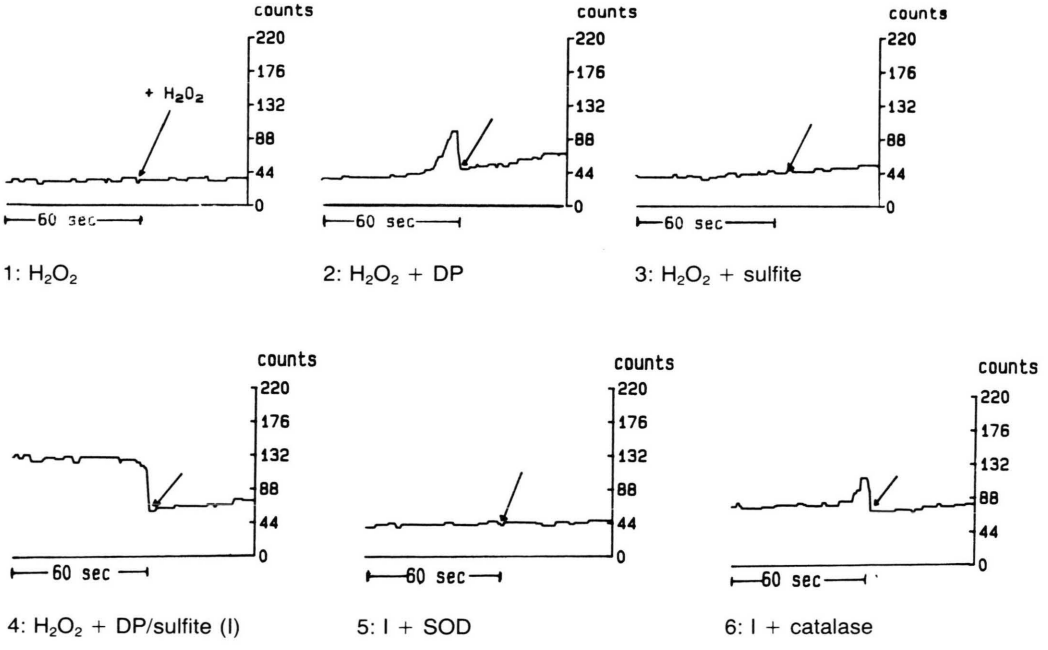


Fig. 14. Kinetics of chemiluminescence produced by H_2O_2, DP and sulfite. The reaction mixture and the combinations (No. 1–6) were as described for Fig. 7. In place of LA, H_2O_2 was used. 0.1 M phosphate buffer had a pH of 6.8.

Our results suggest that the reaction konstant $k1$ is influenced by the catalytic function of DP thus accelerating radical formation.

Iwaoka *et al.* [17] indicated, that chemiluminescence during lipid peroxidation in rat liver microsomes was due to singlet oxygen generation. Further investigations with *t*-BuOOH under anaerobic conditions showed, that the luminescence might be based on the following generalized reaction sequence of hydroperoxide breakdown:

$$\text{LOOH} \xrightarrow{\text{catalysts}} [\text{O}_2^{\cdot -}] \rightarrow {}^1\text{O}_2 \rightarrow h\nu + {}^3\text{O}_2$$

In this sequence $O_2^{\cdot -}$ may play a role in radical-chain propagation under aerobic conditions in the presence of HSO_3^- whereas $^1O_2$ is a light-emitting species operating also under anaerobic conditions.

Cadenas *et al.* reported [18], that chemiluminescence with mitochondrial membranes might be related to lipid peroxidation and singlet oxygen formation *via* the following mechanism:

$$\text{LH} + \text{R}^{\cdot} \longrightarrow \text{L}^{\cdot} + \text{RH}$$
$$\text{L}^{\cdot} + \text{O}_2 \longrightarrow \text{LOO}^{\cdot}$$
$$\text{LOO}^{\cdot} + \text{LOO}^{\cdot} \rightarrow {}^1\text{O}_2 + 2\,\text{LO}$$
$$2\,{}^1\text{O}_2 \longrightarrow 2\,{}^3\text{O}_2 + h\nu$$

[R· = starter radical; LH = unsaturated fatty acid]

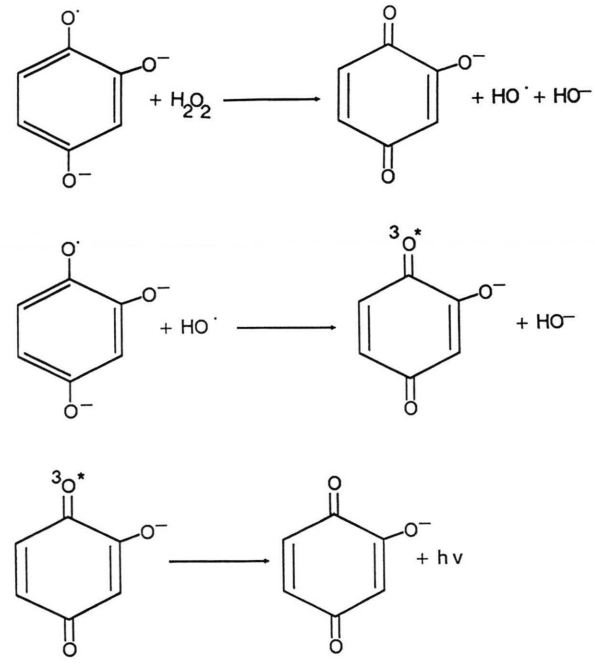
During this sequence, light emission seems also to be due to the decay of intermediarily produced triplet ketones (LO*) according to:

$$\text{LOO}^{\cdot} + \text{LOO}^{\cdot} \rightarrow -\underset{\displaystyle \text{O}-\text{O}}{\text{L}} - \text{L} - + {}^1\text{O}_2$$

$$-\underset{\displaystyle \text{O}-\text{O}}{\text{L}} - \text{L} - \longrightarrow \text{LO} + \text{LO}^*$$

$$\text{LO}^* \longrightarrow \text{LO} + h\nu$$

These findings are in agreement with our results although we cannot identify the light-emitting species with our methods. Superoxide as a reactive intermediate was indicated by decreased luminescence after the addition of SOD. Light emission apparently depends on the content of hydroperoxides: aged LA solutions cause higher rates of light emisions as compared to freshly prepared LA solutions. *t*-BuOOH at the same concentration as pure LA yields 3-fold the light emission in comparison to LA. The light emitting species seems to be generated not exclusively *via* superoxide, since SOD inhibits light emission only partially in certain experiments. Light emission by the system DP, HSO_3^- and H_2O_2 is all but clear, since neither unsaturated fatty acid nor preformed organic peroxide are added. Hydroperoxide formation from a DP-intrinsic compound and/or $^1O_2$ formation must be considered. A most likely mechanism could be outlined by considering the findings reported by Brunmark *et al.* [19]. They suggest that a light emitting species may be formed by the interaction of a hydroxyquinone with H_2O_2 in three steps (1–3) where intermediarily OH· is formed. The light emitting compound would be a hydroxyquinone in the exited state which emitts photons in the range of 490–570 nm.



The cooperative mechanism observed in the presence of DP and HSO_3^- indicates monovalent oxygen reduction and subsequent lipid peroxidation in the presence of an unsaturated fatty acid. In this process, HSO_3^- is supposed to function both as electron donor and radical propagating agent:

$$\text{a)}\ \text{HSO}_3^- + \text{O}_2 \xrightarrow{\text{DP}} \text{HSO}_3^{\cdot} + \text{O}_2^{\cdot -}$$
$$\text{b)}\ \text{HSO}_3^{\cdot} + \text{O}_2 + \text{H}_2\text{O} \longrightarrow \text{SO}_4^{2-} + \text{O}_2^{\cdot -} + 3\,\text{H}^+$$
$$\text{c)}\ \text{O}_2^{\cdot -} + \text{DP} \longrightarrow \text{DP}^{\cdot} + \text{O}_2$$
$$\text{d)}\ \text{DP}^{\cdot} + \text{HSO}_3^- \longrightarrow \text{DP}^- + \text{HSO}_3^{\cdot}$$

e) $DP^- + O_2 \longrightarrow DP^{\cdot} + O_2^{\cdot -}$

f) $2\ DP^{\cdot} \rightleftharpoons DP + DP^-$

g) $DP^{\cdot} + O_2^{\cdot -} + H^+ \xrightarrow{SOD} DPH + O_2$

h) $O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$

As activating principles of DP in reaction a) both nitroaromats and naphthoquinones have to be considered, since both classes of compounds have been shown to undergo redox cycling driving oxidative destructions in the presence of appropriate electron donor molecules [20, 21]. Reactions b)–e) may operate as propagators of the synergistic radical chain reaction observed in the presence of both DP and HSO_3^-. $O_2^{\cdot -}$ plays an important role as mediator between the DP-redox factors and different SO_x-oxidation states including HSO_3^-, $HSO_3^{\cdot}$ where sulfate is the final product. Disproportionation of $DP^{\cdot}$ (f) and SOD-catalyzed dismutations (g, h) represent chain terminating events, where reaction g) would be in agreement with the function of SOD as a superoxide-semiquinone-oxidoreductase [22].

Since both SO_2 and DP are present in significant concentrations in urban air pollution (smog) the indicated model reactions may be seen as a possible explanation for certain respiratory disorders discussed in context with severe air pollution.

[1] W. M. Draper, Chemoshere **15,** 437–447 (1986).
[2] R. O. McClellan, Am. Ind. Hyg. Assoc. **47,** 1–13 (1986).
[3] R. O. McClellan, Ann. Rev. Pharmacol. Toxicol. **27,** 279–300 (1987).
[4] T. R. Henderson, A. P. Li, R. E. Royer, and C. R. Clark, Environ. Mutageneses **3,** 211–220 (1981).
[5] T. Handa, T. Yamauchi, M. Ohnishi, Y. Hisamatsu, and T. Ishii, Environ. Intern. **9,** 335–341 (1983).
[6] U. Heinrich, H. Muhle, S. Takenaka, H. Ernst, R. Fuhst, U. Mohr, F. Pott, and H. Stöber, J. Appl. Tox. **6,** 383–395 (1986).
[7] U. Heinrich, F. Pott, S. Rittinghausen, in: Carcinogenic and Mutagenic Effects of Diesel Engine Exhaust (N. Ishinishi, A. Koizumi, R. O. McClellan, and W. Stöber, eds.), Elsevier Science Publishers B.V., p. 441–455 (1986).
[8] J. Lewtas, Environ. Health Persp. **47,** 141–152 (1983).
[9] P. A. Souththorn, Mayo Clin. Proc. **63,** 390–408 (1988).
[10] J. P. Nachtman, Res. Comm. in Chem. Pathol. and Pharmacol. **51,** 73–80 (1986).
[11] C. Mottley, R. P. Mason, C. F. Chignell, K. Sivarajah, and T. E. Eling, J. Bio. Chem. **257,** 5050–5055 (1982).
[12] G. D. Peiser and S. F. Yang, Phytochemistry **17,** 79–84 (1978).
[13] G. Vogl and E. F. Elstner, Toxicol. Lett., in press (1989).
[14] E. F. Elstner, in: The Biochem. of Plants (P. K. Stumpf and E. E. Conn, eds. in chief, D. D. Davies, ed.), **11,** 253–316 (1987).
[15] J. Friend and A. M. Mayer, Biochim. Biophys. Acta **41,** 422–429 (1960).
[16] R. J. Youngman, G. R. Wagner, F. W. Kühne, and E. F. Elstner, Z. Naturforsch. **40c,** 409–414 (1985).
[17] T. Iwaoka, F. Tabata, and T. Takahashi, Free Rad. Bio. Med. **3,** 329–333 (1987).
[18] E. Cadenas, A. Boveris, and B. Chance, Biochem. J. **188,** 577–583 (1980).
[19] A. Brunmark, D. Mira, G. Buffinton, and E. Cadenas, in: Free Radicals: Methodology and Concepts (C. Rice-Evans and B. Halliwell, eds.), p. 369–391 (1988).
[20] R. J. Youngman, W. F. Oßwald, and E. F. Elstner, Biochem. Pharmacol. **31,** 3723–3729 (1982).
[21] E. Paur, R. J. Youngman, E. Lengfelder, and E. F. Elstner, Z. Naturforsch. **39c,** 261–267 (1984).
[22] E. Cadenas, D. Mira, A. Brunmark, C. Lind, I. Segura-Anguilar, and L. Ernster, Free Rad. Med. Biol. **5,** 71–79 (1988).