Chlorophyll Photobleaching and Ethane Production in Dichlorophenyldimethylurea- (DCMU) or Paraquat-Treated *Euglena gracilis* Cells

Erich F. Elstner and W. Osswald

Institut für Botanik und Mikrobiologie, Technische Universität München, Arcisstr. 21, D-8000 München 2

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Light dependent (35 Klux) chlorophyll bleaching in autotrophically grown Euglena gracilis cells at slightly acidic pH (6.5–5.4) is stimulated by the photosystem II blockers DCMU and DBMIB (both 10^{-5} M) as well as by the autooxidizable photosystem I electron acceptor, paraquat (10^{-3} M).

Chlorophyll photobleaching is accompanied by the formation of thiobarbituric acid – sensitive material ("malondialdehyde") and ethane.

Both chlorophyll photobleaching and light dependent ethane formation are partially prevented by higher concentrations (10^{-4} M) of the autooxidizable photosystem II electron acceptor DBMIB or by sodium bicarbonate (25 mM).

In vitro studies with cell free extracts (homogenates) from *E. gracilis* suggest that α-linolenic acid oxidation by excited (reaction center II) chlorophyll represents the driving force for both ethane formation and chlorophyll bleaching.

Ethane formation thus appears to be a sensitive and non-destructive *"in vivo"* marker for both restricted energy dissipation in photosystem II and, conditions yielding reactive oxygen species at the reducing side of photosystem I.

Introduction

Chlorophyll bleaching is one of the characteristic symptoms for plant diseases introduced by infections, certain physical parameters or by chemicals. Several groups of herbicides cause chlorosis and other symptoms of senescence where both a decrease in biosynthesis and an increased degradation of photosynthetic pigments seem to be involved [1–6]. For the well known weed killers paraquat and DCMU, a correlation between light dependent decrease in leaf pigment levels and lipid peroxidation have been described [4, 7]. Different mechanisms of induction of these connected processes have to be envisaged: a) lack of "energy dissipation" and thus photooxidative destruction of pigments or unsaturated fatty acids in the case of electron transport blockers as DCMU [8] and, b) production of aggressive oxygen species (*e. g.* free radicals) with subsequent lipid attack and membrane destruction in the case of low potential photosystem I electron acceptors as paraquat and other bipyridylium salts [4, 5, 9].

In the present communication, the connection between chlorophyll breakdown and lipid peroxidation, followed by ethane determination [10, 11] from the gaseous head space of illuminated *Euglena gracilis* cells is reported. In comparison to *in vitro* experiments and chemical models reported by others (cited in the discussion), we describe experiments on herbicide – induced photobleaching and unsaturated fatty acid peroxidation *in vivo* initiated by either limited electron flow through photosystem II, or by active oxygen species produced by photosystem I.

Materials and Methods

Euglena gracilis strain *z* was grown autotrophically as described [12]. Cell free preparations (homogenates) were prepared by thawing deep frozen *E. gracilis* cells and homogenization in a glas tube with teflon piston (Potter-Homogenizer). "Malondialdehyde" [13], chlorophyll [14] and ethane [11] were determined, crocin [15], superoxide dismutase [16, 17], mitochondria [18] and spinach chloroplast lam-

Abbreviations: PQ, paraquat (methylviologen): 1,1′-dimethyl-4,4′-dipyridylium dichloride; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; DBMIB, dibromothymoquinone: 2,5-dibromo-3-ethyl-6-isopropyl-*p*-benzoquinone; Chl, chlorophyll.

Reprint requests to Dr. E. F. Elstner.

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ellae [19] were prepared as described. Oxygen concentrations were determined with a "Hansatech" (Fa. Bachofer, Reutlingen) oxygen electrode. Incubations were done with ca. 15 ml Fernbach flasks, sealed with serum rubber stoppers, at 25 °C in a thermostate with illumination (35 Klux) from the bottom.

Results

Chlorophyll bleaching and ethane formation in intact Euglena gracilis cells

Autotrophically grown *E. gracilis* cells were harvested by centrifugation for 10 min at 1.500×*g*, suspended in water and recentrifuged. The pellet was taken up in the indicated buffers (see tables and figures). Illuminated *E. gracilis* cells show bicarbonate-dependent oxygen evolution; if these cells are incubated in acetate buffer with decreasing pH (6.0 – 5.2), an increase in chlorophyll-photobleaching and ethane formation is observed which, at pH 5.2, is ca. 7 to 10 fold stronger in the light as compared to the dark control (data not shown). The stimulation by light of chlorophyll bleaching and ethane formation is somewhat enhanced by 10% methanol (solvent for DCMU) and strongly enhanced by DCMU in 10% methanol or by PQ in 10% methanol, where ethane formation seems to correlate with the production of thiobarbituric acid – sensitive material ("malondialdehyde") (Table I). The time course of chlorophyll bleaching shows a lag of approx. 1 h

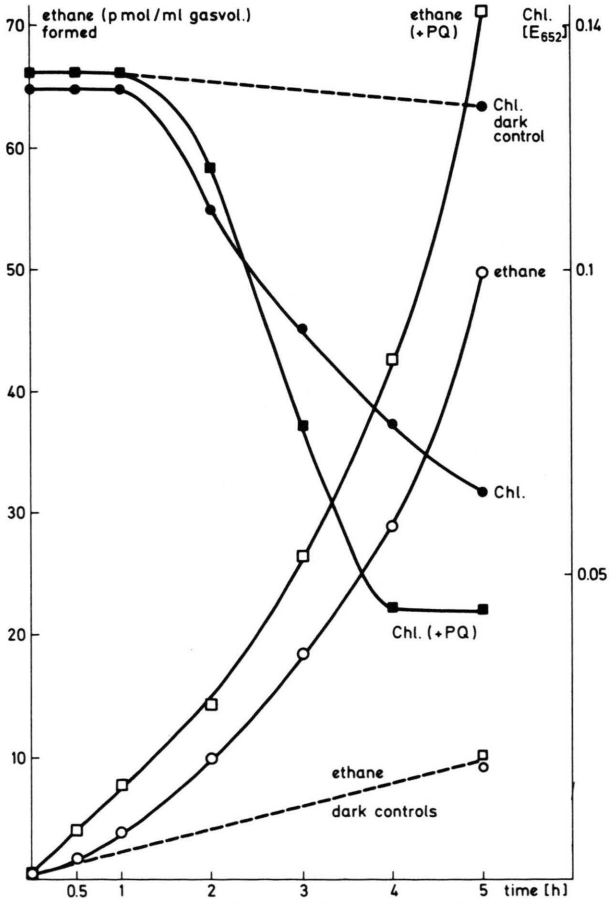

Fig. 1. Comparison of ethane formation and chlorophyll photobleaching in *Euglena gracilis* cells. Heterotrophically grown *E. gracilis* cells (with ca. 2 mg Chl) were incubated in 2 ml 0.1 M acetate buffer pH 5.6 in the light (35 Klux) at 25 °C. At the indicated times, Chl and ethane were determined as described in Materials and Methods, 1 mM PQ was present, where indicated.

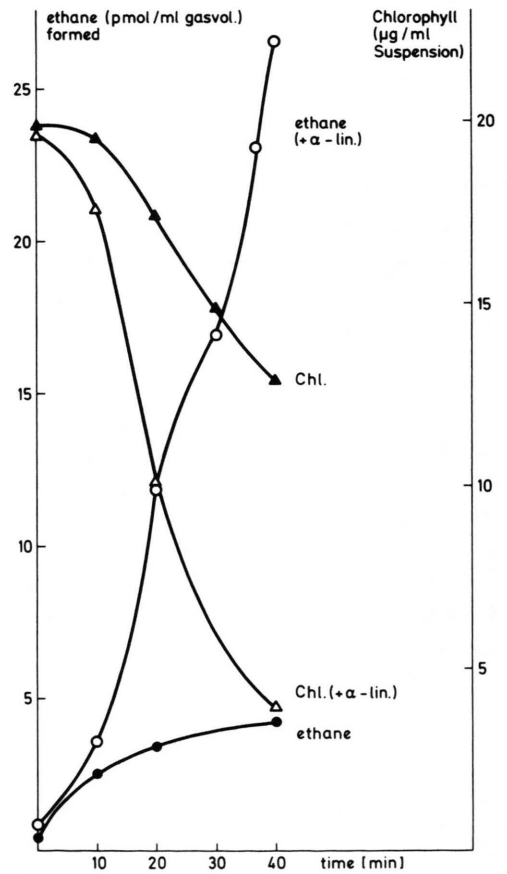

Fig. 2. Effect of α-linolenic acid on chlorophyll photobleaching and ethane formation by *Euglena gracilis* homogenates. Cell-homogenates of *E. gracilis* with ca. 50 µg Chl were incubated in 2 ml 0.1 M acetate buffer pH 6.5. Other conditions as in Fig. 1.

Table I. Effects of light, DCMU and paraquat on ethane- and "malondialdehyde"-formation by *Euglena gracilis* cells. Conditions: 4 h illumination of *E. gracilis* cells (35 Klux, 25 °C) in phosphate buffer pH 7.6. Ethane and "malondialdehyde" were determined as described in Materials and Methods.

Treatment	Ethane formed [pmol/mg Chl]	"Malondialdehyde" formed [nmol/mg Chl]
dark	14	48
light	37	83
light + 2×10^{-5} M DCMU	120	103
light + 10^{-3} M PQ	146	143

while ethane formation seems to start right from the beginning of illumination (Fig. 1). The factor of stimulation of chlorophyll bleaching and ethane formation by light and DCMU at pH values between 5.4 and 7.6 (as compared to the dark controls) changed erratically from 1.2 to 15 in some of the experiments. This may be due to the different age of "random" cultures of *E. gracilis* and also to different CO_2 concentrations during the growth. Hulanicka *et al.* [20] reported that the composition of the lipids in *E. gracilis* (*e. g.* ω-3 unsaturated fatty acids) changes with growth conditions, *e. g.* with the availability of CO_2.

Such a change may be reflected by the capacity of *E. gracilis* cells to bleach chlorophyll and produce ethane: as shown in Table II, light and DCMU-stimulated increase in ethane production and decrease in chlorophyll in the cells correlates with the presence or absence of CO_2 (gassing with 5% CO_2 in air, or air only).

Table II. Dependence of chlorophyll bleaching and ethane formation on the CO_2-concentration during the growth of *E. gracilis*. Conditions: *E. gracilis* cells were illuminated for 5 h (35 Klux) at 25 °C and pH 5.6. Ethane: expressed as pmol/mg Chl. Chlorophyll: as mg/ml susp.

Treatment	*E. gracilis* grown in:			
	air + 5% CO_2		air	
	Chl	ethane	Chl	ethane
dark	0.96	50	0.72	20
light	0.16	80	0.71	67
light + 2×10^{-5} M DCMU	0.04	684	0.68	100

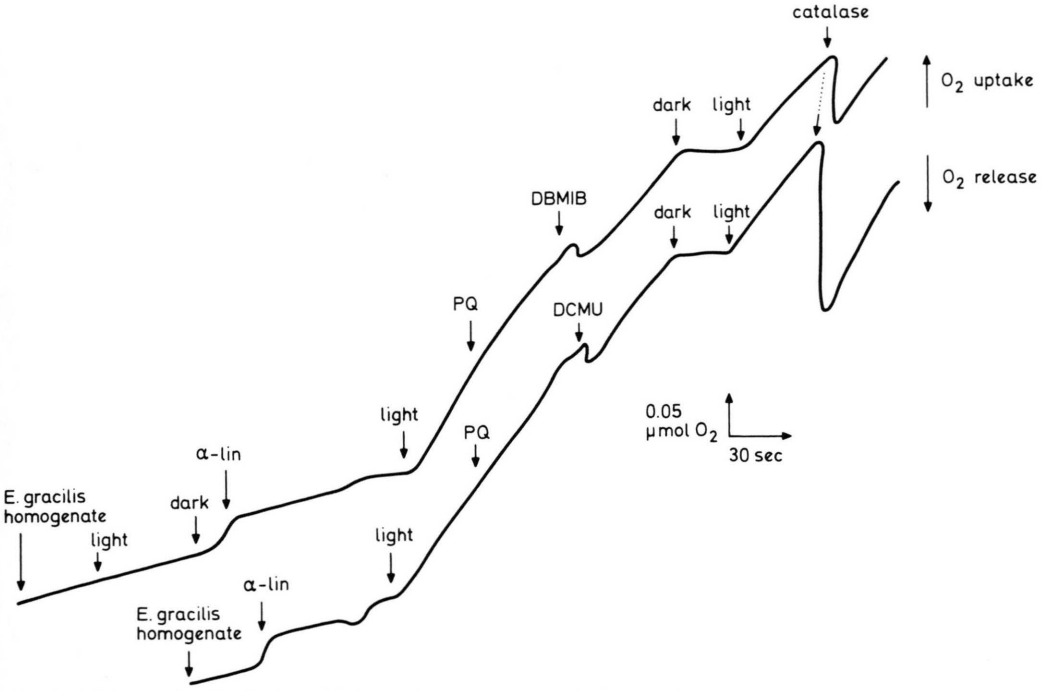

Fig. 3. Light- and α-linolenic acid-dependent oxygen uptake by *Euglena gracilis* homogenates. The reaction mixture contained in 2 ml (oxygen electrode): *E. gracilis* homogenate with ca. 50 µg Chl in 0.1 M acetate buffer pH 6.5 and (where indicated) 20 µmol α-linolenic acid, 10^{-6} M DBMIB, or DCMU and 100 units (Boehringer) catalase. Illumination with 35 Klux white light at 18 °C.

Chlorophyll bleaching and ethane formation with Euglena gracilis homogenates

If *E. gracilis* homogenates (see Materials and Methods) are illuminated in the presence or absence of α-linolenic acid, a strong stimulation of both chlorophyll destruction and ethane formation is observed (Fig. 2). This reaction is accompanied by a light dependent oxygen uptake which is not significantly influenced by acceptors or blockers of photosynthetic electron transport. Hydrogen peroxide seems to be a product of this oxygen reduction as indicated by the oxygen release after addition of catalase (Fig. 3).

Light dependent ethane formation by *E. gracilis* homogenates is not inhibited by a) superoxide dismutase or catalase and b) OH-scavengers as formate, mannitol or α-tocopherol but is inhibited by ca. 60% by 1 mM crocin (the watersoluble digentiobiose ester of the carotenoid acid crocetin). Ascorbic acid or bisulfite strongly stimulate ethane formation by illuminated *E. gracilis* homogenates in the presence of α-linolenic acid (Table IV).

Effects of bicarbonate and DBMIB on chlorophyll bleaching and ethane formation by intact Euglena gracilis cells

a) Effect of bicarbonate

Bicarbonate, in addition to other effects and functions [21], seems to regulate the electron flow between photosystem II and the plastoquinone pool at a site close to (or identical with) the DCMU-sensitive protein(s).

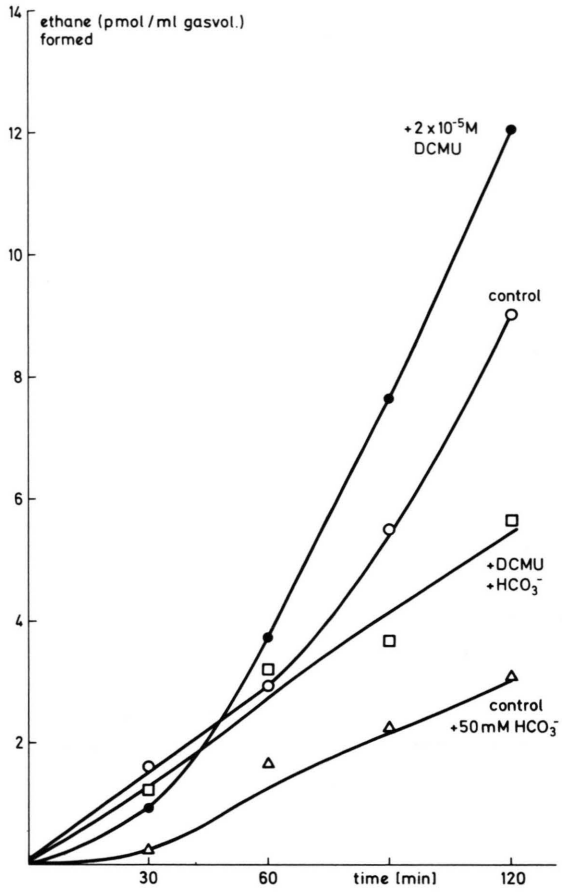

Fig. 4. Effects of DCMU and bicarbonate on ethane formation by illuminated *Euglena gracilis* cells. The reaction conditions were essentially as outlined for Fig. 1. 2×10^{-5} M DCMU and/or 50 mM HCO_3^- were present as indicated.

Additions		Ethane formed [pmol × mg $Chl^{-1} \times h^{-1}$]	Effect
control,	minus α-linolenic acid	90	
control,	plus α-linolenic acid	300	stimulation
control,	plus α-linolenic acid + 63 units (*cf.* ref. [17]) superoxide dismutase + 1300 units (Boehringer)	310	none
	catalase	298	none
10 μmol sodium formate		270	none
10 μmol mannitol		295	none
1 μmol α-tocopheryl-sulfate		328	none
2 μmol crocin		134	inhibition
10 μmol ascorbate		13 200	stimulation
10 μmol bisulfate		127 900	stimulation

Table III. Ethane formation in *Euglena gracilis* homogenates: Effects of inhibitors and reducing agents. Conditions: *E. gracilis* homogenates were incubated in 2 ml 0.1 M acetate buffer pH 6.5 for 4 h at 25 °C under illumination (35 Klux) with 20 μmol α-linolenic acid.

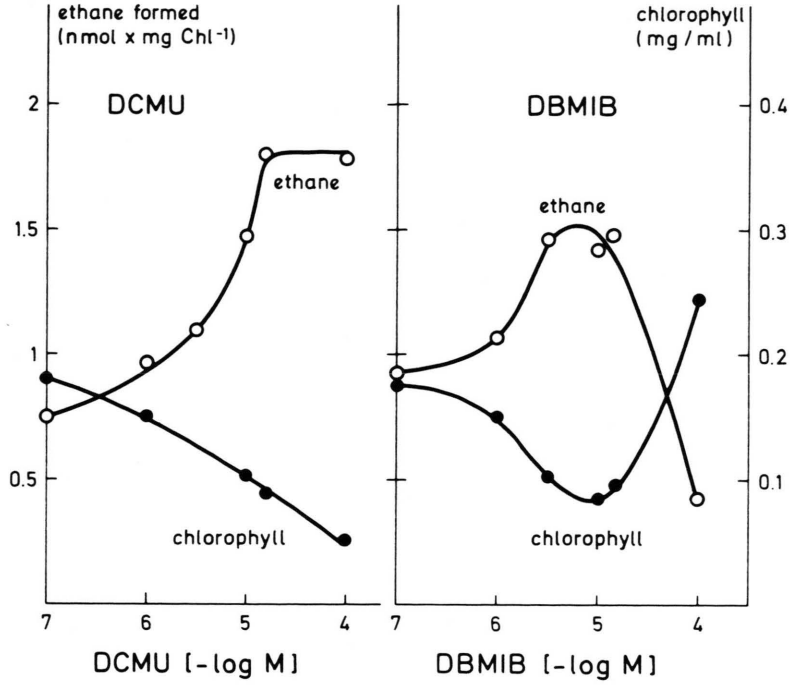

Fig. 5. Effects of increasing concentrations (10^{-7} to 10^{-4} M) of DCMU and DBMIB on ethane formation and chlorophyll bleaching by *Euglena gracilis* cells. Reactions conditions were as outlined for Fig. 1.

Bicarbonate at a concentration of 50 mM, in illuminated *E. gracilis* cells reduces ethane formation both in the presence and absence of DCMU (Fig. 4).

b) Effect of DBMIB

As shown in Fig. 5, DCMU concentrations up to 0.1 mM increase chlorophyll destruction and ethane formation with an apparent saturation at approximately 2×10^{-5} M DCMU. Increasing concentrations of DBMIB up to 0.01 mM cause an increase in both chlorophyll destruction and ethane formation while 0.1 mM DBMIB show no effect on chlorophyll bleaching or ethane formation as compared to illuminated controls. It is interesting to note that isolated spinach chloroplasts in the presence of increasing DBMIB concentrations show an increased inhibition of electron transfer between the two photosystems, measured as monovalent oxygen reduction (yielding $O_2\cdot^-$) by photosystem I. With increasing DBMIB concentrations, however, a two-electron transfer to oxygen (yielding only H_2O_2, but no $O_2\cdot^-$) is increasingly enhanced, indicating that high concentrations of DBMIB catalyse an ionic oxygen reduction, solely driven by photosystem II, since 0.01 mM DCMU inhibit this reaction by 100% [22, 23]. This effect is shown in Table IV.

Table IV. Effect of DBMIB on hydrogen peroxide formation by isolated spinach chloroplast lamellae. The reaction mixture contained in 2 ml: 100 μmol phosphate buffer pH 7.8, 2.5 μmol NH_4Cl, 2.5 μmol $MgCl_2$ and chloroplast lamellae with 100 μg Chl. The reaction was conducted for 10 min at 18 °C in white light (30 Klux). H_2O_2 was determined with the aid of NADH-peroxidase (Boehringer).

DBMIB added (M)	none (control)	10^{-6}	10^{-5}	10^{-4}
H_2O_2 produced (in % of control)	100	30	90	280

Discussion

A connection between chlorophyll bleaching and unsaturated fatty acid oxidation after herbicide treatment of green leaves or algae has been reported by several workers [2, 4, 7–9, 24–26] and, two general mechanisms have been proposed:

a) chlorophyll sensitized photooxidations in the case of limited "energy dissipation" in photosystem II caused by electron transport blockers like DCMU, and

b) formation of reactive oxygen species (radicals) after autoxidation of low potential electron acceptors

of photosystem I like paraquat (see ref. [5]). We recently reported on different mechanisms of oxygen reduction by isolated chloroplast lamellae [9, 22, 23, 27] yielding $O_2 \cdot^-$, H_2O_2 and Fenton-typ oxidants (Fe^{2+}-H_2O_2), but no free OH-radicals [27, 28]. There is also circumstantial evidence that singlet oxygen ($^1O_2$) might be produced by isolated chloroplasts in the presence of DCMU [24].

The present report describes herbicide-induced chlorophyll destruction and unsaturated fatty acid oxidation in autotrophically grown *Euglena gracilis* cells and cell homogenates.

As earlier described [11, 29], the determination of ethane production can be used as a simple method for monitoring unsaturated fatty acid oxidation (*cf.* also ref. [10]). Since α-linolenic acid is a major component of the thylakoid structure imbedding the photosystems of the chloroplasts on the one hand, and ethane is one major product of the decomposition of peroxidized α-linolenic acid [30] ethane determination in addition to the thiobarbituric acid reaction [13] prooved to be an excellent indicator for aerobic lipid destruction (for references see [10, 11, 30]).

As shown in Figs 1 and 4, ethane formation and chlorophyll destruction correlate as far as stimulation by light, pH (not shown), methylviologen (paraquat) and DCMU is concerned. During the time course of events, ethane formation seems to precede chlorophyll bleaching indicating that linolenic acid is peroxidized before chlorophyll is destroyed (Fig. 1). In the presence of high concentrations of CO_2 chlorophyll bleaching and ethane formation are reduced probably due to the regulation of photosynthetic electron flow at a "bottle neck" [21, 31] of electron transport. This especially becomes evident in the presence of unphysiological compounds of the DCMU-type. At 10^{-6} to 10^{-5} M, both DCMU and DBMIB inhibit photosynthetic electron transport. The site of inhibition by DCMU is located between the primary electron acceptor of photosystem II and the quinone pool, that of DBMIB is located after the plastoquinone pool (for a review see ref. [32]). At concentrations higher than 0.01 mM, DBMIB in contrast to DCMU is able to function as an electron redox carrier, transferring two electrons to oxygen, yielding H_2O_2 (Table IV and ref. [22, 23]). This result also indicates that limited electron transport from photosystem II is responsible for chlorophyll bleaching and ethane formation in the presence of DCMU, while H_2O_2 seem to be relatively harmless.

As far as the mechanism of ethane formation is concerned, no convinicing indication for the direct involvement of either H_2O_2, $O_2 \cdot^-$ or the free OH-radical (as postulated in ref. [33]), is obtained from our experiments with *E. gracilis* homogenates. This is concluded from the lack of influence of superoxide dismutase, catalase or OH·-scavengers; only crocin inhibits ethane formation by *E. gracilis* homogenates in the presence of α-linolenic acid to a significant extent (Table III). Ascorbate and bisulfate strongly stimulate ethane formation (Table III). This is in agreement with models presented by Dumelin and Tappel [30] and by Peiser and Yang [26]: Ethyl radical is derived from the hydroperoxide of an ω-3 unsaturated fatty acid [20] via β-scission; reduction of ethyl radical yields ethane. According to the reported results, ethane formation proves to be an indicator and *in vivo* tracer for certain herbicide-induced photooxidations accompanying chlorophyll destruction.

Acknowledgements

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