Assessment of the scavenging action of reduced glutathione, (+)-cyanidanol-3 and ethanol by the chemiluminescent response of the xanthine oxidase reaction[1]

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Summary. The free radical scavenging capacity of reduced glutathione (GSH), (+)-cyanidanol-3 and ethanol was assessed by their interference with the maximal chemiluminescent response produced by the xanthine oxidase reaction. GSH and (+)-cyanidanol-3 induce a progressive inhibition of chemiluminescence when increasing amounts are added to the reaction mixture. GSH and (+)-cyanidanol-3 added together at low concentrations (1 and 0.05 mM respectively) exhibit an additive effect. The addition of ethanol presents a biphasic effect. It inhibits chemiluminescence at low concentrations (10–50 mM) while at higher concentrations (75–500 mM) this effect is reversed. Estimation of the concentrations required to produce half of the maximal inhibition of chemiluminescence by these agents revealed that ethanol is less effective than GSH and (+)-cyanidanol-3 as a free radical scavenger in the system used.

Several enzymatic oxidations have been found to be associated with light emission[2]. Among these, the oxidation of xanthine by xanthine oxidase (EC 1.2.3.2) was shown to be related to the generation of excited states of molecular oxygen[3] (e.g., superoxide radical, $O_2^{\cdot-}$; hydroxyl radical, $HO^{\cdot}$) and to produce low-level chemiluminescence[4]. This view is supported by the inhibition of the light signal of the xanthine oxidase reaction by free radical scavengers such as cysteine, sulfite and superoxide dismutase[4]. Chemiluminescence has also been detected in isolated microsomes, mitochondria[5] and submitochondrial particles[6], and has been suggested to be a useful tool for the assessment of free radical-induced lipid peroxidation both in vitro[7,8] and in vivo[8].

Previous studies by our group have shown that acute ethanol intoxication elicited a marked enhancement in hepatic lipid peroxidation, measured as malondialdehyde[9] or diene conjugate[10–13] formation. This effect of ethanol intake was suggested to be related to the production of oxygen-free radicals[10,13] by its microsomal oxidation[14] and by acetyldehyde oxidation by xanthine oxidase and aldehyde oxidase[15] (EC 1.2.3.1). Ethanol-induced lipid peroxidation in the liver imposes an enhanced demand for hepatic GSH, the main cellular antioxidant[16], the concentration of which is drastically diminished[9,11–13,17]. Liver GSH reduction can be partially explained by its oxidation in the tissue and by its translocation from liver to blood plasma[18]. Aternatively, GSH could be used to form conjugates with ethanol-derived acetaldehyde or as a direct free radical scavenger[18]. Although free radicals involved in lipid peroxidation could be intercepted by thiols such as GSH[19], the scavenging action of GSH has mainly been considered in relation to damage due to radiation-induced free radicals[20]. The purpose of this work was to evaluate the free radical scavenging capacity of cellular concentrations of GSH, assessed by its interference with the maximal luminescent response of the xanthine oxidase reaction[4]. Results obtained with GSH were compared to those elicited by ethanol and (+)-cyanidanol-3, two known free radical scavengers[14,21].

Materials and methods. Measurements of chemiluminescence were carried out in a Beckman LS-3150P liquid scintillation counter in the out-of-coincidence mode[4], using the narrow tritium iso-set module at 25 °C. Readings were taken every 23 sec. In the absence of vials, the counter registered 1200–1600 cpm. Vials alone exhibited an initial signal of 3300–6000 cpm, which decayed to 2600–4000 cpm in 184 sec. Low potassium flasks (13 mm-diameter and 40 mm-height) inserted into 15 ml glass vials (25 mm-diameter and 60 mm-height) and containing 3 ml of buffer presented an initial signal of 9500–12,700 cpm and decayed to 3300–5000 cpm in 184 sec. Backgrounds of the reaction mixture containing the substrate and the agents under study were determined for each vial and were substracted from the signals given by the luminescence reaction.

The reaction mixture consisted of 5 mM tris (pH 7.4), 140 mM KCl, 1 mM EDTA and 1 mM xanthine, kept at 25 °C. The reaction was initiated by the addition of 0.12 units of xanthine oxidase (Sigma units). The scavenging action of GSH, (+)-cyanidanol-3 and ethanol was studied by adding different concentrations of these agents to 3 ml of the reaction mixture followed by the addition of 0.12 units of xanthine oxidase. The peak heights obtained after 138 sec were compared to those found in the absence of scavengers. All reagents used were obtained from Sigma (St. Louis) except for (+)-cyanidanol-3 (Zyma SA, Nyon, Switzerland). Results are expressed as cpm/ml of reaction mixture and values shown represent means ± SEM.

Results and discussion. Figure 1 shows a typical chemiluminescence response when 0.12 units of xanthine oxidase are added to 3 ml of the reaction mixture. As can be observed, chemiluminescence was maximal at 138 sec and presented a signal height of 33 times background. The peak height was linear with respect to either xanthine (0.01–2.0 mM) or xanthine oxidase (0.003–0.05 units/ml of reaction mixture) concentrations (data not shown). Light emission associated with the xanthine oxidase reaction has been suggested to be due to the generation of $O_2^{\cdot-}$ radicals[2–4]. These free radicals can interact among themselves to produce singlet oxygen species which could return to ground state emitting photons[4].

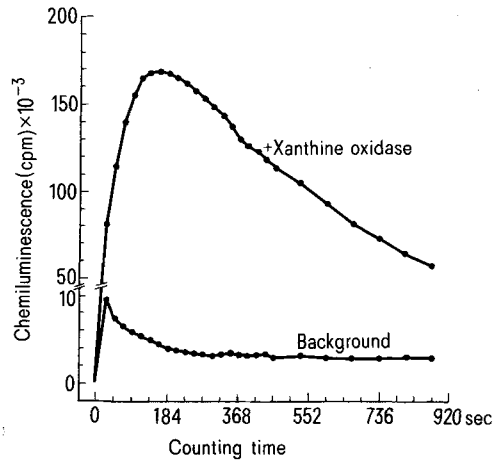

Figure 1. Time course of the chemiluminescent reaction catalyzed by xanthine oxidase. Determinations were carried out in 3 ml of 140 mM KCl, 5 mM tris (pH 7.4), 1 mM EDTA and 1 mM xanthine (background). The reaction was started by adding 0.12 units of xanthine oxidase.

The inhibitory effect of different concentrations of GSH on the xanthine oxidase reaction-induced light emission is shown in figure 2A. Chemiluminescence was progressively reduced with increasing concentrations of GSH, being maximal (90%) when it was added to final concentrations of 5 and 10 mM. It is important to point out that this potent free radical scavenging capacity of GSH was observed at concentrations normally found in cells such as hepatocytes[22] and that it diminished when GSH was below the 2.5 mM level (fig. 2A). Inhibition of xanthine oxidase reaction-induced chemiluminescence by GSH could be explained by its nonenzymatic interaction with O_2^- and/or $HO^\cdot$, forming glutathionyl radicals ($GS^\cdot$) and GSSG by homologous binding[20,23]. Alternatively, GSH can interact with singlet oxygen to produce cysteic acid[23]. The antioxidant properties of GSH, evidenced here by its inhibition of chemiluminescence generated enzymatically, are in agreement with previous studies[24] in which 2 and 4 mM GSH were found to inhibit lipid peroxidation in an in vitro peroxidating system formed by linoleate and Fe^{2+}.

These results support the contention that the reduction of hepatic GSH levels from 4.10–5.75 mM to 1.93–2.75 mM[9-13,17,18], by acute ethanol ingestion, may represent one of the major factors leading to lipid peroxidation, due to a decrease in the antioxidant capacity of the liver tissue in this condition[9]. The addition of (+)-cyanidanol-3 to the xanthine oxidase reaction also resulted in an inhibition of the luminescent signal as found for GSH (fig. 2B), an effect that was maximal (86–94% inhibition) with concentrations ranging from 0.5 to 5 mM. These results are in line with studies in which (+)-cyanidanol-3 was shown to strongly interact with free radical generating systems such as NADH/phenazine methosulfate/O_2/nitro blue tetrazolium and rat liver microsomes supplemented with either NADPH/ADP/ Fe^{2+}/O_2 or NADPH/$FADH_3$/CCl_4[21]. The strong scavenging action of (+)-cyanidanol-3 found (fig. 2B) is also in agreement with reports demonstrating that this flavonoid is able to inhibit lipid peroxidation induced in vitro and in vivo by hepatotoxic chemicals such as CCl_4[21,25-27], $CBrCl_3$[26], galactosamine[27] and ethanol[12]. The simultaneous addition of low concentrations of (+)-cyanidanol-3 (0.05 mM) and GSH (1 mM), which produced 50% and 36% inhibition of chemiluminescence respectively when added alone, showed an additive effect (96% inhibition) (table). This finding is of particular interest in the case of ethanol intoxication in which liver GSH levels are drastically dimished[9-13,17,18]. In fact, if rats are given (+)-cyanidanol-3 prior to ethanol, liver GSH depletion induced by ethanol is reduced by 80%[12]. Thus, (+)-cyanidanol-3 would spare hepatic GSH, a condition in which a better protective effect against the peroxidative action of ethanol would be exerted[12].

In contrast to the pattern of inhibition of the xanthine oxidase reaction-induced chemiluminescence elicited by GSH (fig. 2A) and (+)-cyanidanol-3 (fig. 2B), the addition of different concentrations of ethanol resulted in a biphasic effect (fig. 2C). Ethanol concentrations from 10 to 50 mM inhibited chemiluminescence by 14–39%, while at higher concentrations this effect was reversed, reaching basal light signals with 500 mM ethanol (fig. 2C). Inhibition of chemiluminescence by low ethanol concentration could be related to the scavenging properties of ethanol[14], which has been shown to generate acetaldehyde during the oxidation of xanthine by xanthine oxidase[28]. Reversion of the inhibition of chemiluminescence observed at high ethanol levels could conceivably be due to the production of acetaldehyde, which could be oxidized by xanthine oxidase thus producing free radicals[15] and photons[14]. This possibility could also explain the rather low maximal inhibition of chemiluminescence by ethanol (39%) compared to those produced by GSH (93%) and by (+)-cyanidanol-3 (94%); however, further studies are needed to clarify this view. Finally, the calculated concentrations that produce half of the maximal inhibition of chemiluminescence by (+)-cyanidanol-3, GSH and ethanol were found to be 0.05, 1.2 and 18 mM respectively (from fig. 2), indicating that ethanol is less potent than (+)-cyanidanol-3 and GSH as a free radical scavenger in the system used.

Effect of GSH and (+)-cyanidanol-3 on the maximal chemiluminescent signal produced by the xanthine oxidase reaction

Additions	Chemiluminescence (cpm/ml)	Inhibition (%)	p
-	53,178 ± 2,701 (12)	-	-
(+)-Cyanidanol-3	26,297 ± 375 (5)	50.5	1×10^{-4}
GSH	33,864 ± 1,461 (5)	36.3	2×10^{-3}
(+)-Cyanidanol-3 + GSH	2.054 ± 87 (5)	96.1	1×10^{-8}

Values for chemiluminescence shown correspond to peak signals obtained after 138 sec of the addition of 0.12 units of xanthine oxidase to 3 ml of reaction mixture with and without the agents under study at 25 °C and corrected for background. (+)-Cyanidanol-3 and GSH were added to final concentrations of 0.05 mM and 1 mM respectively. Values shown represent means ± SEM with the number of determinations in parentheses. Significance studies were carried out using Student's t-test for unpaired data.

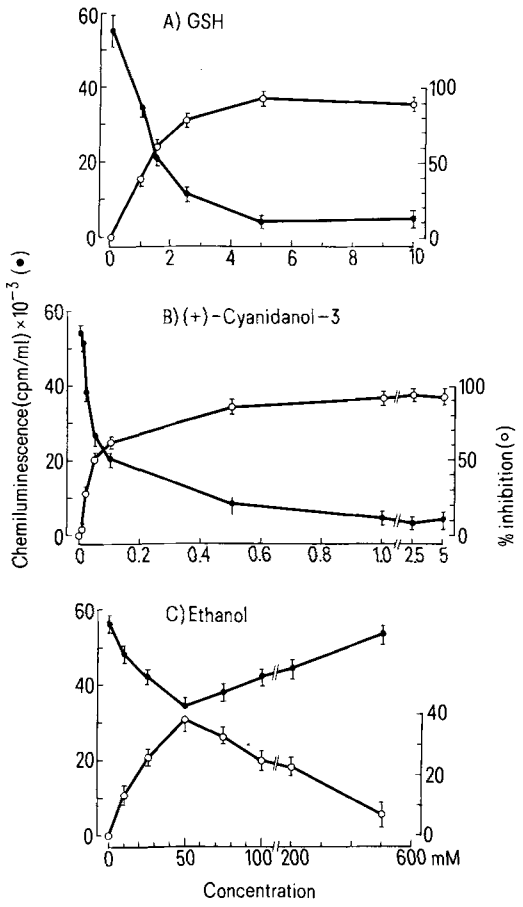

Figure 2. Effect of different concentrations of (A) GSH, (B) (+)-cyanidanol-3 and (C) ethanol on the maximal chemiluminescent response of the xanthine oxidase reaction. Each point represents the mean ± SEM for 5 to 12 determinations.

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Studies on the sex pheromone of *Dacus oleae*. Analysis of the substances contained in the rectal glands[1]

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Summary. 17 substances contained in the rectal glands of *Dacus oleae* have been identified by GC-MS techniques. The synthesis of 3 isomeric spiroketals allows us definitively to assign the structure of 1,7-dioxaspiro-[5,5]-undecane to the most volatile compound.

During the last few years we have developed studies on the substances emitted by *Dacus oleae* (Gmelin), (Trypetidae)[3-8]. This fly (olive fruit fly) is responsible for great damage to olive oil production, especially in the Mediterranean area, and until now, no efficient method for the control of this pest has been found.

We started our work by studying the complex mixture of volatile substances emitted by the female during the period of major sexual activity, and also analyzing the content of the rectal glands of the insect. Morphological studies[9,10] have shown that the anal gland of this insect is the producing and accumulating centre for substances with attractive activity.

In a recent publication[6] we have described all the substances identified in a 'cold trap' condensate of the emission of virgin female *Dacus oleae*, and the attractiveness displayed by some of them. In this paper we want to summarize the results of our analyses on the rectal glands of the insect.

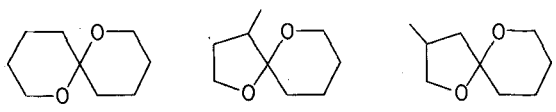
6-day-old virgin females were immobilized by keeping them in a refrigerator (− 10 °C) for some minutes. With the aid of a stereomicroscope the ovipositor was pulled out and the rectal glands excised from the abdominal organs.

3-5 freshly isolated glands were introduced in a glass capillary tube and in a solid-sample injection system connected with a GLC-MS apparatus. Best resolution of peaks was achieved with a SCOT glass capillary column CW 20 M (40 m, 0.25 mm ID) connected with a LKB 2091 mass spectrometer equipped with a Digital LKB 2130 data system.

A typical total ion current plot is shown in the figure. Table 2 shows the main MS fragmentations of the eluted peaks.

All the identified substances, with the one exception of the most volatile one (RT = 17.11 min), are fatty acids with the number of carbons ranging from C_6 to C_{18} esterified with C_1, C_2, C_3, C_4 aliphatic primary alchohols. Their structure was confirmed by comparison with commercially available or easily synthesized samples.

The sharp peak with retention time 17.11 min showed fragmentations (m/z 156 (22%), 128 (18), 111 (18), 101 (100), 100 (53), 98 (87), 83 (30)) which suggested the structure of spiroketal of MW 156[11]. An accurate analysis of the MS-spectrum of this compound in comparison with those described in the literature[12,13] allowed us to propose structures **1**, **2a**, **2b** as the most probable.



(1) **(2a)** **(2b)**