# Inhibition of firefly bioluminescence by scavengers of singlet oxygen, superoxide radicals and hydroxyl radicals

Ch. Richter[1]

*C. N. R. Unit for the Study of Physiology of Mitochondria and Institute of General Pathology, University of Padova (Italy), 5 October 1976*

*Summary.* The participation of highly energetic oxygen species in the ATP-induced bioluminescence of a firefly-extract has been investigated. The inhibition of light emission by a variety of specific scavengers suggests that singlet oxygen, superoxide radicals and hydroxyl radicals are important intermediates in the firefly bioluminescence reaction.

Bioluminescence is an enzyme-catalyzed chemiluminescence. The generic term luciferase refers to the enzyme that catalyzes an oxidation of the substrate luciferin, accompanied by light emission (for a recent review see M. J. Cormier et al.[2]). Some aspects of the bioluminescence are fairly well understood[3] for the firefly system, the luciferase having been purified and crystallized[4], and the stoichiometry of the reaction worked out[5]. In the presence of ATP, luciferin is converted to luciferyl adenylate, which is oxidized to oxiluciferin with 1 mole of $O_2$ consumed per each mole of luciferyl adenylate oxidized. At this stage emission of light occurs[3] with an intensity maximum around 560 nm.

Singlet oxygen has been considered, on theoretical grounds, as a possible intermediate in the oxidation reaction of luciferyl adenylate[6]. Oxygen radicals may lead to the formation of excited peroxides which decompose to an oxidized product in the excited state[7]. In these cases, the standard free energy of the intermediate product formed may be sufficient for the emission of photons of 560 nm. Following the suggestion of Hastings[6], the possible involvement of exited oxygen species and/or radicals has therefore been investigated by studying the effect of a number of known singlet oxygen and radical quenchers on ATP induced light emission in the luciferin/luciferase system. The results reported in this study suggest the participation of singlet oxygen and various radicals in the firefly bioluminescence.

*Materials and methods.* Firefly abdomina and adenosin-5′-triphosphate, grade II, disodium salt, were obtained from the Sigma Chemical Co. Superoxide dismutase was purchased from Miles Lab. Diphenylfuran was obtained from Eastman and recrystallized from ethanol before use. 1,4-diazo-bicyclo-(2,2,2)-octane and β-carotene were purchased from Merck. Dimethoxyethane was a product of Eastman. t-Butanol and mercaptoethanol were from Fluka and reduced glutathione from Zellstoffabrik Waldhof, Mannheim (Federal Republic of Germany). Cysteine was from Hoffmann-La Roche. $Tyr_2$-$Cu^{2+}$ was a generous gift from Prof. U. Weser, University of Tübingen (Federal Republic of Germany) and has been synthesized according to Weitzel et al.[8]. All other reagents were obtained from Merck.

Extracts of firefly abdomina were obtained by the following procedure: 100 mg abdomina were finely ground with quartz sand and taken up in 10 ml phosphate buffer (pH 7.5). After standing on ice for 15 min, the suspension was centrifuged at 3000 × g for 10 min. The supernatant was stored at 0 °C for 1 h and then centrifuged at 4000 × g for 10 min. The resulting supernatant was then frozen and thawed, and the precipitated proteins removed by centrifugation. The extract can be stored at −20 °C in the dark without loss of activity for several months.

A fine dispersion of diphenylfuran was obtained by repeated sonication in buffer under a stream of nitrogen. β-carotene was dissolved in ethanol. Control experiments showed that neither the slight turbidity caused by diphenylfuran, nor the addition of the coloured β-carotene, affected the efficiency of the light detecting system.

For the determination of the light emission, a specially designed apparatus was used (Bioluminiszenz-Messgerät Typ XP 2000, Skan AG, Basel, Switzerland). 0.5 ml buffer (50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$) containing 250 pmoles ATP were pipetted into the cuvette which was then placed in front of the photomultiplier. The light reaction was started by injecting 0.2 ml firefly extract and 1.3 ml. buffer (50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$) into the cuvette. The mixing was completed within 1.3 sec. The time course of the reaction was followed with a recorder and the relative amount of light produced during the first 15 sec of the reaction was printed out by an electronical integrator at the end of the reaction. Experiments were done in triplicates. The SE was ± 2%.

*Results.* When an extract of firefly abdomina is mixed with buffer containing ATP, an instantaneous light flash is observed, followed by a rapid decrease of the light intensity in the first few seconds and by a slower decay which lasts for many minutes. To quantitate the light emission in our experiments, a rapid mixing device was used. Figure A shows the typical time course during the first 15 sec of the reaction (upper curve). In the presence of diphenylfuran, a specific quencher of singlet oxygen[9], less light is emitted (lower curve). In figure B, it can be seen that higher concentrations of the quencher result in a diminution of the emitted light.

In similar experiments, a number of compounds, known to react with singlet oxygen and oxygen radicals, were tested for their ability to inhibit light emission. The table

Inhibition of firefly bioluminescence by scavengers of singlet oxygen, superoxide radicals and hydroxyl radicals

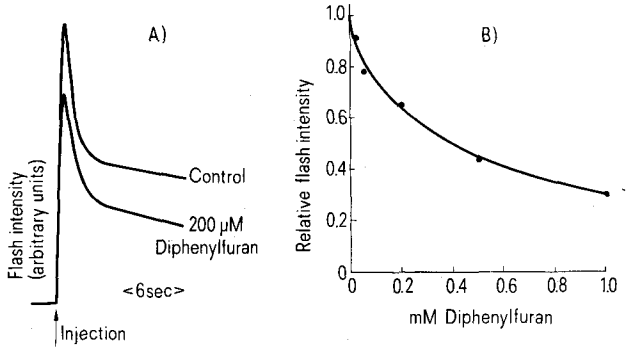
| Compound | Concentration required to give 50% inhibition* |
|---|---|
| Diphenylfuran | 0.4 mM |
| Azide | 1.5 mM |
| 1,4-Diazobicyclo-(2,2,2)-octane | 23 mM |
| $Tyr_2$-$Cu^{2+}$ | 100 μM |
| Glutathione | 7.5 mM |
| Cysteine | 0.75 mM |
| Dimethoxyethane | 3.5 % v/v |
| t-Butanol | 2.5 % v/v |
| Mercaptoethanol | 1 % v/v |
| Ethanol | 7 % v/v |

The relative light intensity in the presence of various scavengers was determined as described in the legend of the figure.
*To give an inhibition of 26% β-carotene (2.5 μM) was added in 50 μl of ethanol. Ethanol alone did not inhibit at this concentration.

reports the concentration of quencher required to give 50% inhibition of the reaction. The table shows that in addition to diphenylfuran, also diazobicyclooctane[10], azide[11] and β-carotene[12], known as singlet oxygen quenchers, inhibit the firefly bioluminescence. It seems unlikely that the inhibition by azide is due to an interaction with a redox component of the enzyme, since the firefly luciferase does not contain heavy metals[13]. This view was also supported by the finding that 10 mM KCN did not inhibit the firefly bioluminescence in our experiment.

Recent evidence suggests that superoxide ions ($O_2^-$) are involved in the bioluminescence of the clam Pholad, and that the light reaction is propagated by a radical chain reaction mechanism[14]. Interestingly, superoxide dismutase, the enzyme known to dismutate superoxide radicals, did not inhibit the reaction, and it was concluded that $O_2^-$ remains tightly bound to the luciferin/luciferase complex[14]. The lack of inhibition of light emission by superoxide dismutase, if $O_2^-$ radicals were involved in the reaction, is consistent with the notion that the high efficiency of light emission during bioluminescence[15,16] requires that the active species (i.e. $O_2^-$, $O_2^*$) is not easily accessible from the environment. As in the case of Pholad also, in our experiments superoxide dismutase (at a concentration of $2 \times 10^{-6}$ M) did not inhibit the light emission. The small and hydrophobic divalent copper complex $Tyr_2$-$Cu^{2+}$, however, which reacts with $O_2^-$ at a rate comparable to that of the enzyme superoxide dismutase[17], was found to be inhibitory at low concentrations.

Dimethoxyethane[18] and t-butanol[19] are considered to be specific quenchers of OH· radicals. The presence of small concentrations of these compounds (3.5 and 2.5% v/v, respectively) inhibits the firefly bioluminescence effectively. Furthermore, compounds which can act as oxygen radical or organic radical quenchers like glutathione, mercaptoethanol, cysteine and ethanol at higher concentrations were tested and found to be inhibitory (table). Similarly it has previously been reported that the bioluminescence of Balanoglossid is inhibited by cysteine and mercaptoethanol[20].




Bioluminescence of a firefly extract induced by ATP and its quenching by a scavenger of singlet oxygen.
0.5 ml buffer (50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$) containing 250 pmoles ATP were mixed with 0.2 ml firefly extract and 1.3 ml buffer (50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$), and the relative light intensity was measured for 15 sec. For details refer to 'materials and methods'.
*A* Time course of the light emission.
*B* Effect on the relative light intensity of increasing amounts of diphenylfuran, a specific quencher of singlet oxygen. Experimental conditions as in A.

*Discussion.* One of the central questions in bioluminescence is how excited molecules are generated whose decay is accompanied by the emission of photons of a minimal energy of 50 to 60 kcal per mole. The experiments reported above indicate the participation of singlet oxygen and oxygen radicals in the firefly bioluminescence. Substances which react specifically with singlet oxygen, $O_2^-$ radicals and OH· radicals inhibit light emission. A direct reaction of some of the scavengers with the excited state of the emitting species, or some other unspecific effect, cannot be excluded. The chemical diversity of the quenchers employed, however, together with their common property of reacting with singlet oxygen or oxygen radicals, suggests that the inhibition of the firefly light emission is related to the scavenging properties of the inhibitors. Singlet oxygen could be produced during the dismutation of $O_2^-$ radicals[21–23], during the interaction of $O_2^-$ with OH·[24] or $H_2O_2$[25], or during the decomposition of peroxides[26]. It is interesting to note that the small copper complex $Tyr_2$-$Cu^{2+}$, but not the relatively large enzyme superoxide dismutase, inhibits

1 Acknowledgments. I thank Prof. R. Bachofen, Institut für Allgemeine Botanik, Abteilung Mikrobiologie, Universität Zürich, Switzerland, in whose laboratory most of these studies have been performed, for his cooperativity. Financial support by the Deutsche Studienstiftung is gratefully acknowledged.

2 M. J. Cormier, J. Lee and J. E. Wampler, A. Rev. Biochem. *44*, 255 (1975).
3 W. D. McElroy and H. H. Seliger, in: Bioluminescence in Progress, p. 427. Ed. F. H. Johnson and J. Hanada. Princeton Univ. Press, New Jersey 1966.
4 A. A. Green and W. D. McElroy, Biochim. biophys. Acta *20*, 170 (1956).
5 M. J. Cormier, J. E. Wampler and K. Hori, Progr. Chem. Org. Nat. Prod. *30*, 1 (1973).
6 J. W. Hastings, A. Rev. Biochem. *37*, 597 (1968).
7 J. Lee and C. L. Murphy, in: Chemiluminescence and Bioluminescence, p. 381. Ed. M. J. Cormier, D. M. Herenles and J. Lee. Plenum, New York 1973.
8 G. Weitzel, F. Schneider, A. M. Fretzdorff and H. E. Heyke, Hoppe-Seylers Z. physiol. Chem. *307*, 14 (1957).
9 D. J. T. Porter and L. L. Ingraham, Biochem. biophys. Acta *334*, 97 (1974).
10 C. Ouanness and T. Wilson, J. Am. Chem. Soc. *90*, 6527 (1968).
11 D. R. Kearns, Chem. Rev. *72*, 395 (1971).
12 C. S. Foote and R. W. Denny, J. Am. Chem. Soc. *90*, 6233 (1968).
13 M. De Luca, G. W. Wirtz and W. D. McElroy, Biochemistry *3*, 935 (1964).
14 A. M. Michelson and F. M. Isambert, Biochimie *55*, 619 (1973).
15 H. H. Seliger and W. D. McElroy, in: Light: Physical and Biological Action. Academic Press, New York 1965.
16 H. H. Seliger and W. D. McElroy, Archs Biochem. Biophys. *88*, 136 (1960).
17 R. Brigelius, H. J. Hartmann, W. Bors, M. Saran, E. Lengfelder and U. Weser, Hoppe-Seylers Z. physiol. Chem. *356*, 739 (1975).
18 L. M. Dorfman and G. E. Adams, Reactivity of the Hydroxyl Radical in Aqueous Solutions NSRDS-NBS No. 46. U. S. Department of Commerce, National Bureau of Standards (1973).
19 P. Neta and L. M. Dorfman, Adv. Chem. Sci. *81*, 222 (1968).
20 L. S. Dure and M. J. Cormier, J. biol. Chem. *239*, 2351 (1964).
21 A. U. Khan, Science *168*, 476 (1970).
22 D. Behar, G. Czapski, L. M. Dorfman, J. Rabani and H. A. Schwarz, J. Phys. Chem. *74*, 3209 (1970).
23 D. Klug, J. Rabani and I. Fridovich, J. biol. Chem. *247*, 4839 (1972).
24 F. Haber and J. Weiss, Proc. Roy. Soc. London A *147*, 332 (1934).
25 E. W. Kellog and I. Fridovich, J. biol. Chem. 250, 8812 (1975).
26 M. M. King, E. K. Lai and P. B. McCay, J. biol. Chem. *250*, 6496 (1975).

the light emission. The high quantum yield of bioluminescence requires a protection from collisional deactivation. This could be achieved by a spacial arrangement in which the active centre is not accessible to the large enzyme molecule.

The observation reported in this study should also be considered of practical relevance for the employment of the firefly bioluminescence as an analytical tool for the determination of very small quantities of ATP, ADP, creatine phosphate and other energy-rich organic phosphates in biological extracts. Since the extracts may contain appreciable amounts of compounds (e.g. azide, cysteine, mercaptoethanol, glutathione, alcohols) which interfer with the light emission, these assays should be interpreted with caution unless the appropriate controls have been performed.

## Carbohydrate composition of the human cataractous lenses

J. F. Alao[1,2]

*College of Medicine, University of Lagos, Biochemistry Department, P. M. B. 12003, Lagos (Nigeria), 15 July 1976*

*Summary.* Specific chemical assays, including carbohydrate, hexosamines and hexuronic acid, were determined on the lens insoluble albumoid. It was noticed that the carbohydrate composition varies with age. The significance of carbohydrate in the lens is discussed.

The structural composition of the insoluble albumoid of the human cataractous lenses has not been clearly defined. It has been hypothesized[3] that cataract formation is associated with the conversion of low molecular weight protein to high molecular weight aggregates, the heaviest of which corresponds to the insoluble protein fraction. Various authorities[4,5] have suggested that the presence of high molecular protein aggregates in the lens can lead to increased scattering of light as a consequence of loss of transparency. Others[6–8] indicated that calcium or glucose may influence the aggregate state of the subunits of α-crystallin protein. The carbohydrate composition of the insoluble albumoid in the human cataractous lens is mostly unknown. The present investigation is concerned with the pattern and composition of the carbohydrate in cataractous lenses removed at surgery.

*Materials and methods.* Human lenses were obtained from patients following cataract operation, usually within 2 h of removal. 8 human cataractous lenses were used in each age group. The procedure for extracting insoluble albumoid and its subsequent treatment with urea has been fully described[9].

Hexuronic acid. Hexuronic acid was determined by a modification of Bitter and Muir[10] of the method of Dische[11]. Hexosamine. Weighed samples in 1 ml were hydrolysed in vacuo in sealed tubes at 110 °C. Total hexosamine was determined by the method of Muir and Jacobs[12] and the distillation procedure of Cessi and Peliego[13]. Hexose. The procedure was that described by Grant and Jackson[14] using Dowex 50 ($H^+$ form) resin hydrolysis. After hydrolysis neutral sugar and amino sugars were separated according to the method of Anastassiadis and Common[15].

Table 1. Carbohydrate composition (quantitative). The neutral sugar content of the urea extracts USF, $UIF_A$ and $UIF_B$ from the human cataractous lenses aged 20–91 years were estimated by the orcinol sulphuric acid reagent method of Tillman and Phillip[18]. Glucose was used to plot a calibration curve

| No. | Average age of patient | Protein USF (%) | $UIF_A$ (%) | $UIF_B$ (%) |
|---|---|---|---|---|
| 1 | 22 ± 2 years | ND | 1.00 | 0.975 |
| 2 | 38.5 ± 0.5 years | 0.800 | 1.208 | 0.980 |
| 3 | 43 ± 2 years | 0.700 | 1.182 | 1.00 |
| 4 | 53 ± 3 years | 0.500 | 1.396 | 0.900 |
| 5 | 65 ± 2 years | 0.698 | 1.533 | 1.100 |
| 6 | 74 ± 4 years | 0.425 | 1.428 | 1.108 |
| 7 | 82 ± 2 years | 0.500 | 1.864 | 1.621 |
| 8 | 90.5 ± 0.5 years | 0.500 | 2.118 | 1.968 |
| 9 | Adult bovine | 0.799 | 1.150 | 0.969 |

ND, not detectable.

Table 2. Distribution of carbohydrate in the insoluble lens extract of human cataractous lenses, average age 20–90 years; using paper chromatography

| Protein | Age (years) | Galactose | Glucose | Mannose | Fucose | Xylose |
|---|---|---|---|---|---|---|
| USF | 20 | + | + | ND | ND | ND |
| | 30 | + | ++ | ND | ND | Trace |
| | 40 | + | ++ | ND | ND | Trace |
| | 50 | + | ++ | ND | ND | ND |
| | 60 | + | ++ | ND | ND | Trace |
| | 70 | + | ++ | ND | ND | ND |
| | 80 | + | ++ | ND | Trace | ND |
| | 90 | + | ++ | ND | ND | ND |
| $UIF_A$ | 20 | + | +++ | ++ | + | + |
| | 30 | + | +++ | ++ | ++ | + |
| | 40 | ++ | +++ | ++ | ++ | + |
| | 50 | ++ | +++ | ++ | ++ | + |
| | 60 | ++ | ++++ | ++ | ++ | + |
| | 70 | ++ | ++++ | +++ | ++ | + |
| | 80 | ++ | ++++ | +++ | ++ | + |
| | 90 | ++ | +++++ | ++++ | ++ | + |
| $UIF_B$ | 20 | + | ++ | + | ND | + |
| | 30 | + | ++ | + | ND | + |
| | 40 | + | ++ | + | ND | + |
| | 50 | + | ++ | ++ | ND | + |
| | 60 | + | ++ | ++ | Trace | + |
| | 70 | + | ++ | ++ | + | + |
| | 80 | + | +++ | ++ | + | + |
| | 90 | + | +++ | ++ | ++ | ++ |

+ Indicates the relative intensity of the spot on the chromatogram after dipping in sodium thiosulphate; quantitative value for + 8%. ND, not detectable.