Lucigenin chemiluminesence

A new approach to study the redox activity of Ehrlich ascetic tumor cells

Ali H. Al-Hashimi, *MSC, PhD,* ***K. V. Ettinger,*** *MSc, PhD.*

ABSTRACT

Objective: To study the mechanism and to measure the oxygenation/reduction activity of Ehrlich ascetic tumor cells in an isotonic solution by means of lucigenin dependent chemiluminesence.

Methods: All the measurements of the chemiluminesence redox activity of Ehrlich ascetic tumor cell samples in suspension medium, were carried out using a photon counting system especially designed to the purpose. The areas under the chemiluminesence kinetic curves were measured at different cell physiological conditions and at different agents concentrations effect.

Results: The rates of lucigenin chemiluminesence redox functional activity of Ehrlich ascetic tumor cells were significantly different from normal oxygenation/reduction activity at different physiological environments of Ehrlich ascetic tumor cells suspension conditions (i.e. cell membrane permeability changes, presence and absence of oxidative or reductive agents at different concentrations and oxy/hydroxy free radical protective agents).

Conclusions: The results explain more fully the mechanism and the value of a lucigenin dependent chemiluminesence probe of the redox functional activity of Ehrlich ascetic tumor cells (at different physiological conditions of suspension).

Keywords: Chemiluminesence-lucigenin probe, Ehrlich ascetic tumor cell function.

Saudi Medical Journal 2001; Vol. 22 (2): 164-169

The activation of metabolism or a respiratory burst that follows stimulation of cells is characterized by an increase in consumption of oxygen (non-metochondrial) and by glucose utilization via dehydrogenases of the hexose monophosphate shunt.[1,2] In the present study, the oxygenation activities of Ehrlich ascetic tumor (EAT) cells were investigated by chemiluminesence (CL) probing. A chemiluminesence probe (CLP) is defined as a substrate whose oxygenation is associated with a high yield of luminesence. These substrate probes are exogenous organic molecules, which are introduced to a biological system and serve as substrates in oxygenation reactions producing electronically excited products with a high yield. The CLP substrate provides a continuous non-distractive and quantitative method for indication of oxygenation activity in cells and must satisfy certain criteria. These include: high CL yield, soluble in water at neutral pH, chemical reactivity suitable for the type of oxygenation measurement and non-toxic to the biological system at the concentration employed. The physical and chemical properties of lucigenin make it ideally suited for a CLP.[3] The knowledge of the emitted wavelength can be used in the selection of the photo multiplier tube with high photo cathode quantum efficiency. Furthermore, the use of band-pass filters may eventually allow for the

From the Department of Physiology and Medical Biophysics (Al-Hashimi), University of Basrah, College of Medicine, Basrah, Iraq and the Department of Bio-medical Physics and Bio-engineering, (Ettinger) University of Aberdeen, Foresterhill, Aberdeen, Scotland, United Kingdom.

Received 9th December 1999. Accepted for publication in final form 18th April 2000.

Address correspondence and reprint request to: Dr. Ali Al-Hashimi, Head of Department of Physiology and Medical Biophysics, University of Basrah, College of Medicine, Basrah, Iraq.

simultaneous measurement of 2 different oxygenation activities with 2 CLP's. The emission of photons during the relaxation of electronically excited oxygen derivatives is, generally, of low intensity; it can, however, be enhanced when CL substrates like lucigenin are present. The sensitivity of the measurement of redox activity of EAT cells by CL can be increased and amplified through introduction of a CL substrate whose oxidation gives a high yield of electronically excited products. lucigenin is introduced in this work as an additional reactant, since lucigenin will emit light upon dioxygenation and serve as an amplifier to detect low levels of various oxidative species. This CLP of Lucigenin is essentially independent of the medium and therefore eliminates possible variation in CL response due to various compositions of the suspension.[3] Also lucigenin CL appears to indicate the presence of oxidized species throughout the duration of EAT cell survival (a dead cell has no CL in the presence of lucigenin).

Methods. Two suspension media have been used in this study, at pH=7.4 Medium A: consists of Hank's Balance salt (BBS) from Sigma Chemical Co, (containing sodium bicarbonate and D-glucose) with 10% added calf serum. Medium B: consists of a simple ionic medium (simple salt solution) with 10% added calf serum. The sustained of EAT cells was carried out and maintained in (Foresterhill Animal House, Aberdeen, Royal infirmary, University of Aberdeen, UK) adult MF1-Swiss mice by weekly intraperitoneal inoculation of 0.1 ml of cells into the peritoneal cavity. Seven days later, EAT cells were withdrawn through an intact peritoneum with a sterile syringe and used directly. Grossly hemorrhagic tumor was discarded. Tumor cells were removed to either BBS medium with added 10% calf serum (medium A) or to simple salt solution with 10% added calf serum (medium B). Both media A and B were at pH 7.4 and at a temperature of 37°C.

Removed tumor cells were washed twice with medium B. This effectively removed any contaminating red cells, which might have been present. Red blood cells inhibit the CL signal in a dose dependent manner.[4] Following that, the ascites cells was resuspended with new fresh medium A, at pH 7.4 and temperature of 37°C. The cells were shaken every 5 minutes to prevent aggregation. Cell survival was measured in triplicate by means of erythrosine B dye. In each experiment conducted in this study, the number of cells kept were constant at $3x10^6$ cells, pH 7.4 and temperature 37°C, unless mentioned to the contrary. A solution of lucigenin (10, 10' -dimethyl-9-9'-biacridinium dinitrate) DBA++ was prepared with either BBS medium or simple ionic salt solution (medium B). The molar concentration of lucigenin used was (10^{-2} m). Other reagent solutions (Ouabain, Superoxide dismutase (SOD), GSH, Adenosine triphosphate (ATP) and CL inhibitors solutions) were made in the individual experiment at the concentration employed there. The BBS medium containing D-glucose of 1g/ml plus 10% calf serum was used. Detailed information and experimental conditions of each designed experiments used are shown under the caption of the experimental figures.

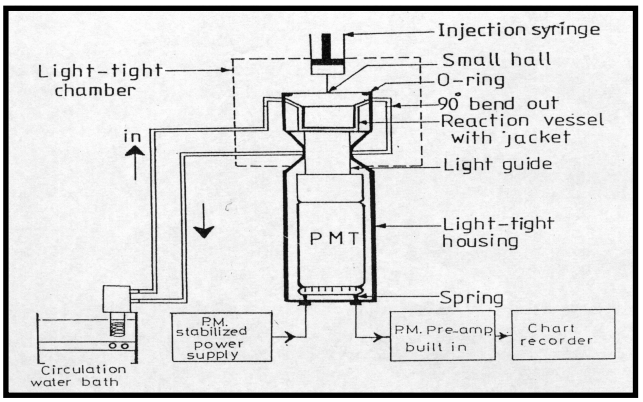

Figure 1 - Schematic diagram of the photon counting system.

The CL light output from the biological system of EAT cells plus lucigenin was measured using a photon counting system, this system was designed and built to be used in this study, Figure 1. The system consists of single photomultiplier (PM) (50 mm diameter, type 9635QB from EMI Electronic Limited, UK), with window passed light to 13-stage bialkali K-Cs photocathod, with spectral response from nearly 300 to 660 nm. The PM tube was mounted inside a box, which was attached by means of coupling to the light tight chamber; the output

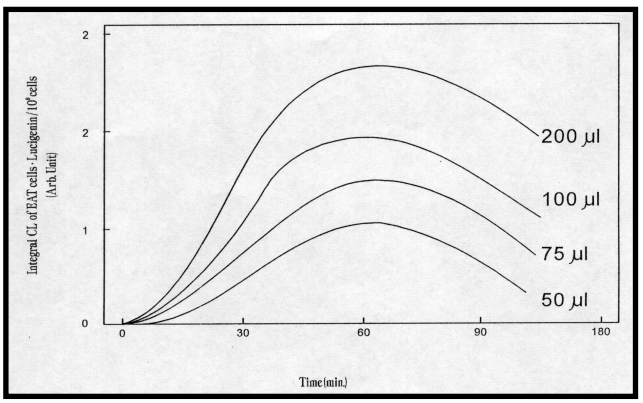

Figure 2 - Typical intensity waveform of EAT cells + lucigenin in the presence of Glucose (1mg/ml) at different lucigenin concentrations and at pH = 7.4 and temperature of 37°C. Lucigenin molar concentration was 10^{-2}M. The arrow shows the moment of lucigenin injection.

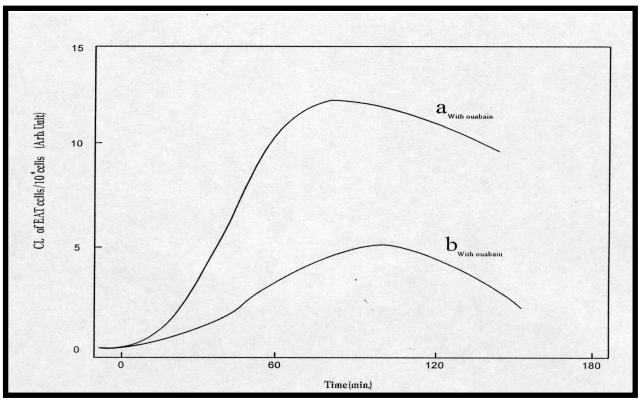

Figure 3 - Chemiluminesence of EAT cells + lucigenin plotted against time (Kinetic plot) in the presence and absence of Ouabain final concentration 10^{-2}M. EAT cells were suspended in BBS medium containing D-glucose (1ug/ml) + 10% calf serum at pH = 7.4 and temperature of 37°C. The lucigenin was added at zero time indicated by the arrow, inital concentration 100 ul (10^{-2}M). Each point represents the mean + SD of 3 measurements.

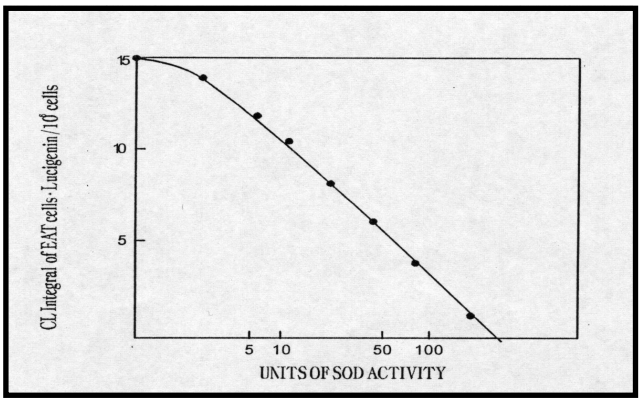

Figure 5 - Chemiluminesence of 2 hours (kinetics) time integral of EAT cells ($CL/10^6$ cells plotted against the unit of SOD activity (log-scale) at 1 mg SOD = 750 units activity. Lucigenin initial concentration 100 ul of 10^{-2}M solution. EAT cells suspended in BBS medium plus 10% calf serum at pH = 7.4 and temperature of 37°C. Each point represents the mean of 3 replicate measurements, SD not shown. (Too small to be shown on the scale of the graph.

from the anode of the PM tube was monitored by electronic chart recorder through a preamplifier.

It has noted that EAT cells in suspension medium plus lucigenin exhibited CL. This CL at different lucigenin concentrations does not begin immediately but after a certain time. In order to quantify the effect of lucigenin concentration on the CL, the number of EAT cells, the volume and the type of medium suspension were all held constant and the concentration of lucigenin varied. Cell membrane permeability changes using a solution of Ouabain was used to study the effect of a K^+, Na^+ dependent ATPase pumping system on the CL redox activity of EAT cells. Inhibition of CL redox activity with different types and concentrations of the inhibitor was studied. Also the protective effect of GSH in the reduced form was investigated. This was carried out after 60 minutes incubation with the inhibitor or the protector, the cells were then washed twice with BBS solution and resuspended with a fresh medium and incubated at 37°C for survival to be determined using erythrosine B dye. The effect of glucose or the ATP level in the medium was investigated, in this case a simple salt solution with 10% calf serum was used for the control. The concentration of both glucose or ATP was 1 mg/ml in the test sample.

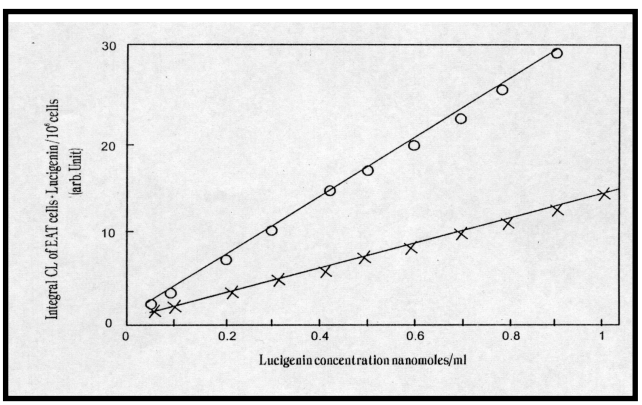

Figure 4 - The chemiluminesence of EAT cells + lucigenin plotted against lucigenin concentration at pH = 7.4 and temperature 37°C. Upper graph: Ouabain presents (initial concentration 10^{-2}M). Lower graph: no ouabain in solution. Each point represents the mean of 3 replicates (integral CL for 2 hours time interval).

Results. The CL of EAT cells plus lucigenin shows significant increases of up to 60 minutes duration followed by gradual decreases in the presence of glucose, Figure 2. However, heat - killed EAT cells (i.e. 60°C for 20 minutes) yield no CL when lucigenin was added. Also no CL above background was detected from EAT cells alone at 37°C. Figure 3 shows the plot of kinetic CL activity from EAT cells plus lucigenin with and without addition of ouabain, the CL redox activity was affected significantly. Also the plot of the integral CL redox activity for 2 hours time interval against the different concentrations of lucigenin was different in the presence and absence of ouabain, Figure 4. Inhabitations of CL redox activity were different using different inhibitors. Figure 5 shows CL, of EAT cells plus lucigenin at different concentrations of SOD (EAT cells were suspended in BBS medium containing 1 mg/ml D-glucose plus 10% added calf serum). An inhibition of the CL kinetic redox activity of EAT cells plus lucigenin was shown in a solution of: glutathione in a reduced form, SOD, 2-mercapto-1methyl imidazole or L-histidine. The degree of the inhibition of CL kinetic

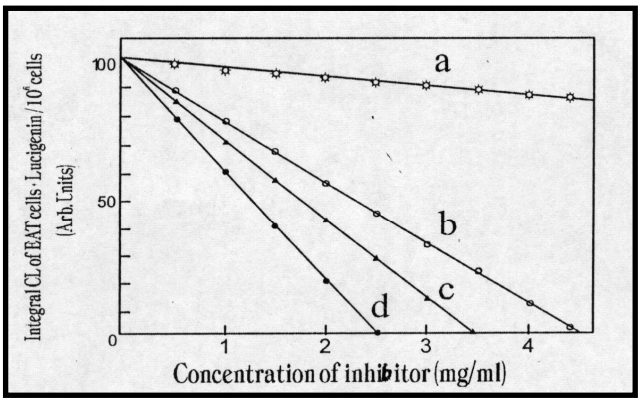

Figure 6 - The integral CL of EAT cells + lucigenin as a function of concentration of different inhibitors used. Condition of experiments: pH = 7.4 and temperature 37ºC , medium of suspension is BBS (sigma) containing 1 mg/ml glucose plus 10% calf serum. Lucigenin concentration in injected solution 100 ul of 10^{-2}M. Each point represents the mean of 3 replicates. SD is too small to be shown on the graph scale. a. L-Histidine. b. SOD, 1 mg/ml (750 units of activity, bovine). c. 2-mercapto-1-methyl imidazole. d. reduced glutathione (GSH).

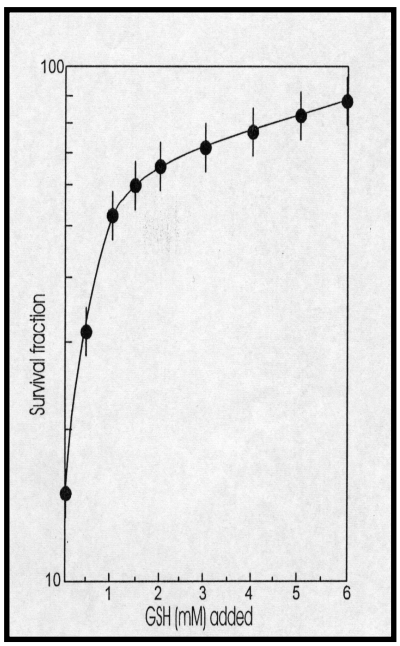

Figure 7 - Effect of glutathione (GSH) concentration on the cytotoxicity of lucigenin (100 ul of 10^{-2} M) towards EAT cells at pH = 7.4 and temperature of 37ºC. Each point represents the mean ± SD of at least 3 replicates.

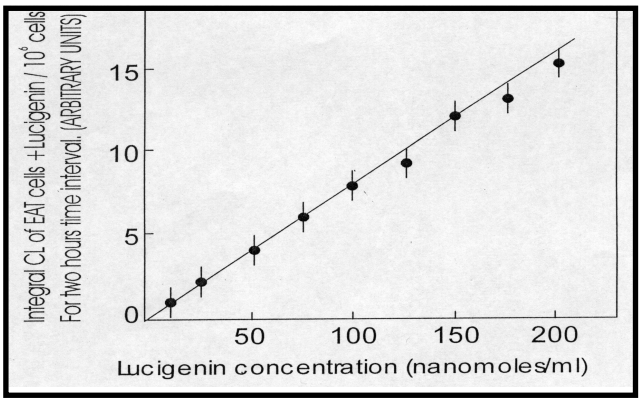

Figure 8 - The plot of integral CL of EAT cells + lucigenin at pH = 7.4 and temperature of 37ºC in glucose containing medium (1 mg/ml). Each point represents the mean ± SD of 3 replicates.

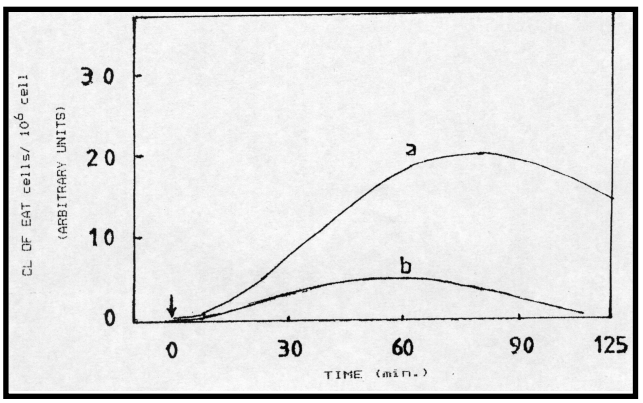

Figure 9 - The CL response of EAT cells + lucigenin in the presence and absence of ATP of final concentration of 1 mg/ml. The suspension medium was a simple salt solution plus 10% calf serum (no glucose). ATP concentration 1 mg/ml. Each point represents the mean + SD of 3 replicate measurements. a. Without glucose (ATP 1 mg/ml). b. Without glucose (no ATP). The arrow shows the moment when 100 ul of 10^{-2} of lucigenin solution was injected.

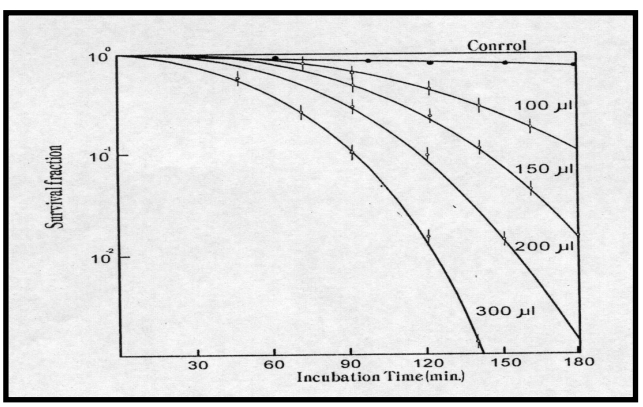

Figure 10 - The toxic effect of various concentration of lucigenin (10^{-2}M) solution on EAT cells for various times of incubation at pH = 7.4 and temperature of 37ºC. Each point represents the mean of triplicate measurements ± SD. (Points without bars, with SD too small to be shown on the graph scale).

redox activity depends on the type and concentration of the inhibitor employed, Figure 6.

GSH in the reduced form shows a protective effect when added to the medium of EAT cells suspension just prior the addition of lucigenin, Figure 7 (the cells were incubated in a suspension medium of BBS containing 1 mg/ml initial concentration of d- glucose plus 10% calf serum). The integral CL for 2 hours time interval were linear function of lucigenin concentration. It is suggested that integral CL can be considered when comparing CL data. The CL redox activity as a function of time of EAT cells plus lucigenin in the presence and absence of ATP, indicated that the addition of ATP instead of D-glucose, restored the level of the CL and acted similarly as glucose, Figure 9.

Discussion. A treatment of EAT cells in suspension with lucigenin at different concentrations (under condition of pH 7.4 in glucose medium at temperature of 37°C) results in a decrease in the shoulder of the cytotoxicity survival curves and indicates little effect on the exponential slopes, Figure 10. Addition of GSH to the medium extends the shoulder of the lucigenin cytotoxicity curve. There is little or no killing after 60 minutes exposure to 100 ul (10^{-2} M) lucigenin, or 240 minutes exposure to 50 ul of 10^{-2} M lucigenin. The Kinetic of cell inactivating by lucigenin may involve some sublethal process and contributing to the ultimate lethal event. It has been noticed that addition of reduced GSH removed the superoxide oxy-radical 02^{-} and lead to suppression of CL of EAT cells plus lucigenin. (The significant protection by GSH against the toxic effect of lucigenin from the exogenous superoxide is shown in Figure 7). The cytotoxicity of lucigenin towards the EAT cells in vitro may be as a result of electron abstraction from the target molecules and the action of free radicals formed in the reduction of the lucigenin. The cytotoxic action may involve irreversible binding of lucigenin radical to the target molecules which leads to a CL rise with time, followed by decaying within 2 hours to about 10% of maximum level. This is evidence that metabolic reduction of lucigenin might involve oxygen of the medium. Lucigenin has been shown in this work to produce CL upon addition to EAT cells in a glucose medium. This CL does not begin immediately with different concentrations of lucigenin (Figure 2), therefore, there must be a triggering mechanism, which eventually turns on the metabolic process and produced light. The possible mechanism for the CL of EAT cells plus lucigenin system, may be categorized in the following ways.

Membrane potential difference and permeability changes. The first event that may occur is the binding of lucigenin to its receptor on the external surface of the EAT cells. Indirect evidence for receptor mediation could come from an early perturbation of the membrane, i.e. depolarization by activators, if a change in membrane potential difference (transmembrane potential) occurs before the generation of superoxide (02^{-}), these changes in electrical potential could be a link between the binding of the stimulant ligament (or perturbance) and the metabolic events and CL.

The linkage between ionic electrical events and metabolic events in the membrane can be traced using oubain as an inhibitor of K^{+}-Na^{+} dependent ATPase, Figures 3,4. In the presence of glucose, lucigenin is believed to stimulate oxygen uptake by EAT cells implying that oxygen is an ultimate electron acceptor leading eventually to CL, which increases with time. The route is via lipooxygenase producing a variety of products as a result of a rapid depolarization followed by a prolonged hyper-polarization of the transmembrane potential.[5] Valid evidence indicates that these shifts in transmembrane potential proceeded the release of superoxide anion or the generation of CL induced by these stimulants.[5] Therefore, it has been suggested that depolarization of the membrane potential acts to trigger the respiratory burst leading to the generation of CL. A hyper polarization occurs, which can be inhibited by the addition of 100 ul of 10^{-2}M solution of ouabain, (Na^{+}, K^{+} pump inhibitor), leading to an increase in the rate of CL of the EAT cells plus lucigenin. Briefly, the addition of the lucigenin may have 2 effects on EAT cells: (i) change in the ionic permeability, which leads to a change in membrane potential. (ii) Depolarization of the cell membrane, which may trigger release of superoxide into the extra cellular fluid that subsequently reacts with lucigenin and gives light.

Non-enzymatic reaction of lucigenin. The dependent-lucigenin CL of EAT cells is found to be inhibited by SOD as shown in Figures 5 and 6. These observation may implicate the product of oxygen metabolism formed during the respiratory burst as mediators of CL in EAT cells. The luminesence may be an indication that EAT cells form singled oxygen ($^{1}0_{2}$) during the respiratory burst. This interpretation is based on the relaxation of singled oxygen $^{1}0_{2}$ to triplet states and the formation of dioxetane by the addition of singled oxygen $^{1}0_{2}$ across carbon bonds (electron-rich doubled bond) which leads to light emission. The spontaneous cleavage of the dioxetane into 2 carbonyl compounds, one of which is an electronically excited state and emits a photon as it decays to the ground state.[6] The source of singled oxygen $^{1}0_{2}$ was postulated to be a superoxide oxy radical 02^{-}, which reacts with itself to form hydrogen peroxide (H_20_2) and can subsequently react with H_20_2 to generate singled oxygen ($^{1}0_{2}$) plus a hydroxyl radical OH.[7]

The addition of histidine (an amino acid known to scavenge singled oxygen) depressed CL emission by

10%, which indicates that a little of the light can be attributed to the oxygen relaxation of single-triplet, Figure 6. Since maximum intensity of CL is reached after about 60 minutes following addition of lucigenin, (Figure 2), this can possibly be explained by the slow accumulation of H_20_2 and spontaneous dismutation of the superoxide oxy-radical 02^{-} produced in this system at very low steady state concentration. The light emission from EAT cells plus lucigenin are inhibited by relatively high concentrations of the SOD enzyme for the same degree of inhibition using the reduced GSH in the low concentration, Figures 5 and 6. Glutathion in the reduced form (GSH) is know to serve as a free radical inhibitor and as a substrate for glutathione peroxidase, keeping the cellular concentration of H_20_2 and hydroperoxidase low, together with radicals such as superoxide oxy-radical 02^{-}. Nicotinamide adenine dinucleotide phosphate (NADPH) acts to maintain GSH in the reduced form, due to its greater affinity for superoxide oxy-radical 02^{-} compared with lucigenin.[7] The rapid inhibitory action of GSH on the CL in this system, favors the assumption of extra cellular scavenging of oxy-radicals in a non-enzymatic process.[8] The thiol group (GSH) does not react rapidly enough with the hydroxy radical OH.[9]

Enzymatic reaction of lucigenin. The enzyme responsible for the manifestation of respiratory burst in the cells is NADPH which catalyses the following reaction.[10,11]

$$NADPH + 2O_2 \text{ ------- } NADP^{+} + 20_2^{-}$$

The oxy-radical 0_2^{-} reacts with itself to give singled oxygen.[12] The singled oxygen quencher (histidine) used in relatively high concentrations, does not inhibit completely the CL of EAT cells plus lucigenin, Figure 6. As described earlier, the CL was inhibited by SOD and diminished by GSH. These experiments implicate the superoxide anion rather than the singled oxygen as a reaction intermediate. However this conclusion is somehow complicated by 2 facts. Firstly, the superoxide ion can be apparently oxidized to give singled oxygen.[13,14] Secondly; superoxide dismutase can remove the superoxide by catalyzing the reaction:

$$20^{-}_2 + 2H \text{ ---------- } H_20_2 + 0_2$$

No attempt has been made to analyze the spectrum of the emitted light and hence to indicate the extent of possible involvement of singled oxygen in EAT plus lucigenin CL.

Acknowledgments. We would like to greatly thank all the people in the Department of Bio-medical physics and Bio-engineering, University of Aberdeen, UK, especially Professor J.R. Mallard for the opportunity to carry out the research and to friends and colleagues, Mrs. Duff and Dr. Sandra Dizon for their generous help and advice. We are grateful to all members of the electronic and mechanical workshop, especially Mr. Dick, Mr. J. Hallidy, Mr. George, and Mr. Eddy for their valuable help in the maintenance and building of the experimental devices. Also, we would like to express our appreciation and gratitude to all staff in the Animal House at Foresterhill for their help on the technical side and for preparing the EAT cell lines, especially Miss Moreen. Finally, great thanks to the University of Basrah, Iraq for the grant of research.

References

1. Greger EA, Drum AS, Elston RA. Measuring of oxidative activity in hemocytes of the pacific razor clam, siliqua spatula and the oyster crassostrea gigas, using lucigenin and luminol - dependent chemiluminesence. J Invertebr Pathol 1995; 65: 48-60.
2. Friedl FE, Alvarez MR. Oxygen uptake, oxidant production and luminol-enhanced chemiluminesence by hemocytes of eastern oysters. J Shellfish Res 1993; 12: 131.
3. Robert CA. Biochemiexcitation chemiluminesence and the study of Biological oxygenation reactions. In: Waldemar A and Giuseppe C, editors. Chemical and Biological Generation of Excited states. London: Academic Press; 1982. p. 309-344.
4. Al-Hashimi AHM, Mohamed FH, Lazim SK. A comparative study of Hemoglobin estimation by the traditional (WHO) technique and chemiluminesence Method. Dirasat Medical and Biological science 1997; 24: 161-177.
5. Know VD, Cythia VD, David P, Maria M. Preliminary events leading to the production of luminol-dependent chemiluminesence by human granulocytes. In: Deluca and McElroy, editors. Bioluminesence and Chemiluminesence: Basic Chemistry and Analytical Application. New York (USA): Academic Press; 1981. p. 45-52.
6. Waldemar A. Determination of chemiexetation yield in the generation of electronic excitation from dioxetanes. In: Wakdemar A, Giuseppe C, editors. Chemical and Biological Generation of Excited States. London: Academic Press; 1982. p. 115-152.
7. Osamu S. Mechanism of Biolominescence. In: Waldemar A, Giusepp C editors. Chemical and Biological generation of Excited States. London: Academic Press; 1982. p. 249-276.
8. Lash LH, Rutt DA, Zalups RK. Role of extra cellular thiols in accumulation and distribution of inorganic mercury in rat renal proximal and distal tubular cells. J Pharmacol Exp Ther 1998; 285: 1039-1050.
9. Arthur IG, Gerald C. Microsomal metabolism of hydroyle radical scavenging agent. In: Rodgers MAN, Powers EL, editors. Oxygen and Oxy-radical in Chemistry and Biology. London: Academic press; 1981. p. 612-614.
10. Zubay G. Nucleotides. In: Kane K, editor. Biochemisry. 3rd ed. Vol 2 (Catabolism and Biosynthesis). London: WC Brown; 1993.
11. Bellative P. The superoxide forming enzymatic system of phagocytes. Free Radi Biol Med 1988; 4: 225-261.
12. Bgchi K, Puri S. Free radical and antioxidant in health and disease. Eastern Mediterranean Health 1988; 4: 550-560.
13. Cross CE, Halliwell B, Borish ET, Pryor WA, Saual RL, McCord JM et al. Oxygen radical and human diseases. Annals Intern Med 1987; 107: 526-545.
14. Halliwell B, Grootveld M. The measurement of free radical reaction in humans. Febs Lett 1987; 5213: 9-14.