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TITLE: Mechanisms of Bioluminescence, Chemiluminescnece and of Their Regulation

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PROGRESS REPORT: One year period through March, 1976.

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A. During the past year the following papers have been written, accepted for publication, or presented at scientific meetings:

Ward, W. W. and H. H. Seliger COO-3277-24
Action Spectrum and Quantum Yield Determinations for Photoinactivation of Mnemiopsin, A Bioluminescent Photoprotein from the Ctenophore Mnemiopsis sp. Photochem. & Photobiol. 23 (1976)

Seliger, H. H. COO-3277-28
The Origin of Bioluminescence
Photochem. & Photobiol. 21, 355-361 (1975)

Seliger, H. H. COO-3277-31
A Common Enzyme Mechanism for the Production of Reactive Chemical Species of Carcinogenic Polycyclic Aromatic Hydrocarbons, Paper presented at Ann. Mtg. FASEB, Atlantic City, N.J. April, 1975; Fed. Proc. 34, 623 (1975).

Hamman, J. P. and H. H. Seliger COO-3277-36
Chemiluminescence from Liver Microsomes during the Hydroxylation of the Carcinogen Benzo[α]pyrene. Paper at the Ann. Mtg. Amer. Soc. for Photobiol., Denver, Colorado, Feb., 1976.

Hamman, J. P., Biggley, W. H., and H. H. Seliger COO-3277-37
A Kinetic Model for the Photoinhibition of Mechanically Stimulable Bioluminescence in Marine Dinoflagellates and the Implications for the Measurement of Action Spectra. Paper at the Ann. Mtg. Amer. Soc. Photobiol., Denver, Colorado, Feb., 1976.

Seliger, H. H. COO-3277-38
Chemiluminescence from Liver Microsomes during Hydroxylation of Carcionogens. Paper at the Michael Kasha Symposium on Energy Transfer in Organic, Inorganic and Biological Systems, Tallahassee, Florida, Jan., 1976.

Hamman, J. P. and H. H. Seliger COO-3277-39
The Chemical Formation of Excited States During Hydroxylation of the Carcinogenic Hydrocarbon Benzo[a]pyrene by Liver Microsomes. Biochem. Biophys. Res. Commun. accepted.

Seliger, H. H. and J. P. Hamman COO-3277-30
The Chemical Production of Excited States: Chemiluminescence of Carcinogenic Hydrocarbons Accompanying Their Metabolic Hydroxylation and a Proposal for Common Active Site Geometries for Hydroxylation. J. Phys. Chem. submitted.

Seliger, H. H. Environmental Photobiology. COO-3277-40
Chapter 6,in PHOTOBIOLOGY, ed. K. C. Smith to be published.

B. Research Summary through March 1976.

1. The underlying theme of this research program is the study of the production and role of excited states in biological systems.

A basic assumption has been that there exist common oxygenated intermediates in the mechanisms for the conversion of chemical free energy into electronic excitation energy. Four corollaries of this assumption follow:

a. Bioluminescence (enzyme-catalyzed chemiluminescence) in all species should be the result of similar chemical pathways involving molecular oxygen as a direct electron acceptor.

b. The active sites of luciferases (although not necessarily the stereospecificity) and the reactive sites of luciferin molecules should be similar for all bioluminescent species.

c. From the knowledge of the mechanism of chemiluminescence one can infer the catalytic functions of luciferase molecules and also their role in the subsequent light emission from the excited state luciferin product molecule.

d. Since oxygenase reactions by hydroxylase enzymes and O_2^- released during osidase reactions can produce product molecules in excited electronic states, there should be an Adventitious Chemiluminescence from all biological systems. This Adventitious Chemiluminescence differs from Bioluminescence in that it is incidental to the biochemical function in the organism.

A second basic assumption is that there exist similar membrane mechanisms for achieving initial triggered responses to external stimuli, whether these stimuli be light in the case of vision, stereospecific

chemicals in the case of olfaction, pressure in the case of hearing or shear forces in the case of mechanically stimulable bioluminescence of dinoflagellates.

The research objectives are therefore:

i) to examine the detailed mechanisms of chemiluminescence, bioluminescence, and Adventitious Chemiluminescence using techniques of absolute measurement of light intensities and spectral composition.

ii) to develop the techniques for measuring absolute and relative spectral intensities of ultraweak sources of luminescence as well as of rapid flashes of chemiluminescence.

iii) to ascertain the relevance of Adventitious Chemiluminescence from microsomal extracts, from the action of leukocytes on phagocytized bacteria and from rapidly growing cells, to hydroxylase enzyme function. More specifically to ascertain whether the Adventitious Chemiluminescence emitted during the in vitro enzymatic hydroxylation of polycyclic aromatic hydrocarbons (PAH) is the result of an enzyme-catalyzed internal oxidation ring splitting or a non-specific attack by superoxide radicals.

iv) to study the role of luciferase excited state product binding on the color (energy levels) of the bioluminescence.

v) to study the relationship between the chemistry of the bioluminescent reaction, the membrane triggering system for bioluminescence and the action of external light intensities on the regulation of the triggering mechanism. All of this is possible in the single celled dinoflagellates.

We can ask a number of questions:

a). Can the general concept of "photoprotein" be applied to systems other than Johnson's aequorin and halistaurin? Is this a semantic term related only to the stability (binding) of an intermediate required for chemiluminescence? Can this explain the apparent lack of a requirement for molecular oxygen?

b) Is the 'environment' of a chemically-formed product molecule the same as a photo-excited product molecule? Are there cage effects in bioluminescence or in chemiluminescence?

c) Is it necessary that the stepwise bioluminescent oxidation mechanism be identical with the chemiluminescent oxidation e.g., for the latter in aprotic solvents?

d) What is the mechanism of photoinhibition of a shear-sensitive receptor? Is this a general mechanism that can also be applied to taxis and tropism? How do excited molecules react in enzyme-coupled systems?

e) How do primitive "nerve" networks (as in the single-celled dinoflagellates) trigger the bioluminescent system? How are primitive "nerve" networks themselves triggered? What is the mechanism of the Ca^{++} activation of a photoprotein?

f) What is the mechanism of photoinhibition of bioluminescence in the marine dinoflagellates? How is this related to photo-synthesis? Where are the pigments located?

g) What is the relationship of particulate bioluminescent systems to soluble bioluminescent systems? Are soluble bioluminescent systems in vitro artifacts of the purification procedure? Are

the articulate vesicles themselves an artifact of the extraction procedure?

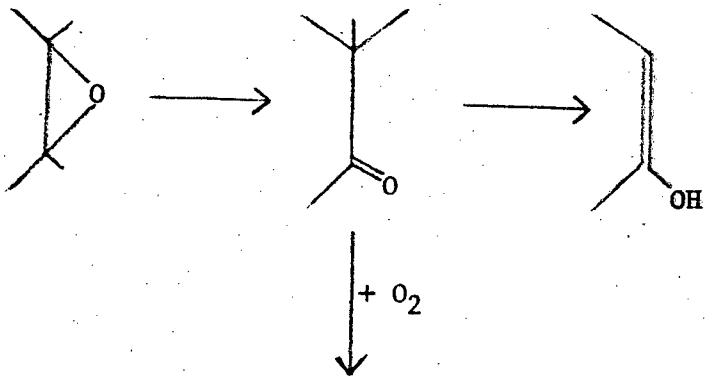
h) How can we compare ground state (vibrational) chemistry excited state (electronic level) chemistry and enzyme catalyzed (charge or group transfer) chemistry?

i) The detection of light emission as the result of chemical reactions permits the observation of as few as 50-100 photons per second. Under optimum conditions this amounts to chemical reactions of the order of 10^{-22} mole sec^{-1}. This sensitivity is sufficient to observe light emission from almost any chemical reaction. It is even possible to observe the spontaneous chemiluminescence of reactants which by analytical chemical assays are considered to be stable. It is possible to observe low-level luminescence in biological systems. In a recent paper I suggested that the measurement of the low-level chemiluminescence accompanying chemical reactions might have industrial applications in process control or in feed back, regulating complex synthesis steps (Seliger (COO-3277-14)). Could not this same approach be used to monitor some aspects of the biological system? What is the origin of these low-level luminescent reactions?

2. a) I can now report what I consider to be a major achievement of our research program. Based on our work over the past 10 years on the mechanisms of bioluminescence and chemiluminescence I developed a new hypothesis for the origin of bioluminescence, the central focus of which is the mixed function oxygenase reaction. On this basis I predicted a

chemical mechanism for the low-level chemiluminescence observed for oxidizing reactions in cells and tissue extracts. This led further to the proposal for common geometries for hydroxylation of carcinogenic aromatic hydrocarbons and the prediction that chemiluminescence should accompany the metabolic hydroxylation of carcinogens such as benzo[a] pyrene.

As can be seen from the attached preprints COO-3277-30 and COO-3277-39 we have been able to verify the prediction of chemiluminescence of carcinogenic polycyclic aromatic hydrocarbons as the result of enzymatic hydroxylation. The mechanism of oxygenation as well as the geometries for the original hydroxylations are completely consistent with the present evidence for the mutagenicity of the epoxides. In both of the above papers we show that the kinetics of the chemiluminescence are consistent with the spontaneous oxygenation of a fraction of the epoxides which rearrange to the phenol.



Excited State Product

For a long time I have wondered whether biochemical reactions could be different from homogeneous chemical reactions. Obviously specificity -- charge and geometry -- are basic to enzyme reactions. It is obvious also

that the standard condensation, hydration, oxidation reactions carried out by enzymes can be reproduced in the non-enzymatic test tube. The difference is a subtle one and only becomes obvious when we consider the complete enzyme mechanism. If we want to add group G to a particular atom of a substrate S chemically, we place removable protecting groups G' at the other reactive sites on S, react G with S and then remove the G' groups, leaving SG. In many cases the enzyme E can bind G and S sterically so that G can only react with the correct atom of S. In order to do this the enzyme is "designed" around the function to be performed. E is put together so that a binding site on E will bind a binding site on S, and likewise for group G, so that the reactive sites of S and G are favored to react to form SG. This is nothing new. However, start at this point and ask the mode of action of primitive luciferases, the present day oxygenases, the oxidative carboxylyases and the amino acid oxidases. A few of the latter two are quite substrate specific. A carboxylyase from Proteus vulgaris works on valine, isoleucine and nonpolar amino acids. In animal tissues there are carboxylyases which operate on aromatic amino acids. Most amino acid oxidases are generally non-specific. The primitive luciferases and the detoxifying oxygenases were not designed to be extremely specific. There is a lack of specificity of bacterial luciferase for aldehyde chain length (we can even fool firefly luciferase with 6-amino luciferin).

The requirement for metabolic oxidation of a parent carcinogenic aromatic hydrocarbon relates both to the binding to the aryl hydrocarbon hydroxylase and to the subsequent reaction or reactions with molecular oxygen.

The quantum mechanical calculations for K regions of PAH molecules may not necessarily be correlated with carcinogenicity. It is the site for attack by oxygen, after the K region has bound to the oxygenase, which relates to the carcinogenicity of a PAH. In homogeneous non-enzymatic test tube chemistry and in photochemistry the reaction with oxygen will generally be at the K region site. Here then is the major distinction between chemistry and biochemistry.

b) Relative Spectral Analyzer

We have used this technique to demonstrate that during the spontaneous chemiluminescence (CL) of cigarette smoke extracts in dimethylformamide (DMF) there are at least two components to the CL. The blue emission decays at a more rapid rate than the green emission. The instrument has been calibrated absolutely for photon detection using the Luminol chemiluminescent reaction (Lee and Seliger, Photochem. Photobiol. 15:227 (1972)). We have developed a diffusing geometry so that we can use the RSA for measuring the bioluminescent emission spectra of single dinoflagellates.

c) Bioluminescence

We have developed a simple set of kinetic linear equations which are consistent with all of the observations on the light-inhibition of the mechanically stimulable bioluminescent system in the dinoflagellates. With these we can account for a lag, a saturation effect, a threshold of intensity and most important of all, the dependence of the inhibition on the logarithm of the light intensity. We have found a minimum of four different types of response of dinoflagellate species to inhibiting light. Our major effort has been on the Pyrocystis (Dissodinium) lunula species and an action spectrum paper is in preparation.

d) We have set up to re-measure the emission spectra of single Jamaican chick beetles and to compare by HPLC the luciferins extracted from the individual light organs. It has been postulated by J. W. Hastings at Harvard University and ourselves that the in vivo bioluminescent system was the particulate system. The observations that supported this hypothesis were 1) the kinetics of the in vitro acid stimulable particulate flash and the in vivo flash are similar, 2) the emission spectrum of the in vitro particulate reaction and the in vivo emission are the same, 3) the in vitro particulate can be recharged after acid stimulation by incubation with soluble luciferin at pH 8, 4) a soluble luciferase, luciferin, and luciferin binding protein that binds luciferin at pH 8 but not at pH 6 can be extracted from the particulate system from G. polyedra, 5) image intensification showed the light emitted from discrete points in vivo. New observations we have made lead us to believe that the in vitro particulate system may be an artifact of the extraction procedure. These observations are summarized below. 1) An acid stimulable soluble system can be extracted from morphologically altered D. lunula with yields 100 to 1000 times the particulate yield, 2) luciferin binding protein is only found in the Gonyaulax species, 3) from protease digestion and radiolabeling with ^{125}I and lactoperoxidase, the luciferase appears to be located on the outside of the particulate vesicle extracted from D. lunula. Our present view of the in vivo bioluminescent system has aggregates of luciferase molecules attached to membranes in the cell with luciferin weakly bound to nonspecific sites. The light emission is controlled by the availability of hydrogen ions which activate the luciferase and release the luciferin.

e) We have isolated by high pressure liquid chromatography the active fractions of cigarette smoke extracts which give rise to the observed chemiluminescence (see Seliger et al., Science 185:253(1974)). We are in the process of characterizing these by chemiluminescence emission spectra and fluorescence.

We have been able to isolate by HPLC the metabolites of the microsomal oxidation of benzo[a] pyrene, in agreement with what has been reported in the literature. These isolated metabolites exhibit a further low-level spontaneous chemiluminescence which can be enhanced in strong base. Since we can identify metabolic products by HPLC our next step is to produce and isolate the specific products referred to in our paper COO-3277-30 and to compare the chemiluminescence and fluorescence emission spectra with those observed from microsomal extracts directly.

f) We have learned the techniques for and are now using the Ames' strains of Salmonella histidine mutants to perform what we consider to be definitive experiments comparing the mutagenicity of epoxides with the mutagenicity of that small fraction of those same epoxides which are spontaneously oxygenated and which produce excited states.

g) We have continued with the measurement of the spontaneous and base-stimulated chemiluminescence of urine. Our indications are that these values, normalized to creatinine concentrations, are different for smokers and non-smokers.