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RENEWAL PROPOSAL AND TRI-ANNUAL SUMMARY REPORT *

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"Chemical Production of Excited States in Biology:

Mechanism, Regulation and Function"

Principal Investigator: H. H. Seliger

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Period Covered: July 1, 1974 through June 30, 1977

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"Chemical Production of Excited States in Biology: Mechanism, Regulation and Function" 1974-1977

TABLE OF CONTENTS

A. During the past 3 years, the following papers have been published or are in process of publication:

1. Insect Bioluminescence (Chapter 8)

   W. D. McElroy, H. H. Seliger, and M. De Luca

   in The Physiology of Insecta Vol. II, Academic Press, N.Y. 1974 pp. 411-460.

2. Long-Lived Chemiluminescence in Cigarette Smoke.

   H. H. Seliger, W. H. Biggley, and J. P. Hamman

   Science 185, 253-256 (1974).

3. Extraction and Purification of Calcium-Activated Photoproteins from the Ctenophores Mnemiopsis sp. and Beroë ovata

   W. W. Ward and H. H. Seliger

   Biochemistry 13, 1491-1499 (1974)

4. Properties of Mnemiopsin and Berovin, Calcium-Activated Photoproteins from the Ctenophores Mnemiopsis sp. and Beroë ovata

   W. W. Ward and H. H. Seliger

   Biochemistry 13, 1500-1510 (1974)

5. The Origin of Bioluminescence

   H. H. Seliger

   Photochem. Photobiol. 21, 355-361 (1975)

6. Action Spectrum and Quantum Yield for Photoinactivation of Mnemiopsin, A Bioluminescent Photoprotein from the Ctenophore Mnemiopsis sp.

   W.W. Ward and H. H. Seliger

   Photochem. Photobiol. 23, 351-363 (1976)

7. The Chemical Formation of Excited States During Hydroxylation of the Carcinogenic Hydrocarbon Benzo[a]pyrene by Liver Microsomes
J. P. Hamman and H. H. Seliger
Biochemical Biophysical Research Communications 70, 675-680 (1976)

8. Chemical Production of Excited States. Chemiluminescence of Carcinogenic Hydrocarbons Accompanying Their Metabolic Hydroxylation and a Proposal for Common Active Site Geometries for Hydroxylation
H. H. Seliger and J. P. Hamman
Journal of Physical Chemistry 80, 2296-2306 (1976)

9. A New Type of Biological Chemiluminescence: The Microsomal Chemiluminescence of Benzo[a]pyrene Arises from the Diol Epoxide Product of the 7,8-Dihydrodiol
J. P. Hamman, D. R. Gorby and H. H. Seliger
Biochemical Biophysical Research Communications 75, 793-798 (1977)

10. Chemical Production of Excited States: Adventitious Biological Chemiluminescence of Carcinogenic Polycyclic Aromatic Hydrocarbons
H. H. Seliger and J. P. Hamman
in Tenth Jerusalem Symposium on Excited States in Bio and Organic Chemistry. Eds. B. Pullman and N. Goldblum, D. Reidel, Holland (1977).

11. Environmental Photobiology (Chapter 6)
H. H. Seliger
in The Science of Photobiology, ed. K. C. Smith, Plenum Press, N.Y. (1977) p. 143-173

12. Evidence that the Microsomal Chemiluminescence of Benzo[a]pyrene Results from the Spontaneous Oxidation of Diol Epoxide I
J. P. Hamman, D. R. Gorby and H. H. Seliger
Cancer Research (in preparation)

13. Comparison of the Effects of Inducers and Inhibitors on the Microsomal Chemiluminescence of Benzo[a]pyrene and Its Metabolites
J. P. Hamman, D. R. Gorby, S. Massa, P. Heiter and H. H. Seliger
Cancer Research (in preparation)

14. The Mechanism of Chemical and Mechanical Stimulability in the Bioluminescent Marine Dinoflagellates
J. P. Hamman and H. H. Seliger
Journal of Cellular Physiology (in preparation)

15. Action Spectrum for the Photoinhibition of the Mechanically Stimulable Bioluminescence of Dissodinium lunula
J. P. Hamman, W. H. Biggley, B. F. Kozlovsky and H. H. Seliger
Photochemistry and Photobiology (in preparation)

16. The Joshua Experiment: The Effect of Different Ambient Spectral Light Intensities on Mechanically Stimulable Bioluminescence in Marine Dinoflagellates
J. P. Hamman, W. H. Biggley, and H. H. Seliger
Photochemistry and Photobiology (in preparation)

B. During the past 3 years the following papers have been presented at scientific meetings:

1. Long-Lived Chemiluminescence in Cigarette Smoke

   H. H. Seliger, W. H. Biggley, and J. P. Hamman

   Presented at Biochemistry Section of Fed. Amer. Societies for Experimental Biology - Biophysical Society Annual Meeting, Minneapolis, Minnesota. June, 1974.

2. Spectroscopic Properties of the Spontaneous Chemiluminescence of Cigarette Smoke

   W. H. Biggley, J. P. Hamman, and H. H. Seliger

   Presented at Second Annual Meeting, American Society for Photobiology, Vancouver, British Columbia. July, 1974.

3. Photobiology and Marine Ecology.

   H. H. Seliger

   Invited paper presented at Second Annual Meeting American Society for Photobiology, Vancouver, British Columbia, July, 1974.

4. The Origin of Bioluminescence and the Low Level Luminescence of Cells and Tissue Extracts

   H. H. Seliger

   Presented at Second Annual Meeting, American Society for Photobiology, Vancouver, British Columbia, July, 1974.

5. A Soluble Aggregate Dinoflagellate Bioluminescent System Exhibiting the Acid Stimulable Kinetics of the In Vivo Flash

   J. P. Hamman and H. H. Seliger

   Presented at Third Annual Meeting, American Society for Photobiology Louisville, Ky. June, 1975.

6. Measurement of Absolute and Relative Spectral Intensities

   H. H. Seliger

   Invited paper presented at Third Annual Meeting, American Society for Photobiology, Louisville, Kentucky, June, 1975.

7. A Common Enzyme Mechanism for the Production of Reactive Chemical Species of Carcinogenic Polycyclic Aromatic Hydrocarbons

   H. H. Seliger and J. P. Hamman

   Presented at FASEB Meeting, Atlantic City, April, 1975

   Fed. Proc. 34, 623 (1975).

8. Chemiluminescence from Liver Microsomes During Hydroxylation of Carcinogens

   H. H. Seliger and J. P. Hamman

   Invited paper presented at Michael Kasha Symposium on Energy Transfer in Organic, Inorganic and Biological Systems, Tallahassee, Florida.

9. A Kinetic Model for the Photoinhibition of Mechanically Stimulable Bioluminescence in Marine Dinoflagellates and the Implications for the Measurement of Action Spectra

   J. P. Hamman, W. H. Biggley, and H. H. Seliger

   Presented at Fourth Annual Meeting, American Society for Photobiology, Denver, Colorado, February, 1976.

10. Chemiluminescence from Liver Microsomes During Hydroxylation of the Carcinogen Benzo[a]pyrene.

    J. P. Hamman and H. H. Seliger

    Presented at the Fourth Annual Meeting, American Society for Photobiology, Denver, Colorado, February, 1976.

11. The Microsomal-Mediated Chemiluminescence of Benzo[a]pyrene Results from the Metabolism of 7,8-Dihydro-7,8-dihydroxy Benzo[a]pyrene.
J. P. Hamman and H. H. Seliger
Presented at the 61st Annual Meeting, Federation of American Societies for Experimental Biology, Chicago, Illinois, April, 1977.

12. Chemical Production of Excited States: Adventitious Biological Chemiluminescence of Carcinogenic Polycyclic Aromatic Hydrocarbons.
H.H . Seliger and J. P. Hamman
Invited paper presented at the Tenth Jerusalem Symposium on Excited States in Bio and Organic Chemistry, Jerusalem, Israel, March, 1977.

13. A New Type of Adventitious Biological Chemiluminescence
H. H. Seliger and J. P. Hamman
Presented at the Fifth Annual Meeting, American Society for Photobiology, San Juan, Puerto Rico, May, 1977.

C. During the past 3 years the following graduate students have completed their dissertations and have been awarded their Ph.D. degrees:

W. W. Ward	"Purification and Characterization of the Calcium-Activated Phosphoproteins from the Bioluminescent Ctenophores, Mnemiopsis sp. and Beroë ovata"
J. P. Hamman	"Regulation of Stimulable Bioluminescence in Marine Dinoflagellates"

D. Research Accomplishments and Their Significance

Included as Section H.1 in this summary report and renewal proposal are copies of the publications listed in section A. When these are referred to in the text they will be starred so that the reviewer may examine them in detail.

1. Molecular mechanisms of bioluminescent reactions

a) Fifefly

A review of the physiology and the biochemistry of firefly bioluminescence is presented in McElroy, Seliger, and DeLuca (1974)* One of the remaining intriguing problems in bioluminescence is the "control", apparently by the enzyme, of the color of light emission by the excited state product molecule. For example, extracted and purified or chemically synthesized firefly luciferin appears to have the same structure (White et al., 1961; 1963). The in vitro color of bioluminescence produced from natural or synthesized luciferin reacted with extracted and purified luciferase from a particular species will be identical to the in vivo bioluminescence color of

that species. However the color, which is correlated to the peak of the bioluminescence emission spectrum will depend on the species (Seliger and McElroy, 1964; Seliger et al., 1964; McElroy, Seliger and DeLuca, 1965). If we arbitrarily denote $(\lambda_{max})_j$ as the peak of the in vivo emission spectrum for species j the general rule is

$$(\text{Luciferin})_i + (\text{Luciferase})_j \qquad (\lambda_{max})_j \qquad (1)$$

A number of insects emit two different colors of bioluminescence from different light organs. For example, Pyrophorus plagiophthalamus, the Jamaican click beetle, emits a different color of bioluminescence from its ventral light organ than from its two dorsal light organs (Seliger et al., 1964a; Biggley, Lloyd, and Seliger, 1967). Phrixothrix, the South American "railroad worm", has 11 pairs of light organs on its abdominal segments that emit a yellow green bioluminescence and 2 light organs on its head that emit a fiery red bioluminescence. This we have measured quantitatively (see Fig. 1 of McElroy, Seliger, and DeLuca, 1974)*. We have verified relation (1) for Pyrophorus, but for lack of material we do not know whether the deep red bioluminescence of Phrixothrix is the result of luciferin or luciferase. In P. phagiophthalamus we have measured that among a population of insects captured within a one or two mile square area the dorsal colors fall into three statistically significant λ_{max}'s corresponding to 550.1 $\pm$ 1.3, 556.8 $\pm$ 1.4 and 562.4 $\pm$ 1.0 nm. The standard deviations of the means were 0.21, 0.19 and 0.14 nm respectively so that the Student t test gave ratios greater than 20 in all cases (Biggley, Lloyd, and Seliger, 1967). No correlation was observed between dorsal bioluminescence color and the bioluminescence color of the ventral light organ.

The differences in color of bioluminescence present a number of interesting questions, (a) in the molecular mechanism of enzyme-excited state product binding and its effect on the energy level of the excited state; (b) in the possibility of sensitized bioluminescence, for which the only example conclusively demonstrated is in the Sea Pansy, Renilla reniformis (Cormier et al., 1973; Ward and Cormier, 1976). (c) Is there a selective evolution for an emission spectrum that matches the visual spectral sensitivity of the firefly species? (d) Why should Pyrophorus and Phrixothrix emit two different colors? (e) How precisely do members of a population such as P. plagiophthalamus retain their color of bioluminescence? (f) Since signal pattern appears to be the means by which fireflies attract one another for mating (Seliger et al., 1964b), is there a function for specific color in firefly bioluminescence?

b) Ctenophore photoproteins

We have isolated and purified two calcium-activated photoproteins from the Ctenophore Mnemiopsis sp. and a single calcium-activated photoprotein from Beroë ovata and have characterized their physical and spectral properties (Ward and Seliger, 1974a*; 1974b*). We have measured the emission spectra of bioluminescence from the two components of mnemiopsin and from berovin. We have verified that the two component photoproteins are not due to species differences by isolating both photoproteins from a single organism. This was quite a feat when one considers that 30,000 adult specimens collected by hand nets from the Chesapeake Bay, totalling more than 600 kg wet weight, yielded 2 mg of purified photoprotein. We have been able to demonstrate that the mnemiopsin activation is specific for Ca^{2+} and that activation by other cations including Sr^{2+} are artifacts of the assay method. Since the reactions are run in EDTA, 16 cations found "effective" activate mnemiopsin indirectly by replacing Ca^{2+} bound to

EDTA. The molecular weights of the photoproteins were determined by gel filtration and by polyacrylamide gel electrophoresis with excellent agreement. The active photoprotein has a long wavelength peak in the blue at 435 nm that disappears and is replaced by a peak at 335 nm subsequent to the bioluminescent reaction upon addition of Ca^{2+}. This is very similar to the Renilla bioluminescent system and is strong indirect evidence that the substrate of the photoprotein is the same as (or very similar to) that for Renilla. We have measured the bioluminescent quantum yield to be 0.12 (Ward and Seliger, 1976*). The action spectrum for photoinactivation of the photoprotein matches exactly the 435 nm absorption peak of the chromophore [Fig. 4 of Ward and Seliger (1976*].

The significance of our photoprotein work, aside from the aesthetic beauty of enzyme purification and characterization, is that we have been able to demonstrate for the first time that a photoprotein (Shimomura and Johnson, 1966) which was proposed to be a new type of biological macromolecule, not requiring oxygen and requiring only Ca^{2+} for light emission, is in reality a luciferase molecule with both oxygen (as peroxide) and its luciferin chromophore tightly bound. Ctenophore bioluminescence shouth therefore be a monooxygenase reaction as will be discussed in section D.4.a. The chromophore can be removed by 8 M urea. The Ca^{2+} requirement is analogous to the H^{+} requirement for the dinoflagellate system to be discussed next. The oxygen appears to be bound as a peroxide. The photoinactivation has also been shown to be the means by which Mnemiopsis bioluminescent potential is physiologically inhibited in vivo since the photoinactivation of the photoprotein releases H_2O_2 but does not destroy the luciferin substrate.

c) Dinoflagellate acid stimulable system

We have been able to demonstrate that for Dissodinium lunula (formerly Pyrocystis lunula) we can extract up to 100% of the total dark

phase bioluminescence as an acid-stimulable soluble system. This soluble system consists of a luciferase-luciferin complex and luciferin. An acid flash consists of an initial flash due to the oxidation of the bound luciferin with a first-order decay identical with the initial in vivo flash of dinoflagellates when they are mechanically stimulated, followed by a less rapid decay indicating enzyme turnover. We have found that only the armored dinoflagellates contain luciferin binding protein (LBP) while the vacuolar dinoflagellates such as Dissodinium and Pyrocystis do not contain luciferin binding protein.

The significance of this research is that, taken with our previous work with Dr. E. Swift (Swift, Biggley, and Seliger, 1973) that correlated total photons emitted during bioluminescence with total cellular protein for 6 species of dinoflagellates, we can now unify the mechanisms of stimulable bioluminescence in all the genera of dinoflagellates.

1) The in vivo physiological bioluminescent system is membraneous (vesicular) and H^+ stimulable. The removal of luciferase from membrane i.e. the soluble system, is an artifact of the extraction.
2) The 100-1000 X greater bioluminescence emission of the vacuolar dinoflagellates as compared with the armored dinoflagellates (Seliger, Biggley, and Swift, 1969) cannot accommodate a luciferin binding protein mechanism as this would require in some cases more than 100% of the total cellular protein as luciferase and luciferase binding protein. Therefore rapid turnover is selected for. In the armored dinoflagellates the total bioluminescence emission can be accommodated by 1-2% of the total cell protein and does not therefore require the assumption of turnover.

2. Photoregulation and Chemicalregulation of Bioluminescence

Bioluminescent marine dinoflagellates emit brief flashes (50-100 msec duration) of light when they are mechanically stimulated (in nature by waves, by fish swimming through the water, or by being seized by predators. From observations on the mechanical and chemical stimulability of light emission and the effects of ions, pharmacological agents, and light on the mechanical stimulability using a number of different species, we have developed a mechanism for the regulation of bioluminescence in marine dinoflagellates. Central to the development of the mechanism was the development of a method to quantitate the mechanical stimulability. The mechanical stimulability was defined as the rate of light emission during the application of a controlled mechanical stimulus.

The sequence of molecular events for the mechanical stimulation of bioluminescence from those species of marine dinoflagellates that do not have a large vacuole was shown to be:

a) An increase in the permeability of the membrane to Ca^{2+} due to a deformation of the membrane by the mechanical stimulus

b) The movement of Ca^{2+} into the cell down its electrochemical potential gradient resulting in a depolarization of the membrane potential

c) The change in potential increases the permeability of the membrane delimited particulate bioluminescent system to H^{+}

d) H^{+} moves down its electrochemical potential gradient to the outside of the vesicle

e) The increase in H^{+} concentration releases luciferin from the luciferin binding protein and activates the luciferase both of

which are structurally associated in the membrane on the outside of the vesicle

f) The luciferase catalyzes the oxidation of the luciferin by molecular oxygen to a product in an electronically excited state which returns to the ground state with the emission of a photon of light.

The sequence of molecular events for the mechanical stimulation of bioluminescence from those species of marine dinoflagellates that have a large vacuole is proposed to be:

a) An increase in the permeability of the vacuolar membrane to cations as a result of mechanical stimulation depolarizes the potential across the vacuolar membrane.

b) The graded depolarization of the vacuolar membrane potential (with respect to the cytoplasm) increases the permeability of the vacuolar membrane to Ca^{2+} when a threshold potential is reached.

c) A Ca^{2+} action potential is propagated along the vacuolar membrane.

d) The local depolarization due to the action potential increases the permeability of vesicle membrane to H^{+}.

e) H^{+} moves out of the vesicle down its electrochemical potential gradient.

f) The increase in H^{+} concentration releases luciferin from sites on the vesicle membrane and activates the luciferase associated with the membrane.

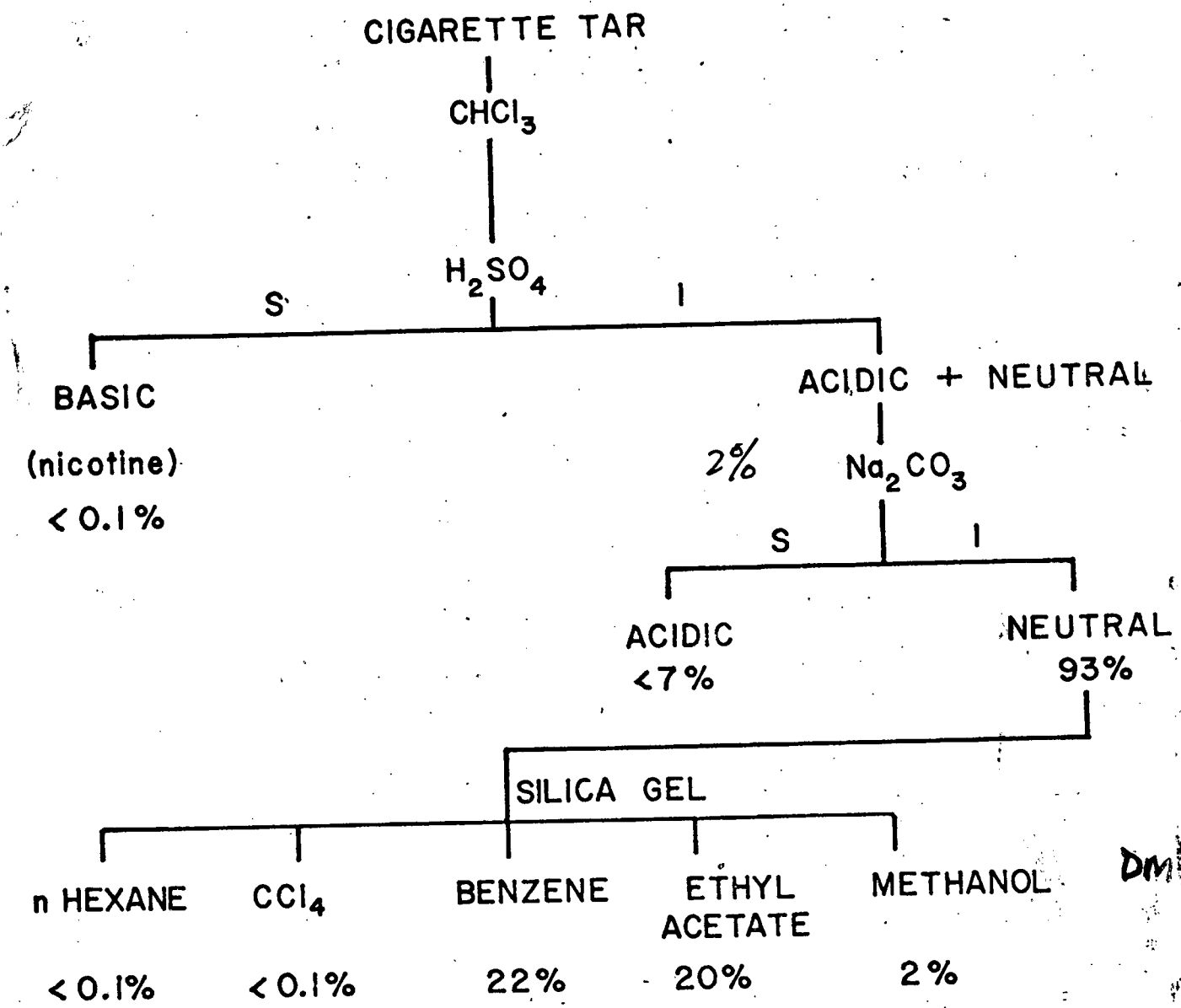
g) The luciferase rapidly catalyzes the oxidation of all the released luciferin resulting in light emission.

In all species of photosynthetic bioluminescent marine dinoflagellates we have found that the mechanically stimulable luminescence is photoinhibitable. There was no photoinhibition of stimulable luminescence for the non-photosynthetic species. In all species of photosynthetic bioluminescent dinoflagellates light was found to decrease the bioluminescence capacity of the cell resulting in a decrease in the total stimulable luminescence. This effect can be mediated by changing the activity of the biochemical components, chainging the association of the enzyme and substrate binding protein on the membrane or changing the hydrogen ion gradient across the membrane of the in vivo particulate bioluminescent system. In some species, light also has been shown to affect the mechanical stimulability of the cell resulting in a decreased rate of light emission, by increasing the threshold for mechanical stimulation. The increased threshold results from a hyperpolarization of the potential across the active membrane. In those species with large vacuole light can decrease the total mechanically stimulable luminescence by affecting the propagation of the Ca^{2+} action potential along the vacuolar membrane. We found the action spectrum for photoinhibition of the signal transmission in D. lunula to be similar to the action spectra reported for "blue" light effects in a variety of green plants, fungi and bacteria. These action spectra have peaks suggestive of riboflavin as the receptor pigment. This photoinhibition was inhibited by reducing agents and mimicked by methylene blue and irradiation with red light (Hamman, Biggley, Kozlovsky, and Seliger, in preparation).

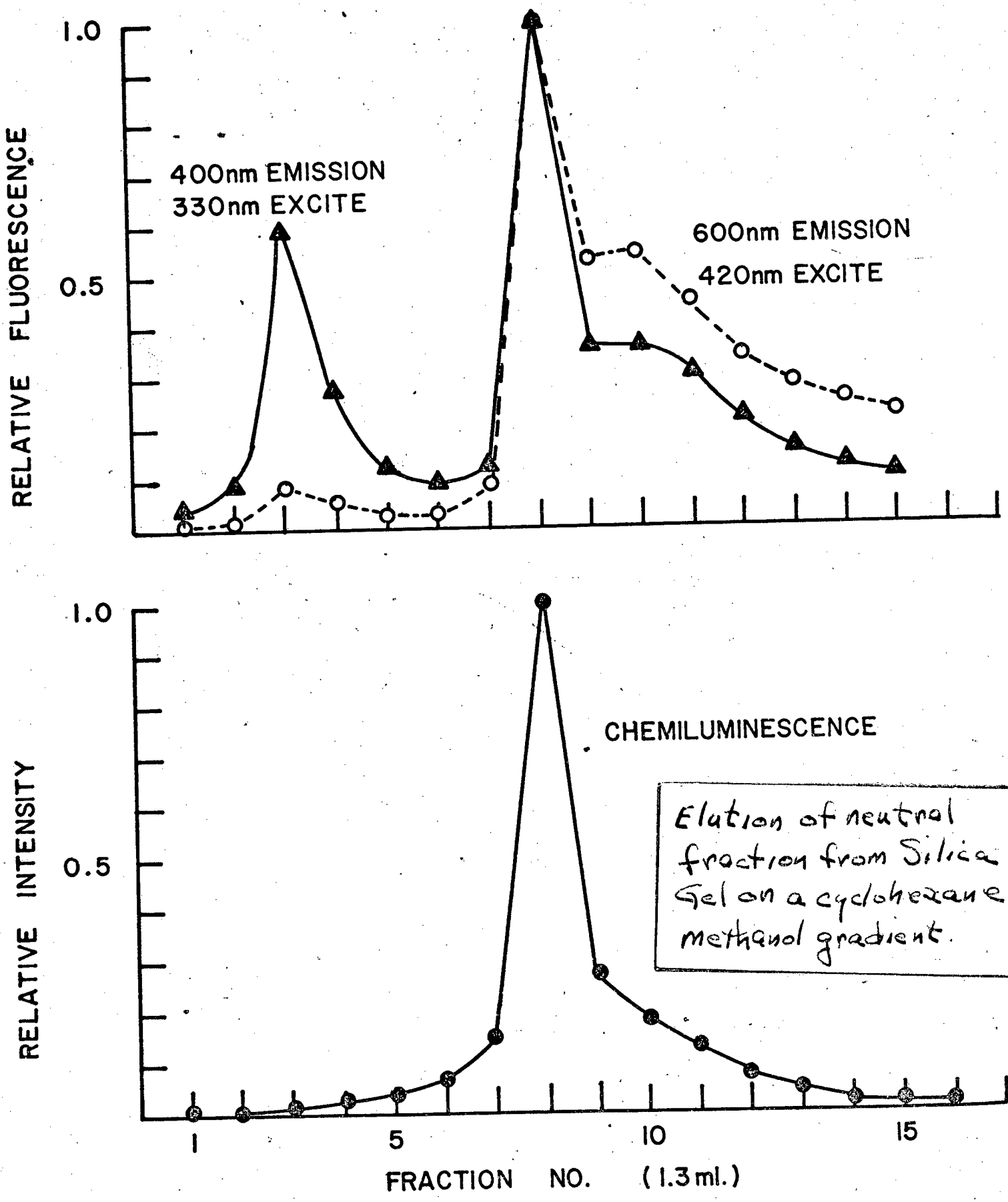
3. Spontaneous Chemiluminescence of Cigarette Smoke

The pyrolysis of organic compounds in tobacco leads to the presence of literally hundreds of products in cigarette smoke (Van Duuren, 1958a; 1958b; Van Durren et al., 1966; Lyons et al., 1958; Lyons and Spence, 1960; Marsden and Collins, 1963; Takeshita and Ohe, 1964). The smoke aerosal of tobacco is spontaneously chemiluminescent. Molecular oxygen is required for chemiluminescence and the active precursors to chemiluminescence can be extracted into organic solvents (Seliger, Biggley, and Hamman, 1974*). We have measured the emission spectrum of this spontaneous chemiluminescence.

We have attempted to characterize the spontaneous chemiluminescence of cigarette smoke aerosal and of extracts of this aerosal in absolute untis of photons and to identify the products and precursors of the chemiluminescence by emission spectroscopy and chromatographic separation techniques (Papers No. 1 and 2 under Section B). Using spontaneous chemiluminescence as the assay we have traced the chemiluminescent precursors using the following protocol and with the following results:



Extraction protocol for smoke extracts. Elution solvents from hexane to methanol were sequential



Elution of neutral fraction from Silica Gel on a cyclohexane - methanol gradient.

In summary we have found using chemiluminescence as the assay technique that

i) most of the nicotine is removed in the basic fraction which does not contain the major chemiluminescent precursors

ii) the acidic and neutral fraction retains the chemiluminescence

iii) the neutral fraction retains approximately 93% of the chemiluminescent precursors.

iv) However, these do not elute from silica gel in the hexane fraction which is where the PAH parent carcinogens elute. Therefore, the chemiluminescence is not the spontaneous oxygenation of parent PAH's.

v) In a cyclohexane-ethanol gradient the chemiluminescence is eluted in a sharp peak, characteristic of the elution of free radicals, phytosteroids and aromatic phenols

vi) The chemiluminescent precursors can be stabilized in chloroform solution in the cold.

The measurement of this significant chemiluminescence from extracts of cigarette smoke aerosal implies the presence of relatively high concentrations of reactive products of pyrolysis, since this spontaneous chemiluminescence is not present in tobacco extracts. The spontaneous reaction with molecular oxygen to produce chemiluminescence is strictly fortuitous and indicates the presence of reactive cytotoxic intermediates. Presumably only a small fraction reacts with oxygen. The CL is useful as a tracer assay technique to follow the kinetics of decay of the precursors and to identify the precursors by column chromatography and possibly from the emission spectra of their excited state products. The major implications

of the chemiluminescence are to confirm the presence of large quantities of cytotoxic products in tobacco smoke, to correlate the chemiluminescent intensities observed with the tar content of cigarettes, and the sensitivity of the assay. For example, all of our chemical separation of fractions can be performed starting with smoke extracted from a single cigarette.

4. Adventitious Biological Chemiluminescence

a) In Vitro Microsomal Chemiluminescence of Carcinogenic Environmental Hydrocarbons

In an attempt to synthesize our research in mechanisms of bioluminescent reactions, I wrote an article on "The Origin of Bioluminescence" (Seliger, 1975*) in which I showed that the bioluminescent systems for which the detailed chemical mechanism had been established were monooxygenase reactions. The implications of this generalization were that precursors to the oxygenation step should have common reactive centers independent of the total structure of the molecules and that as the result of less than 100% coupling of reduced oxygen-enzyme complexes to oxidizable substrates, there could be a release of $O_2^{\cdot-}$ or H_2O_2 during flavin oxidase and peroxidase reactions. This latter release was proposed as the source of the radicals for the low intensity luminescences of rapidly oxidizing cells, organelles and tissue extracts (Barenboim et al., 1969; De Ment, 1945; Hodgson and Fridovich, 1976; Howes and Steele, 1971; 1972; Quickenden and Que Hee, 1974; Stauff and Ostrowski, 1967; Stauff and Wolf, 1964).

Since drug detoxification reactions are also monooxygenase reactions it was predicted that some of the intermediates produced during the metabolism of polycyclic aromatic hydrocarbons could be subject to a spontaneous oxygenation by molecular oxygen, to produce a product electronically excited state that would have a finite probability for light

emission. Since the spontaneous oxygenation was not itself an enzyme-directed oxygenation the probability of this adventitious chemiluminescent reaction should depend upon the reactivity of the PAH metabolite and the concentration of these specific metabolites. Therefore we used the potent PAH carcinogen benzo[a]pyrene (BP) and induced rat liver microsomes to look for this predicted chemiluminescence. At the same time, based on the distribution of carcinogenic and noncarcinogenic PAH metabolites isolated by high pressure liquid chromatography an empirical "Common Geometry" mechanism was proposed for the enzymatic oxygenation of all PAH's (Seliger and Hamman, 1975; Seliger and Hamman, 1976*). Thus far all of the predictions and correlations of the production of chemiluminescent metabolites with the Common Geometry mechanism have been verified (Hamman and Seliger, 1976*; Hamman, Gorby and Seliger, 1977*; Seliger and Hamman, 1977*). In particular we have shown that the observed microsomal chemiluminescence of BP arises from the diol epoxide product of the 7,8-dihydrodiol of BP, the proposed ultimate carcinogenic metabolite of the parent carcinogen. We have been able to demonstrate that the enzyme pathways leading to the diol epoxide do not correlate directly with the pathway leading to the non-mutagenic 3-OH-BP metabolite. Although both the enzyme activities leading to 3-OH-BP and the enzyme activities leading to reactive epoxides (the 7,8-diol 9,10-epoxide of BP) increase upon induction, they can be induced and inhibited to different degrees by different inducers and inhibitors (Hamman et al., in preparation). Therefore the chemiluminescence assay may be a more precise assay for the enzyme activities leading to reactive epoxides (ultimate carcinogenic metabolites) than the currently used fluorescence assay for the production of non-carcinogenic phenols. However, in view of some of our

concepts on the mechanism of transport of carciongens to the nuclear material *in vivo* it may be that both assays are equally important. It would be of great value to know whether the results of Kellerman et al., 1973a; 1973b; 1975; 1976; and Kouri et al., 1973; 1974) on variation of AHH inducibility in human populations would have been demonstrated also by the chemiluminescence assay. It also would be interesting to know whether the seasonal variation in AHH activity reported by Paigen et al. (1977) would be corroborated by the chemiluminescence assay.

As a result of our efforts thus far, we have been able to show how the previous electronic theories of carcinogenesis that do not consider the biochemistry of drug metabolism are only partial and sometimes incorrect explanations of the carcinogenicities of PAH's. This is summarized in Seliger and Hamman (1977*).

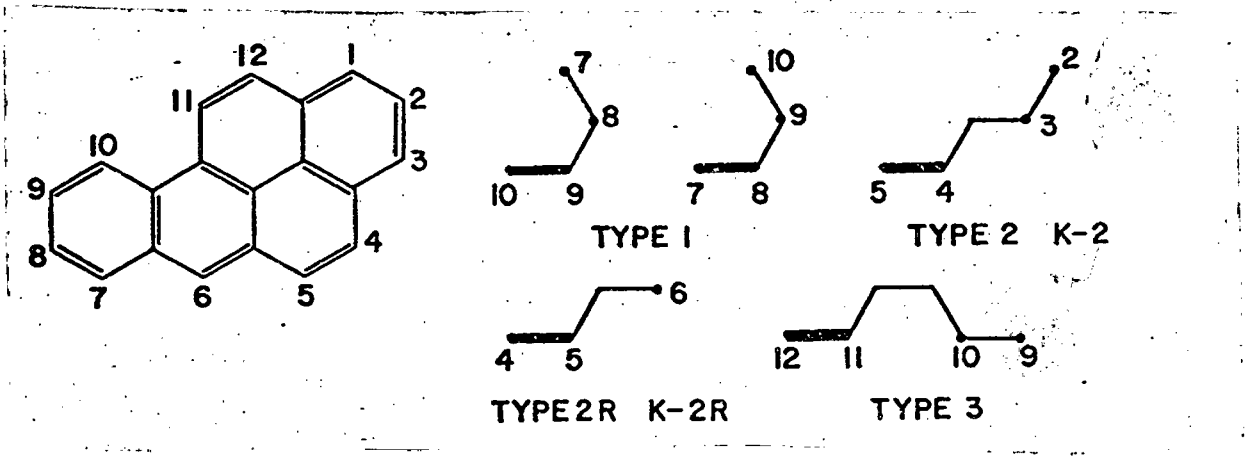
The significance of this research to the study of environmental carcinogens can be summarized as follows:

We have discovered a new type of specific adventitious biological chemiluminescence (Hamman and Seliger, 1976*; Hamman, Gorby and Seliger, 1977*), applicable to reactive epoxides or carbonium ions. This may have wide applicability in epidemiologic studies to the precise measurement of enzyme pathways leading directly to carcinogenic metabolites of PAH's different from pathways leading to noncarcinogenic metabolites. It may be possible to use exogenous chemiluminescent or fluorescent probes to assay for strong electrophiles that may not themselves be chemiluminescent. The study of the mechanisms of oxygen attack and the separation into specific and non-specific adventitious biological chemiluminescence provide possible experimental probes for examination of the in vivo production of reactive

metabolites. The mechanism also is related to the more general question of chemi-excited states in biology which, aside from bioluminescence, has no recognized functon. The possibility of functional chemi-excited states in biology is introduced by Seliger and Hamman (1977*) and may have significant biological importance. These aspects are discussed more carefully in Section F.

b) Common Geometry Mechanism

The original binding site-metabolitic site model of Anderson (1947), in which the Pullmans' K region of a PAH was the binding site rather than the reaction site for epoxidation, has been extended to the remaining bonds of the molecule, since binding is a statistical function governed by an association constant measured at equilibrium. The K region, by virtue of its highest electron density, would still be the most probable binding site. However, from examination of the spectrum of the products reported (Selkirk et al., 1974; 1975) for the metabolism of BA and BP Seliger and Hamman (1976*) proposed a minimum of three linear geometries that could account for the relative yields of all of the known metabolites. These were, in relation to the benzo[a]pyrene molecule:



where the thick solid line represents the binding site and the solid dots represent the sites for oxygenation (epoxidation). Where binding coincides with a K region the geometry is called K-2. The restricted (R) Type 2 geometry represents all of the L region reactions.

The Common Geometry Mechanism is an empirical theory that was constructed to fit the observed distributions of metabolites of PAH's with only two initial criteria: (a) metabolic oxygenation of the parent carcinogen must occur and therefore tht binding site of the substrate to the enzyme must be different from the site for oxygenation. (b) the large number of PAH's in the environment are not produced biochemcally. They are the result of high temperature pyrolysis, the burning of carbonaceous material-volcanic action, forest fires, the industrial revolution, cigarette smoking. They therefore arose late in evolution subsequent to the emergence of green plants from the seas onto the land. Therefore the first step in detoxification, the oxygen step, should be culled from those chemical reactions already evolved for biological aromatic hydrocarbons, i.e. sterols. The optimum efficiency in handling the myriad of PAH's would be achieved if, due to the structures of the PAH's, they exhibited common geometrical relationships between the binding sites and the sites of oxygenation. This would permit a degree of nonspecificity for the detoxifying enzymes that would be consistent with the observations that induction of AHH systems by any specific carcinogen such as BA or MC results in the ability of the AHH system to metabolize a wide range of carcinogenic PAH's. This nonspecificity would be reflected in an economy of binding-active site configurations for the detoxifying enzymes, i.e. a selection for a minimum number of common geometries.

A corollary of this second criterion is that there are some enzymatic oxygenation reactions that, for certain PAH's, result in strongly electrophilic metabolites. These PAH's would be the carcinogenic PAH's. Since these same detoxification reactions would not be expected to have been selected during evolution for producing carcinogenic metabolites from their natural biochemical substrates, there must be something "different" about the structure of carcinogenic PAH's that permits the fortuitous production of carcinogenic metabolites during the "normal", predominant production of soluble, noncarcinogenic detoxification products. It is this difference, the structure of the parent carcinogenic PAH, that provides the key to the partial correlations between electronic theory parameters and carcinogenicity.

The development of this Common Geometry mechanism and a number of experimental confirmations and predictions are found in Seliger and Hamman, 1976*; 1977* copies of which are included in this proposal in Section H.1. For example, the 7,8-oxide of BP is carcinogenic which the 9,10-oxide is not (Levin et al. 1972). This is completely consistent with the Common Geometry mechanism which permits production of 7,8-diol-9,10 epoxide BP from the 7,8-oxide but does not provide an enzymatic pathway to produce a diol epoxide beginning with 9,10-oxide BP (see p. 2303 of Seliger and Hamman, 1976*).

It must be reemphasized, lest the reader attempt to infer more from Section D.4.a and this present section than is justified, that we are involved here with the molecular mechanism of the production of metabolites from carcinogenic PAH's by oxygenase reactions. What we have come up with is a generalization of information theory to provide for the relative non-

specificity of induced aryl hydrocarbon hydroxylases for a variety of PAH carcinogens and a sensitive means (chemiluminescence) of looking at the kinetics of specific enzyme reactions leading to reactive epoxide metabolites of PAH carcinogens. The requirement for such a specific assay relative to the fluorescence assay for 3-OH-BP has recently been emphasized by McLemore et al. (1977, p. 1180) and Pelkanen et al. (1977, p. 20). However this should permit more selective questions to be asked about the induction and inhibition of aryl hydroxylases by a variety of compounds.

c) Selective Inhibitors of Microsomal Chemiluminescence

The results of D.4.a and b imply that there should be selective inhibitors of the carcinogenicity of PAH's. We have just begun experiments correlating absolute microsomal chemiluminescence measurements of BP and its purified metabolites with the 3-OH-BP product fluorescence assay, together with their covalent binding to DNA and to poly-G. The chemiluminescence of the product of 7,8-diol BP, used as substrate in rat liver microsomal extracts in the presence of DNA or poly-G, is directly correlated with binding. Inhibitors of chemiluminescence also inhibit binding. Our preliminary results on the kinetics of inhibition by different inhibitors indicate that the nature of the inhibitions observed is different for the 3-OH-BP formation as measured by fluorescence than for the 7,8-diol-9,10-epoxide formation as measured by chemiluminescence. It should therefore be possible in principle to selectively inhibit the chemiluminescence relative to the 3-OH-BP production, i.e. to selectively inhibit an enzyme pathway leading to the production of a carcinogenic diol epoxide thereby increasing the probability of production of other soluble

contamination by chemiluminescent organic molecules, buffers; for example, this is an excellent way of detecting the presence of chlorine in drinking water. We are investigating this approach to detecting low levels of chlorine as HOCl or ClO^- by chemiluminescence techniques.

5. Detection and Spectral Analysis of Chemi-Excited States

a) 6-Channel Relative Spectral Analyzer (RSA)

In order to measure the precise relative spectral distribution of firefly bioluminescence and dinoflagellate bioluminescence, we have designed and built a 6-channel relative spectral analyzer for the precise comparision of emission spectra from single light organs of fireflies and with the sensitivity to measure the relative spectral intensity from a crude luciferase preparation from the same light organ that was used for the in vivo measurement. This crude luciferase preparation, luciferin free is then reacted with synthetic luciferin and exogenous ATP to be able to examine the validity of relation (1) for a large number of individual samples of different firefly species that will be sent to us by Dr. J. E. Lloyd during the course of our collaboration efforts.

We have set the 6-channel RSA up in conjunction with a fast (10 msec response 10-90% full scale) Brush 6-channel recorder so that the relative emission spectra can be determined for reactions that decay rapidly due to substrate limitation. The instrument has been calibrated absolutely using the Luminol chemiluminescent reaction (Lee and Seliger, 1972). We have been able to measure reproducibly the relative emission spectra from single dinoflagellates held vertically by surface tension in the tip of a 50 microliter plastic micropipette tip and stimulated with a small 5 microliter drop of acetic acid. We have compared this with the relative emission

spectrum of the soluble enzyme system and preliminary results indicate a difference between the two. The sensitivity and precision of the RSA will permit me to ask whether the bioluminescence emission spectra of individual members of a species are truly identical. The principle of the measurement is that independent of amplitude or time the ratios of the intensities of different wavelength bands of a bioluminescent or chemiluminescent emission spectrum should be invariant. This technique increases the precision of our spectral comparisons by more than a factor of 10 over our capability using our f/3 photoelectric grating spectrometer and in addition allows us to examine rapidly decaying light intensities. It should have a wide range of use for low intensity chemiluminescent reactions.

b. Emission Spectra of Low Intensity Biological Chemiluminescence

We have compared the sensitivities of our photoelectric spectrometer for measurement of the emission spectra of low intensity chemiluminescent reactions with the apparatus of Dr. G. Reynolds of Princeton University. The two systems were designed to do different things -- ours for light collection at the expnse of time resolution and Dr. Reynolds' for rapidly changing light intensities. We used the chemiluminescence of cigarette smoke extracts, a radioactive phosphor and the Luminol chemiluminescent reaction. All of these have or can have a reasonably constant light emission over a minute. Under these conditions our instrumentation could measure emission spectra at intensities not detectable by the Reynolds spectrometer.

There is a need for highly sensitive rapid complete spectral detection by vidicon techniques and Dr. Reynolds and I intend to collaborate to develop such instrumentation based on the readout and analysis programs

he has already developed for X-ray crystallography.

We have been able to measure the spontaneous chemiluminescence emission spectrum from a single puff (30 cc) of cigarette smoke extract into dimethyl formamide (DMF), an improvement over that reported in Seliger, Biggley, and Hamman (1974*). These techniques have also been used to follow the emission spectra of chemiluminescence from the various fractions into which the smoke extracts have been separated (see Section 3.D.3).

Despite this sensitivity we have not been able to measure the emission spectrum of the microsomal chemiluminescence of benzo[a]pyrene with the photoelectric spectrometer. However at the slight expense of wavelength resolution we have developed a prototype of a simple continuous interference filter spectrometer with higher light collection efficiency and we have measured the emission spectrum of the microsomal chemiluminescence produced when 7,8-diol BP is reacted with methylcholanthrene induced rat liver microsomes (see Section D.4.a). We have used the chemiluminescent reaction of Luminol (Lee and Seliger, 1972) both for spectral calibration in the blue region and for determining the absolute sensitivity of the interference filter spectrometer.

This technique will now permit us to measure the precise emission spectra of all of the low intensity adventitious biological chemiluminescences, such as xanthine oxidase, myeloperoxidase, horseradish peroxidase, mitochondria, etc. (see introductory paragraphs of D.4.a). The knowledge of whether the emissions are due to an oxidized product, a sensitized chemiluminescence, of the carbonate ion dimer or of singlet oxygen, is essential to the understanding of the mechanism and possible role of chemi-excited

states in biological systems. In particular, the mechanism of the chemiluminescence of the carcinogenic diol epoxide of BP should provide additional verification of the Common Geometry mechanism (see Section D.4.b). For example, knowledge of the environment of the excited state--e.g. hydrophobic environment retains bond structure of the chemiluminescence (fluoresecence) of the product molecule--hydrophilic environment broadens the emission bands to a continuum--retention of the diol epoxide in the membrane system may give rise to a sensistized chemiluminescence in which case the emission spectrum should be independent of the chemical structure of the carcinogenic metabolite. We should also be able to measure the emission spectrum of the "residual light" in the in vitro firefly system, again in an effort to establish the mechanism for this reaction which appears to be independent of ATP.

E. Plans for Future Research

The following description of our proposed research activities is a distribution of our ideas on how to proceed along the directions opened up by our previous findings. These are:

α Chemiluminescence of metabolites of environmental PAH carcinogens

β Mechanism of chemical production of excited states in biological systems

γ Photo regulation of dinoflagellat bioluminescence

δ Instrumentation for measurement of low intensity photon emission from UV through $^1\Delta g(O_2)$ in the infra-red.

ε Development of chemiluminescence measurements for use in large scale screening assays

It should be obvious that some of the experimental directions while open ended, can furnish answers to specific questions within a reasonable time period. For example, in Sections E.1.c and d we should be able to determine in a reasonably short time whether the microsomal chemiluminescence observed is a direct or a sensitized chemiluminescence, and whether it is feasible to attempt to reduce the effective carcinogenicity of ingested environmental carcinogens, while for Section E.1.e, which is basic to the mechanism of carcinogenesis by PAH's, the information in the subject headings required for the formulation of any self-consistent mechanism is a long range program.

1. Chemiluminescence of Carcinogenic Metabolites of Environmental Carcinogens

a. In Vitro Microsomal Chemiluminescence.

We plan to extend our microsomal chemiluminescence measurements to a variety of carcinogenic and non-carcinogenic environmental

hydrocarbons and their derivatives. In particular we shall attempt to test the predictions in Seliger and Hamman (1976*)

i) that arylamines should produce chemiluminescence concomitant with microsomal metabolism and that the non-specificity of AHH systems induced by PAH carcinogens such as BA or MC should extend to the arylamines and their N-oxidized metabolites;

ii) that the non-alternant carcinogenic hydrocarbons, the benzofluoranthenes and the ace-benzanthracenes should also give rise to microsomal chemiluminescence;

iii) that the methylations and hydroxylations of BP that interfere with the production of the 7,8-diol-9,10-epoxide also eliminate the microsomal chemiluminescence; the predictions in Seliger and Hamman (1977*) that

iv) the covalent binding of carcinogenic PAH's to DNA or to poly G should be proportional to the time integral of the chemiluminescent intensity and that therefore the inhibition of microsomal chemiluminescence should be proportional to the inhibition of binding. Thus for any substrate PAH parent or metabolite, the chemiluminescence can be used to assay for the effects of inhibitors on the specific enzyme reactions leading to the reactive diol epoxide.

v) by the use of double label and label and non-label experiments to ask whether the non-specificity of MC or BA-induced liver microsomes leads to competitive binding between the various carcinogenic PAH's predicted by the simple Common Geometry mechanism.

vi) that the mutagenicity of a PAH metabolite in the Ames' Salmonella system (Ames, 1973) should be proportional to the time integral of the observed chemiluminescence and conversely that inhibitors of the chemiluminescence should inhibit mutagenicity. If this correlation holds it should be possible to correlate the microsomal chemiluminescence of a PAH to its potential mutagenicity and thus to develop a simple, rapid screening assay for potentially carcinogenic hydrocarbons and for environmental samples.

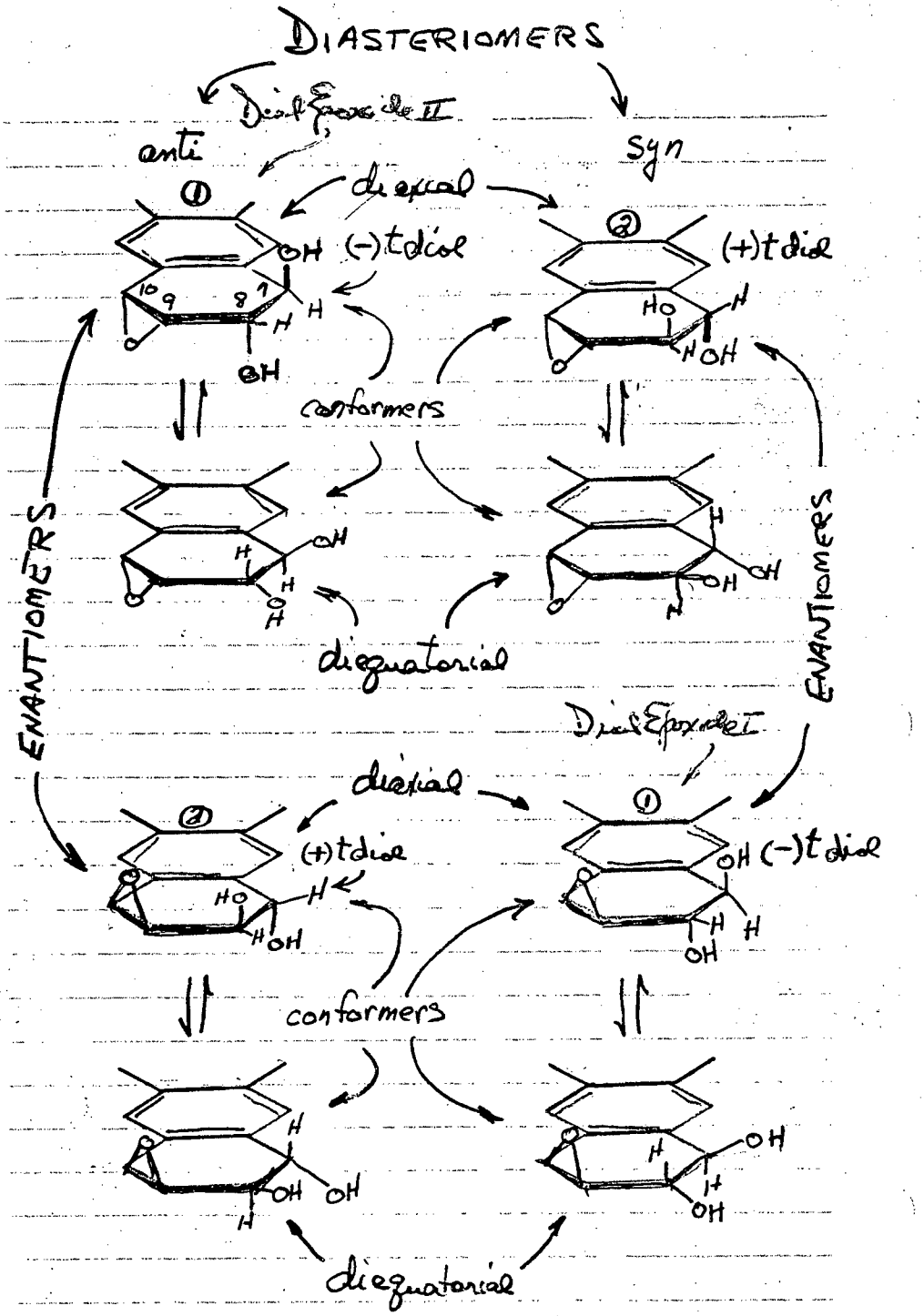
vii) that the microsomal chemiluminescence of mono-methylated benzo[a]anthracenes should correlate with their reported mutagenicities and carcinogenicities. For example, the 1,2,3,4 monomethyl-BA derivatives should exhibit negligible microsomal chemiluminescence compared with that of 7-Me-BA, 12-Me-BA, 7,12-diMe-BA, and significantly lower chemiluminescence compared with BA, 6-Me-BA, 8-Me-BA, 9-Me-BA, 10-Me-BA, and 11-Me-BA. As we shall propose under the binding studies, it is possible that, in addition to the inhibition of mesoanthracenic attack by the 7- and/or 12-Me derivatives of BA, the methyl groups add to the bulkiness of the molecule, making it appear closer in shape to a steroid and therefore making it more probable that it will be "mistaken" statistically for a steroid by the carrier proteins of the cell.

viii) We have already demonstrated that the ratios of the rate of production of the 3-OH-BP metabolite, measured by fluorescence of phenols (Nebert and Gelboin, 1968) to the intensity of

chemiluminescence are different for different inducers (Seliger and Hamman, 1976*). Since it has already been established that the radiometric assay for phenols and dihydrodiols correlated well with the fluorimetric assay for 3-OH-BP (Pelkonen et al., 1977) it should follow that the rate of production of reactive (mutagenic or carcinogenic) metabolites is not necessarily directly correlated with the phenol fluorescence assay. Therefore we want to correlate the two assay techniques with binding to poly G and with our studies on the inhibitors of chemiluminescence and binding.

ix) According to the Common Geometry mechanism 9,10-diol-BP should exhibit weak microsomal chemiluminescence if at all, should not result in the 8,9,10-triols or 7,8,9,10-tetrols. However, it is possible that the 7,8 bond can be non-enzymatically oxygenated by virtue of the $O_2^{\cdot-}$ and H_2O_2 released non-specifically during in vitro micorosomal metabolism. Thus triol and tetrol formation from 9,10-diol-BP should be strongly inhibitable by SOD whereas triols and tetraols from 7,8-diol-BP should not be affected significantly. In addition the kinetics of production should be different and in fact oxygenation of the 9,10-diol should proceed just as efficiently in the presence of other enzymic generation of $O_2^{\cdot-}$ and H_2O_2 such as xanthine oxidase. We therefore want to examine the distributions of the tetraols formed from microsomal metabolism of 7,8-diol-BP and 9,10-diol-BP and from $O_2^{\cdot-}$ and H_2O_2 generating systems. The HPLC technique makes it possible to separate the tetrols formed from the different enantiomers of

the diol epoxides. For example microsomal metabolism of (-)t 7,8-diol of BP results in the greater than 90% production of the anti 1 diol epoxide II, and 10% of syn 1 diol epoxide I, so that the tetrols should be mainly $\frac{7,10}{8,9}$ tetrol and $\frac{7}{8,9,10}$ tetrol, while the xanthine oxidase $O_2^{\cdot-}$ and H_2O_2 generating system should produce equal amounts of anti 1 diol epoxide and syn 1 diol epoxide. In this case the tetrols should be equally distributed among $\frac{7,10}{8,9}$ tetrol, $\frac{7}{8,9,10}$ tetrol, $\frac{7,9}{8,10}$ tetrol and $\frac{7,9,10}{8}$ tetrol. The enantiomers, conformers and diasteriomers of the diol epoxides of BP are shown in the drawing below (see Yagi et al. (1977))



DIOL EPOXIDES OF BP
FORMED FROM trans 7,8 DIOLS

b. Chemiluminescence in Cell Culture

We want to extend the measurement of carcinogen chemiluminescence to ask whether the time sequence of metabolism of BP and other carcinogenic PAH's to reactive metabolites can be determined in vivo in cell culture, in cooperation with Dr. E. N. Moudrianakis in this department. We will need to adapt the geometry of the cell culture system to our highly sensitive photon counting system and to develop techniques for manipulation in the dark. Hopefully, we will be able to work in far red light (λ>700 nm). The chemiluminescence measurement, combined with radioactivity labeling and the pulse chase technique of following the radioactive label and its subsequent metabolites should yield basic information about the movement of the parent carcinogen and/or its metabolites across the cell and into the nucleus. Except for shop work we have all of the instrumentation for these experiments.

c. Sensitized Chemiluminescence

We plan to improve the signal-to-noise capability of our instrumentation for measurement of emission spectra of microsomal chemiluminescence of PAH carcinogens and to extend the measurements to the metabolites of BP and BA. The questions to be asked are: Is the chemiluminescent emission spectrum of BP characteristic of the fluorescence of the pyrene product as predicted by Seliger and Hamman (1976*)? Is the chemiluminescence emission spectrum characteristic of a hydrophilic or a hydrophobic environment? (For example a non-aqueous environment should retain the vibronic band structure of the product molecule). Are the observed chemiluminescent emission spectra the same, independent of the residual structure of the product excited state molecules? (This would be evidence of a sensitized chemiluminescence). If the observed emission is indeed a sensitized chemiluminescence is it the

fluorescence of a fluorophore on the AHH system or on some other protein system?

Thus by being able to follow not only the kinetics of the chemiluminescence (the production of reactive epoxides) but also the emission spectra it may be possible to infer the location of the reactive epoxide subsequent to its production.

By comparison of the chemiluminescent emission spectra of microsomal extracts with those produced by purified cytochrome systems (in collaboration with Dr. Henry Strobel of The University of Texas) it should be possible to separate any effect on the excited state product molecule by the membranous AHH system from that of the cytochrome itself.

d. Possible Selective Inhibition of Specific Enzyme Pathways Leading to Carcinogenic Metabolites

The work we intend to do in this area is in its initial stage. It should not be inferred that any selective inhibition of diol epoxide pathways of BP <u>in vitro</u> in microsomal extracts that we may find is immediately applicable to the much more complex transport and tissue specificity problems involved in <u>in vivo</u> carcinogenesis. With this preamble we intend to continue our microsomal chemiluminescence -- phenol fluorescence - poly G- binding correlation studies to examine the inhibition kinetics of a number of possible inhibitors of reactive epoxide formation, predicted by the Common Geometry mechanism. The preliminary results stated in Section D.4.c. show that selective inhibition of pathways of metabolism is indeed possible. The measurement of microsomal chemiluminescence is the key to our ability to screen the range of possible selective inhibitors of reactive epoxide production.

e. Correlations of Metabolic Chemiluminescence with:

i) Mutagenicity in Bacterial Systems and Equivalence with the Ratiation Equivalent Man (rem).

We plan to correlate the time integral of the microsome activated chemiluminescence of PAH carcinogens with mutagenicity in the Ames Salmonella system (Ames et al., 1973, 1975; McCann et al., 1975) in collaboration with Dr. Philip Hartman of this department. We are particularly interested in whether the inhibition of microsomal chemiluminescence and binding to poly G can be correlated to the observed mutagenicity. We shall begin with BP, BA and their methyl derivatives as predicted by the Common Geometry mechanism (Seliger and Hamman, 1976*; 1977*).

As separate experiments we intend to compare the mutagenicity directly with rads using the ^{137}Cs in the Biology department. We possess all of the necessary experience and dosimetry equipment for making these comparisons. Drake et al. (1975) have suggested the term REC, the "dose" of chemical mutagen that produces an amount of genetic damage equal to that produced by 1 rem of chronic irradiation. We will look at the Salmonella bacterial system initially, but we hope, depending on the results of these experiments, to correlate these measurements with the mammalian cell culture studies of E.1.b. and with the selective inhibitor studies of E.1.d.

ii) Binding to DNA and Analogs, Chromatin Fractions, "Hormone" Carrier Proteins and Temporal Sequence of Transport of Carcinogens Into Nucleus

We want to extend our studies of the use of microsomal chemiluminescence of BP and the correlation of inhibition of chemiluminescence with inhibition of poly G binding to the chromatin of specific target tissues

of BP rather than rat liver. The questions asked are: How closely can the measurement of the rate of production of reactive epoxides reflect the interaction of these epoxides with the target material? Does the correlation exist for any of the other potent carcinogens (DMBA, methyl cholanthrene)? Does the correlation exist for a specific chromatin fraction (Moses et al., 1976)?

We propose to determine if BP or any of its metabolites will bind specifically to hormone carrier proteins in target tissues. We will look initially at corticoid receptors because they are found in a variety of cell types and elicit a variety of responses through control of cellular metabolism (Frieden and Lipner, 1971) and a number of possible BP metabolites superficially resemble corticoids. DEAE-cellulose filters which retain the protein-ligand complex while the unbound ligand is removed by washing (Santi et al., 1973) and radiolabeled carcinogen and unlabeled steroid (or labeled steroid and unlabeled carcinogen) will be used to assay for specific binding of BP or its metabolites to hormone receptor proteins. We want to ask the following questions: Does the parent hydrocarbon bind? Which metabolites bind? What is the "error" frequency for the binding of the non-steroids? Can the bound hydrocarbon be further metabolized by the microsomal AHH system or the nuclear AHH system?

Using pulse labeling and subsequent cell fractionation and fluorescence microscopy with a spatial resolution of 1.0 μ^2 and the capability for the detection of single molecules (Hershfeld, 1976) we plan to determine the temporal sequence of BP entry into the nucleus of target tissue cells. This data along with the kinetics of the chemiluminescence of BP in cell culture (see E.1.b.) may provide answers to the following questions: Is transport of the hydrocarbon into the nucleus implicated? Is metabolism required for transport?

2. Methodology for Chemiluminescence Assay of Environmental Carcinogens for Genetic and Epidemiologic Studies of Aryl Hydrocarbon Hydroxylases

The measurement of low intensity emission of single photons as the result of microsomal chemiluminescent reactions is not a routine procedure in most laboratories. A great deal of care must be exercised to prevent "contaminating" light from a large variety of sources, light-induced long lived radicals, triboluminescence, crystalloluminescence, phosphorescence of glassware, trace contaminants of efficient chemiluminescent molecules that can give rise to non-specific chemiluminescence; modification of existint instrumentation, selection of low noise phototubes, etc.

Fortunately it should be possible to use existing liquid scintillation counters in the non-coincidence addition mode and with specially selected low dark noise phototubes at room temperature to measure the microsomal chemiluminescence of BP and other PAH carcinogens. Almost all laboratories have the potential for this sensitive assay technique, once the precautions and control experiments are described in detail. Therefore when the mechanism of specific adventitious biological chemiluminescence (Hamman, Gorby and Seliger, 1977*) is more completely understood (E.3.a) and has been demonstrated to be correlated with inhibition of binding (E.1.d.) and with the binding and mutagenicity of a range of other PAH carcinogens (E.1.a. and E.1.e) it ought to be the method of choice for routine assay of environmental PAH carcinogens.

a. Intercomparison with Other Laboratories

We plan to design and construct a portable photon counting instrument for measuring microsomal chemiluminescence of BP and

visit a number of laboratories where AHH activity measurements are carried out. We plan to intercompare our photon counting measurements on their samples with their measurements using their own liquid scintillation instrumentation. We hope in this way to avoid some of the pitfalls that usually occur when one laboratory tries to repeat the observations of another laboratory. In this way the samples will be identical and the usual problem of induction, microsomal preparation, concentrations, specific activities, etc., can be separated from instrumental and photon detection problems.

b. Use of chemiluminescent Standards for Internal Calibration

At the same time we plan to develop and specify reaction conditions for chemiluminescence and to distribute to interested laboratories standard solutions of Luminol (Lee and Seliger 1970; 1972) for calibration of their low intensity photon detection instrumentation. In this way microsomal chemiluminescence of carcinogenic PAH's can be specified in absolute units of photons emitted. At the present time the fluorescence quantum yield of the emitting molecule is not known (Hamman and Seliger, 1976*). However it should still be possible to report data in terms of specific photon emission of the AHH system and retain internal consistency among all of the laboratories using these standards. Luminol is an appropriate chemiluminescent standard because its chemiluminescent emission in aprotic solvents is within the wavelength range of the observed microsomal chemiluminescence of BP.

3. Mechanism of Oxidation by Molecular Oxygen to Produce Excited States

In Section F.3. we have summarized some of the problems involved in understanding the activation of oxygen, chemically and photochemically. In Hamman, Gorby and Seliger (1977*) we have reported that the microsomal chemiluminescence of BP is a specific chemiluminescence as differentiated from non-specific chemiluminescence (see Seliger, 1975* and Seliger and Hamman, 1977*). It has also been observed that light-induced chemiluminescence of Luminol persists for up to a minute, so that $^1\Delta g(O_2)$ cannot be involved as the oxygenating precursor.

a. Roles of One Electron Reduced Oxygen, $O_2^{\cdot-}$

Two Electron Reduced Oxygen, H-O-O-H

Singlet Oxygen, O_2^*

Halogen Ions

We plan to investigate the photo-induced chemiluminescence of Luminol and compare this with the sensitized photochemical oxidation of Luminol by a series of sensitizers. The absolute quantum yields and the kinetics of these reactions will be measured as well as the dependence upon wavelength (action spectra) and intensities of exciting light. These will be compared with the chemi-induced oxidation of Luminol by NaOCl + H_2O_2. It is expected that by these means it should be possible to distinguish between $O_2^{\cdot-}$ radical reactions and $^1\Delta g(O_2)$ reactions of Luminol.

We plan to measure the quantum yields of the specific chemiluminescence of a number of carcinogenic PAH's and to use inhibition by catalase and by superoxide dismutase as criteria for participation by H_2O_2 and $O_2^{\cdot-}$ respectively.

We plan to use Luminol as a sensitive chemiluminescent probe for $O_2^{\cdot-}$ and/or H_2O_2 in non-specific chemiluminescent reactions of superoxide and peroxide generating systems--xanthine oxidase, horseradish peroxidase, myeloperoxidase and light and flavins. In section F.3.a. and b. we have discussed a possible role for chloride ion in bactericidal action. We plan to investigate this role in the chemiluminescence of Luminol and in microsomal non-specific chemiluminescence, by comparing Cl^- with Br^- and I^- under a variety of oxidizing conditions using the chemiluminescence quantum yield as a normalization factor.

4. Biological Functions for Chemically Produced Excited States

a. Bioluminescence

We want to examine the following: the role of luciferase excited state product binding on the color (energy levels) of the bioluminescence emission in the case of Pyrophorus plagiophthalamus where there are 2 different colors of bioluminescence. 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? Is it necessary that the stepwise bioluminescent oxidation mechanism be identical with the chemiluminescent oxidation e.g., for the latter in aprotic solvents? How can we compare ground state (vibrational) chemistry, excited state (electronic level) chemistry and enzyme catalyzed (charge or group transfer) chemistry?

Using the 6-channel RSA we want to determine if the in vitro and in vivo bioluminescent emission spectra are identical for the 9 species of dinoflagellates we have in culture. Is the "environment" of the chemically-formed excited state product the same for the in vitro reaction of luciferin from one species and luciferases isolated from different species even though the

enzymes differ in their single chain molecular weights (Schmitter et al., 1976). What is the structure and the function of "protected" luciferin that we have isolated from the vacuolar dinoflagellates? Can we determine by ^{125}I or photoaffinity labeling and subsequent fractionation where the biochemical components of the particulate bioluminescent system are located?

b. Other Possible Functions - Photochemical Analogs

In Section F.3.c. the possibilities for functional excited states in biology produced by chemical reactions other than bioluminescence is discussed (see Seliger and Hamman, 1977*). The most likely place for a chemi-excited state in biological systems that would be the analog of the photoexcited state would be in the photoreactivating system, where an excited state is a specific requirement for reversal of UV damage to DNA and to chromosomes (Rupert et al., 1958; Griggs and Bender, 1973). The general idea is to use bacterial or mammalian cells where repair of UV damage occurs and attempt, by gentle disruption of the repair systems, to insert a fluorescent acceptor molecule that might be excited to sensitized fluorescence. The fluorescent probe could be a singlet (diphenylanthracene) or a triplet (dibiomanthracene) acceptor.

If the excited state aromatic aldehydes are in any meaningful way participants in high activation energy-requiring reactions of PAH carcinogens with nucleic acids or proteins it might be possible to differentiate between the in vitro binding to chromatin or DNA fractions or to specific proteins of microsomal diol metabolites as a function of oxygen concentration. Since the excited state production requires an additional oxygenation the rate should be dependent upon oxygen to higher order. The analogous experiments can be run with the Salmonella his^+ revertants. However since the bacterial mutagenicity technique was selected for high efficiency it is likely that the ground state carbonium ion reactivity and consequent mutagenicity would mask any possible small addition due to excited state reactions.

It should be possible to synthesize model aromatic aldehydes based on those predicted by the common geometry hypothesis. It would be of extreme value to examine the photochemical *in vitro* reactions of the model compounds with DNA or chromatin or nuclear proteins, and to ask whether these aromatic aldehydes were photochemically mutagenic or could induce transformation in cell culture when excited by light. In general 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 (Seliger, 1973), I suggested that the measurement of the low-level chemiluminescence accompanying chemical reactions might have industrial applications in process control or in feedback, regulating complex synthesis steps. In the same application this spontaneous non-specific chemiluminescence might be used to monitor the activity of *in vitro* mitochondrial activity.

5. Possible Correlation Between Smoking and Spontaneous Chemiluminescence of Urine

We plan to continue our experiments which have shown a statistically significant difference in urine spontaneous chemiluminescence between smokers and non-smokers of cigarettes. It appears possible that an initial simplification can be made in the sampling so that a complete set of samples over 24 hours may not be necessary for the initial screening. We can therefore obtain a much larger statistical sample in order to test our initial results.

We use the same photon counting equipment for these assays as in the microsomal chemiluminescence.

There are two major sets of internal controls that we plan to institute. One is to have some subjects volunteer to stop smoking and to trace the possible decrease in spontaneous chemiluminescence. The second is to measure smokers and non-smokers alike plus and minus vitamin C, which does appear to inhibit the spontaneous chemiluminescence of urine.

6. Regulation of Membrane Permeability by Light

a. Stimulable Bioluminescence of Dinoflagellates

Bioluminescent marine dinoflagellates emit flashes of light when they are mechanically stimulated. In many species the intensity of mechanically stimulable luminescence, MSL, is photoinhibitable. Photoperiodic variations of mechanically stimulated luminescent intensity have been observed in laboratory cultures (Sweeney and Hastings, 1957; Biggley et al., 1969; Hamman and Seliger, 1972; Esaias et al., 1973; Hamman and Seliger, 1977), in plankton samples (Harday and Kay, 1964; Kelley and Katona, 1966), and in the natural environment (Seliger et al., 1962; Clark and Kelly, 1965; Soli, 1966).

In addition to the photoinhibition of MSL, there are endogenous circadian rhythms in MSL as evidenced by differences in "day-night" amplitudes of MSL when some species are kept in constant darkness or in constant dim light (Zacherias, 1905; Kofoid and Swezy, 1921; Harvey, 1952; Hastings and Sweeney, 1957; Christianson and Sweeney, 1972).

For the bioluminescent dinoflagellates, the mechanism for the regulation of the circadian rhythms in sensitivity to mechanical stimulation has been proposed to involve ion gradients across the cell membrane (Sweeney, 1974; Njus et al., 1974; Njus et al., 1976). Agents which affect membrane permeability, valinomycin and ethanol, have been

shown to affect the bioluminescence rhythms of Gonyaulax polyedra (Sweeney, 1974). A circadian rhythm in the intracellular level of K (Sweeney, 1974) and membrane potential as assayed by the K^+, valinomycin, and cyanine dye fluorescence technique (Adamich et al., 1976) have been reported.

Hamman and Seliger (1977) have found that for the different species light can regulate 1) the bioluminescence capacity of the cell only; 2) both the bioluminescence capacity and the degree of mechanical stimulability; 3) the bioluminescence capacity, the degree of mechanical stimulability and the propagation of an action potential proposed to be involved in stimulus-response coupling of light emission from the Pyrocystis sps. We proposed that the degree of mechanical stimulability is decreased as the result of hyperpolarizing the membrane. The mechanism for the hyperpolarization of the membrane by light was proposed to be the result of a decrease in the intracellular concentration of K^+. This proposal was based on the lower intracellular concentration of K^+ found in G. polyedra during photophase (Sweeney, 1974) and the interpretation of the K^+-valinomycin-cyanine dye data of Adamich et al. (1976) as indicating a membrane hyperpolarization during the circadian time period corresponding to photophase. The hyperpolarizing response to light of Aplysia giant neurons (Brown and Brown, 1972) and the scallop eye (McReynolds and Gorman, 1974) have been shown to be due to an increase in the K^+ conductance of the membrane.

The decrease in the bioluminescence capacity in response to light may involve several components. A change in the activity of the luciferase, luciferin binding protein (in those species that have it) or in the amount of luciferin as shown for G. polyedra during the circadian time corresponding to photophase (Hastings and Bode, 1962; McMurry and Hastings, 1972b, Gooch

et al., 1974) would decrease the bioluminescence capacity. However, no changes in luciferin yield or luciferase activity have been found for D. lunula and P. noctiluca (Schmitter et al., 1976). A decrease in the proposed hydrogen ion gradient across the particulate membrane would also decrease the bioluminescence capacity. If the gradient was decreased a depolarization and subsequent increase in H^+ permeability would result in a decreased flux of H^+ to the active site and fewer luciferase molecules would be activated or luciferin molecules released. Chemical stimulation would be no more effective than mechanical stimulation and the rate of light emission upon mechanical stimulation would not change. Hamman and Seliger (1972) proposed that the decrease in bioluminescence capacity might be due to a change in the intracellular pH during photosynthesis. This change in pH would alter the H^+ gradient across the particulate membrane. Other possible mechanisms for altering the H^+ gradient include a specific membrane chromatophore which controls membrane permeability, light induced electron translocations, or switching cofactors required for active transport to other metabolic pathways.

Regulation of the action potential propagation in the vacuolar species by light involves, for low exposures, a chromatophore with an absorption maximum of 450 nm in the visible region of the spectrum (Hamman et al., 1977a). At high exposures red light is also effective in reducing the signal transmission indicating that photosynthesis may also play an indirect role (Hamman et al. 1977b).

Sweeney et al. (1959) reported an action spectrum for the photoinhibition of MSL of G. polyedra with two peaks, one in the blue (440 nm) and the other in the red (700 nm) regions of the spectrum. This was similar

but not identical to their action spectrum for photosynthesis. However, it is not clear which component of the bioluminescent system was assayed, i.e. whether the inhibition was of the degree of mechanical stimulability or the bioluminescent capacity. Esaias et al. (1973) reported a single yellow peak (562 nm) in the action spectrum for the rapid photoinhibition of the degree of mechanical stimulability for G. catenella, G. acatenella and G. tamarensis. The above data and the absence of photoinhibition in the non-photosynthetic dinoflagellates (N. miliaris, Polykrikos schwartzii and Peridinum pentagonium) raises the questions of what is the role of the accessory pigments of photosynthesis or photosynthesis itself in the photoinhibition of MSL and what are the molecular mechanisms for the photoregulation of MSL in marine dinoflagellates?

We have developed the assays to separate the three effects of light on MSL and have designed and constructed an apparatus for the controlled irradiation of dinoflagellates with light of defined spectral quality. We propose to determine the absolute action spectra for the photoinhibition of the degree of mechanical stimulability, photoinhibition of bioluminescence capacity and extend our action spectrum for the photoinhibition of signal transmission in D. lunula into the near ultraviolet. We also have the capability to determine the action spectra for photosynthesis for the different species of bioluminescent dinoflagellates (Owens and Seliger, 1977). Since we have found that the photoinhibition of D. lunula depends on the ambient photophase intensity and the time irradiated in scotophase, we will examine the photosynthetic capacity as a function of ambient light intensity and time during the photoperiod.

We also plan to test our proposed mechanisms for the regulation of MSL in dinoflagellates on a molecular level. We have a Leitz fluorescence microscope equipped for photodetection, that is capable of determining the cellular distribution of bioluminescent "microsources" in the dinoflagellates either by emitted luminescence or fluorescence. Using chemical modification techniques the localization of the components of the bioluminescent system on or in the purified particulate membrane will be determined. The proposed active H^+ transport of the particulate bioluminescent system will be examined using radioactive isotopes. The proposed electrical excitability of the vacuolar membrane in the Pyrocystis sps. will be examined by microelectrodes and intracellular electrical recording.

7. Instrumentation for Detection and Spectral Analysis of Chemi-Excited States in Biological Systems

We plan to use simple microprocessing techniques for extraction of signal from noise in an improved interference filter spectrometer in order to be able to measure the emission spectra of microsomal chemiluminescence from the complete range of carcinogenic or mutagenic metabolites of BP and BA. At the present time we can measure only the emission spectrum of the microsomal metabolism of 7,8-diol-BP. When these state of the art improvements are made it will be possible to measure the chemiluminescent emission spectra of all of the carcinogenic PAH metabolites as well as the non-specific reactions such as xanthine oxidase, myeloperoxidase and horseradish peroxidase. The importance of this instrument development is discussed in Section E.1.c.

We plan to use the same sensitive spectrometer to look at the fluorescence spectrum of DNA-bound products of BP and BA. In this case