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BIOLUMINESCENCE *and* CHEMILUMINESCENCE: INSTRUMENTS *and* APPLICATIONS

Volume I

Knox Van Dyke

CRC PRESS

Bioluminescence and Chemiluminescence: Instruments and Applications

Volume I

Editor

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CRC Press, Inc.
Boca Raton, Florida

Library of Congress Cataloging in Publication Data
Main entry under title:

Bioluminescence and chemiluminescence.

Bibliography: p.
Includes index.
1. Chemiluminescence—Measurement. 2. Bioluminescence
—Measurement. 3. Biological chemistry—Technique.
I. Van Dyke, Knox, 1939- . [DNLM: 1. Luminescence.
QH 641 B6153]
QP517.C54B56 1985 574.19'125 84-15561
ISBN 0-8493-5863-9 (v. 1)
ISBN 0-8493-5864-7 (v. 2)

Direct all inquiries to CRC Press, Inc., 2000 Corporate Blvd., N.W., Boca Raton, Florida, 33431.

International Standard Book Number 0-8493-5863-9 (v. 1)
International Standard Book Number 0-8493-5864-7 (v. 2)

Library of Congress Card Number 84-15561
Printed in the United States

¥425.93元

PREFACE

If these two volumes serve to introduce to the uninitiated the importance of using chemiluminescence (CL) and bioluminescence (BL) as an analytical endpoint, they will have served their purpose. It is clear to most workers in the field that the measurement of the production of light will continue to challenge absorbancy, fluorescence, and radioactivity as methods of detection of substances. Since it is most difficult to encompass all of the important uses of chemiluminescence and bioluminescence, we hope that the various chapters in these volumes give a clear representation of the length and breadth of this field. We have tried to give outstanding examples of the uses of CL and BL and to clarify the difficulties one may encounter in performing these measurements. It should be noted that the efforts herein have an international flavor which reflects the interests and concerns of scientists everywhere.

Knox Van Dyke

These books are dedicated to my late daugher Cynthia Joyce Van Dyke who was so helpful in their preparation.

THE EDITOR

Knox Van Dyke, Ph.D., is a professor in the Department of Pharmacology and Toxicology at West Virginia University, Morgantown, West Virginia.

He obtained his A.B. in chemistry at Knox College, Galesburg, Illinois in 1961, and his Ph.D. in endocrinological biochemistry in 1966 under Philip A. Katzman, professor in the Edward A. Doisy Department of Biochemistry, St. Louis University.

After completion of his graduate studies, Dr. Van Dyke joined Dr. Leroy Saxe at West Virginia University in the development of novel drug screening systems for malaria under a U.S. Army contract. The culmination of this work led to the discovery of mefloquine as an important antimalarial drug against *Plasmodium falciparum* and the fundamental difference between purine and pyrimidine metabolism in malarial parasites. A purine metabolite, hypoxanthine, was found to be a key endogenous nucleic acid precursor for malarial parasites and it forms the basis of present screening systems developed against the deadly *P. falciparum* form of human malarial parasites.

As part of the drug screening system for malarial parasites, the first automated system for the measurement of adenosine triphosphate (ATP) by light production was developed which measured drug toxicity to the erythrocyte. This led to the development of the Aminco Chem-Glo Photometer using a flowing stream.

A collaboration with Dr. Madhu Manandhar produced the first research into the phenomenon of "delayed luminescence" which allows the use of almost any purine and pyrimidine nucleotide to produce light using luciferase from *Photinus pyralis.*

In 1975, luminol-dependent chemiluminescence was developed in the laboratory of Dr. Van Dyke. Multiple works using this system for the study of chronic granulomatous disease, neonatal sepsis, and neutropenic states were done by his students, Dr. Michael Trush, Dr. Mark Wilson, and collaborator, Dr. Paul Stevens. The first published work with alveolar (lung) macrophages producing cellular chemiluminescence was accomplished in the era of 1975 to 1980. Myeloperoxidase CL and drugs were studied in collaboration with Dr. Gary Pekoe.

Currently Dr. Van Dyke is applying cellular CL to the various disease states of cancer, arthritis, genetic deficiences, and environmental problems in collaboration with Dr. Vincent Castranova and Dr. David Peden.

In addition to his duties as a professor of Pharmacology and Toxicology, Dr. Van Dyke maintains an active research laboratory fundamentally interested in cellular CL and CL immunoassay in disease states. He is a member (past or present) in the following societies: American Chemical Society, National Association for the Advancement of Science, Sigma Xi, Society of Pharmacology and Experimental Therapeutics, American Society of Photobiology, Who's Who in the Eastern United States, and Who's Who in the Frontiers of Science.

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Chapter 1

INTRODUCTION

Knox Van Dyke

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I. CHEMILUMINESCENCE AND BIOLUMINESCENCE AS ALTERNATIVES TO RADIOACTIVITY

There is a general need for methods to assay a variety of chemicals at low concentrations (10^{-9} to 10^{-15} molar). Radioactive labels such as ^{3}H, ^{14}C, ^{32}P, and ^{125}I have been used to meet this need. However, the increasing costs of obtaining radiolabeled materials as well as those of surveying and maintaining these relatively unstable compounds have brought their use into question. Further, most states and nations have enacted legislation against the dumping of even low-level radioactive waste, making disposal a difficult problem to solve. Also, once a suitable site is found, waste materials must be packaged to government standards, stored until time of shipment, and eventually shipped across state lines by truck or rail, all incurring great expense ($500 per barrel). An alternative to the use of radioactivity is the use of a chemical label which produces light by a particular mechanism. Light produced from a chemical or biological source is referred to as chemi- or bioluminescence, respectively. ("Luminescence" by itself is often used to describe light production regardless of origin; hence, many instruments to detect light are luminometers.) Since luminescence detection is similar to scintillation counting, either instrument can be used to detect light.

Certain chemicals can be detected in the 10^{-9} to 10^{-15} range using luminescence methods. Since these assays are generally nontoxic, there is no waste disposal problem and the autodegradation found with radiolabeled compounds does not occur. All these factors combine to make luminescence detection a useful assay for a variety of compounds.

II. LIGHT PRODUCTION AND MEASUREMENT OF FREE RADICALS

The production of light from chemicals emanates from excited states. Therefore, certain chemicals, sensitive to free radical attack, can be added to biological samples to produce light. This light production can be used as a measure of free radical attack and/or presence. This can be used both externally in a biological system or internally within cells of biological organelles. These site-directed chemical probes have shown to be important in measurement of peroxidation and oxygen-radical attack. Excited states can be detected without adding chemicals to biological systems and this in vivo analysis of body tissues or breath analysis will prove to be important in assessing toxicity. (See Chapter 8, Volume II.)

III. GENERAL PRINCIPLES OF LIGHT PRODUCTION FOR CHEMI- AND BIOLUMINESCENCE

A. Production of Excited States

A chemical substance that absorbs energy can undergo a variety of changes. An increase in temperature, for example, can excite the electrons of the substance. In this "excited" state, the electrons may become unstable and they must lose the excess energy to regain their original stability (known as ground state). The energy released into the environment can take a variety of radiation forms, including heat.

B. Discussion of Luminescence (Bio- and Chemi-)

An unusual way for excited molecules to release excess energy is by emission of light. Processes in which excited state molecules release light are termed luminescent and can be produced from a chemical or biological source. Therefore, the terms *chemiluminescence* and *bioluminescence* have been developed. Since both means of production are ultimately chemical in origin, bioluminescence can be considered a form of chemiluminescence.

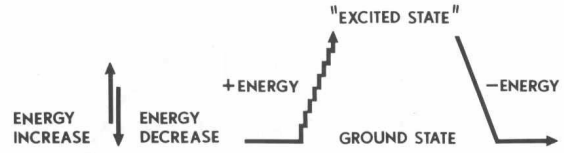

FIGURE 1. Schematic diagram depicting the idea that as certain molecules absorb energy they are transformed from a basal ground state of energy to an excited state which can return to ground state with the loss of energy.

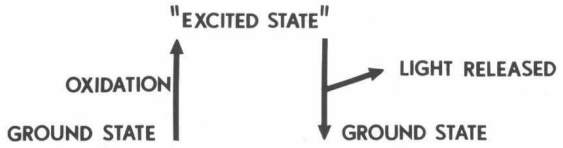

FIGURE 2. Schematic diagram depicting the production of light from certain molecules in the excited state. The same change in energy levels occurs as in Figure 1 with the exception that upon return to ground state light is released. The initial change from ground state to an excited state could be accompanied by an oxidation of the initial molecule in ground state.

1. Chemiluminescence

A few chemical reactions can produce excited states with a wide variety of different substances. In order to produce light, the energy released during the reaction must be high enough. Therefore, prime candidates are oxidation reactions in which high energy release occurs. The color of the emitted light is dependent on the molecule oxidized and the energy released. In general, the more energetic reactions give light of short wavelength, e.g., blue, whereas a less energetic reaction will produce green or (even weaker) red light.

2. Bioluminescence

Many light-producing reactions which occur in, or are derived from, living organisms are called bioluminescent. These reactions are catalyzed by enzymes called luciferases or certain photoproteins. The luciferases are enzymes which react with a substrate (luciferin) and molecular oxygen to produce an intermediate excited state which emits light upon rearrangement and returns to ground state. This reaction is identical to the chemiluminescence mechanism above except that luciferase and luciferin are involved. The luciferin-luciferase mechanism has been isolated from different species of insects, bacteria, snails, worms, fungi, etc. Photoproteins are precharged bioluminescent proteins that are triggered to emit light by binding inorganic ions, e.g., calcium ion (Ca++). (See Chapter 14, Volume II.)

C. Analytical Use of Chemi- and Bioluminescence

With chemiluminescence (CL) or bioluminescence (BL) as the analytical endpoint, the rate of light (or photon) production is measured. The intensity of light (or photon) emission is dependent on the rate of the oxidative reaction or change in photoprotein. Therefore, light measurements quantitate the concentration of reaction molecules. If one of the reactants or substrates (Sa) is held constant, then light production will vary with the concentration of a second substrate (Sb).

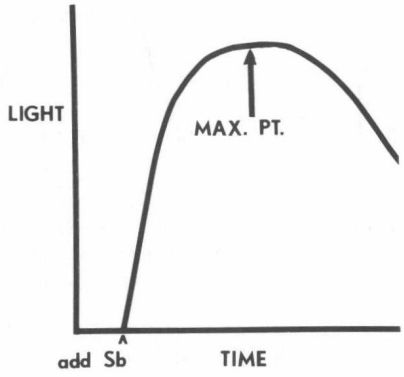

FIGURE 3. The production of light with time after the addition of substrate b (Sb) with substrate a (Sa) in saturating concentrations. Under these conditions, the rate of the reaction, $S_a + S_b \rightleftharpoons P_c + P_d$ + light, where S_a = substrate a, S_b = substrate b, P_c = product c, and P_d = product d. The rate of the reaction or the light produced will be dependent upon the concentration of substrate b (Sb). A maximal point of light emission will be reached at a point in time when the reaction slows down.

$$Sa + Sb \rightarrow Pc + Pd + light$$

Therefore, Sb can be quantitated specifically if the concentration of Sa is in excess. The reaction would produce a curve as shown in Figure 3. Initially, Sa and enzyme are present but not Sb, so that no reaction takes place. As Sb is added, the intensity of light (reaction rate) increases up to a maximal point and then declines. The concentration of reaction molecules changes as the reaction proceeds.

The concentrations of Sa and Sb decrease while the products Pc and Pd increase near the equilibration point. When the reaction rate increases, the light produced increases, and when the reaction rate decreases, the light decreases.

There are a variety of ways to use light emission curves to quantitate reactions. These are summarized in Chapter 4.

D. Calculations from Light Measurements

A method to make quantitation easier is to change the concentrations of the reactants so that a constant production of light is achieved over the time of measurement (Figure 4B). This can be done by using low concentrations of substrates so that the concentration of reactants changes little during measurement. It would be difficult to measure and reproduce the light peak shown in graph A of Figure 4, which was produced over a short period of time. Whereas in graph B of Figure 4, the constant level of light production makes measurement more convenient and precise.

IV. SELECTED APPLICATIONS OF BIO- AND CHEMILUMINESCENCE

A. Bioluminescence — Direct and Coupled Assays

1. Measurements with Direct Reactions

1. ATP + luciferin — firefly luciferase, Mg++ → AMP + PPi + oxyluciferin + CO2 + light + O_2

2. 2 NADH + FMN — bacterial reductase → FMNH2 + 2 NAD+

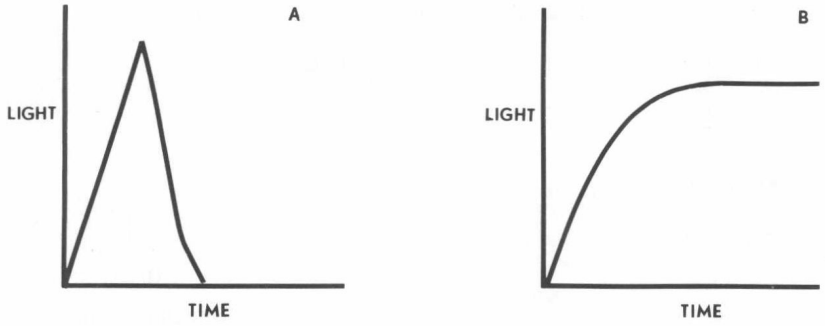

FIGURE 4. The production of light from chemical reaction that produces a light peak or flash is depicted in (A) while a steady-state production of light is depicted in (B). In general, the measurement of light in state (B) is more convenient and reproducible than in state (A).

3. O_2 + luciferin — clam luciferase → light

4. Precharged photoprotein — Ca++, other inorganic ions → blue photoprotein + light

Advantages of these assays are the extreme sensitivity, e.g., 10^{-12} *M* or less, and the selectivity of most reactions.

2. Measurements with Coupled Assays (Indirect)

The range of biochemical reactions is greatly extended using reactions coupled to substrates of the direct reactions (1 through 4).

a. Measurement of Creatine Kinase

5. ADP + creatine phosphate — creatine kinase → ATP + creatine

6. ATP + luciferin + O2 — luciferase → AMP + PPi + oxyluciferin + CO2 + light

b. Measurement of Cyclic AMP

7. cAMP — phosphodiesterase → AMP

8. AMP + ATP — pyrokinase → 2 ATP

9. 2 ADP + 2 phosphoenolpyruvate ↔ 2 pyruvate + 2 ATP

10. ATP + luciferin + O2 — luciferase → AMP + PPi + CO2 + oxyluciferin + light

B. Chemiluminescence

1. Hydrogen Peroxide or Coupled Assays (Base Catalyzed)

A variety of methods have been developed to measure hydrogen peroxide using luminol, isoluminol, or lucigenin.

$$\text{Luminol} + H_2O_2 \xrightarrow{\text{Peroxidase, or base + metal or heme}}$$