AD-A007 924

RESEARCH ON BIOLUMINESCENCE

Frank H. Johnson

Princeton University

Prepared for:

Office of Naval Research

1 April 1975

REPORT DOCUMENTATION PAGE

READ INSTRUCTIONS BEFORE COMPLETING FORM

1 REPORT NUMBER	2 GOVT ACCESSION NO.
3 RECIPIENT'S CATALOG NUMBER	AD-A007924
4 TITLE (and Subtitle)	Final Report: Research on Bioluminescence
5 TYPE OF REPORT & PERIOD COVERED	Final; 1970-1974
6 PERFORMING ORG. REPORT NUMBER	
7 AUTHOR(s)	Frank H. Johnson
8 CONTRACT OR GRANT NUMBER(s)	N00014-67-A-0151-0025
9 PERFORMING ORGANIZATION NAME AND ADDRESS	Biology Department Princeton University Princeton, N. J. 08540
10 PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS	NR 108-860
11 CONTROLLING OFFICE NAME AND ADDRESS	Office of Naval Research Biochemical Branch, Code 442
12 REPORT DATE	April 1, 1975
13 NUMBER OF PAGES	iii + 70
14 MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office)	
15 SECURITY CLASS. (of this report)	Unclassified
15a DECLASSIFICATION/DOWNGRADING SCHEDULE	

16 DISTRIBUTION STATEMENT (of this Report)

Approved for public release
Distribution unlimited

17 DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18 SUPPLEMENTARY NOTES

19 KEY WORDS (Continue on reverse side if necessary and identify by block number)

Bioluminescence, Luciferin, Luciferase, Biological Light, Biochemistry of Luminescence

20 ABSTRACT (Continue on reverse side if necessary and identify by block number)

This report reviews the research on bioluminescence under the subject contract, briefly listing the luminescence systems of the various types of bioluminescent organisms investigated with the objectives of isolating and determining the properties and the chemical structure of the essential reactants; also, the reaction mechanisms and specific products involved in the process of light emission and factors influencing the photon yield. Brief discussions are included concerning accomplishments under previous contracts, as well as the significance of bioluminescence and background of research in this area.

II

Office of Naval Research

Contract N00014-67-A-0151-0025

Task Number NR 108-860

FINAL REPORT: RESEARCH ON BIOLUMINESCENCE

by

Frank H. Johnson, Principal Investigator

Biology Department, Princeton University

Princeton, New Jersey 08540

CONTENTS

		Page
	ABSTRACT	i
I.	INTRODUCTION	1
II.	SIGNIFICANCE OF BIOLUMINESCENCE	1
	A. As Basic Research	2
	B. For Support by Office of Naval Research	
	(1) Basic Research Aspects	3
	(2) Strategic Military Aspects	5
	C. As Unique Reaction Rate Tool	5
III.	REVIEW OF ONR SPONSORED RESEARCH BY F. H. JOHNSON AND COLLEAGUES, 1954-74	29
	A. Bacterial Luminescence	31
	B. The New Zealand luminescent species: The limpet *Latia*, glowworm *Arachnocampa*, and earthworm *Octochaetus*	35
	C. The ostracod crustacean *Cypridina hilgendorfii*	37
	D. The hydromedusan jellyfish *Aequorea*	40
	E. The "paddle worm" *Chaetopterus*	50
	F. The deep-sea euphausiid shrimp *Meganyctiphanes norvegica*	52
	G. The "fireworm" *Odontosyllis enopla*	54
IV.	SUMMARY OF EXTRACTED SYSTEMS AND REACTION SCHEMES, AS OF 1973-74	56
V.	REFERENCES, PART I (References cited without relation to ONR aid at Princeton)	61
	REFERENCES, PART II (Publications by F. H. Johnson and collaborators, aided by ONR)	66

ABSTRACT

Research with special reference to the use of bacterial luminescence as a "tool" in the study of fundamental mechanisms involved in the biological action of temperature, hydrostatic pressure, and narcotics or other chemical factors, led to a rational basis for the effects of all these factors, and the quantitative data were, on the whole, satisfactorily accounted for in terms of Eyring's theory of absolute reaction rates. A common theoretical basis thus became evident for what had previously been considered to be three independently acting factors -- temperature, pressure and narcotics -- and at the same time some previously unknown relationships were discovered, including the reversal of effects of certain narcotics by increased hydrostatic pressure, as well as the determining effect of temperature on the amount and even the direction, i.e., inhibition or "stimulation", of the over-all effects of increased hydrostatic pressures up to some 10,000 psi. The results of research on the luminescence of intact bacterial cells anticipated very similar results in regard to pressure-temperature relations of bacterial luminescence in cell-free extracts, as well as in regard to the pressure-reversal of narcosis in tadpoles and other animals. The theory arrived at on the basis of bacterial luminescence also anticipated diverse phenomena in regard to other processes, including rates of bacterial growth and disinfection, virus multiplication and inactivation, denaturation of enzymes and other proteins, bacterial oxidative metabolism, molecular basis of narcosis, etc.

The more purely biochemical advances that have resulted chiefly through the collaborative research of Johnson, Shimomura and colleagues are summarily reviewed with reference to the light-emitting systems of various types of bioluminescent organisms listed below.

(1) The ostracod crustacean Cypridina hilgendorfii (and the seemingly identical systems of certain self-luminous fishes, Parapriacanthus beryciformes and Apogon ellioti, which give reciprocal "cross-reactions" with the luciferin and luciferase of Cypridina); determination of the chemical structure of the luciferin; purification and properties of the luciferase; pathway of luciferin oxidation in aqueous solution catalyzed by luciferase, and differences in aprotic solvents; origin of the oxygen in the carbon dioxide produced; structure of the oxyluciferin and etioluciferin products, and analysis of the mechanism of action of factors influencing the quantum yield of luciferin.

(2) Discovery of a new ("photoprotein") type of luminescence system, at first in the hydromedusan jellyfish Aequorea aequorea, extracts of which were found to contain a single-organic-component system, i.e., a conjugated protein that was given the name "aequorin" which in aqueous solution would emit light on addition, practically specifically, of a trace of Ca^{2+}, thereby furnishing a means of testing for the presence of this cation in biological systems; determination of molecular weight and other properties of aequorin; separation, elucidation of chemical structure and synthesis of the light-emitting group; pathway of changes in the luminescence reaction, and of regeneration of active, Ca^{2+}-triggerable aequorin from the spent protein moiety plus the synthetic light-emitter.

(3) Extraction and purification of a second photoprotein system from the "paddle worm" Chaetopterus variopedatus and determination of the properties of the crystallized protein, the cofactor requirements and quantum yields.

(4) Extraction and purification of a third photoprotein type of system from the euphausiid shrimp Meganyctiphanes norvegica; identification of the purified light-emitter as a recycling, fluorescent molecule; and quantum yield of the photoprotein.

(5) Practically complete purification, though only in microgram yields, of the luciferin of the "Bermuda fireworm" Odontosyllis enopla, and high purification of the luciferase; determination of major properties of the luciferin and its chief reactions, including certain aspects of the luminescence reaction and the activating effects of small concentrations of CN^-.

(6) Extraction, purification and properties of the luminescence system of the New Zealand fresh water limpet Latia neritoides; synthesis of the luciferin; identification of the light-emitting complex, the pathway of the luminescence reaction, including the structure of the products and the quantum yield.

(7) Evidence that fatty aldehydes, required in the luminescence of the bacterial system in vitro, are oxidized to the corresponding organic acid, with a quantum yield of 0.017 ± 0.01, independently of chain length, and that the energy supplied in the oxidation of these acids, added to the energy of oxidation of reduced flavine mononucleotide is sufficient for radiation of luminescence at the observed frequencies; the aldehydes are thus seen as functional equivalents of the luciferin in systems such as that of Cypridina.

(8) Extraction of functional aldehydes from luminescent strains of luminous bacteria, but in only insignificant traces from "aldehydeless" dark mutants that produce no visible light except by adding exogenous aldehydes, and demonstrating that the amounts present in the luminous strains are only enough to sustain the steady-state luminescence for about half a second, indicating that the aldehydes in living cells are consumed about as fast as they are normally produced.

(9) Discovery of the quantitative significance of photoprotein and luciferin-luciferase types of system among various coelenterates, and the identical product of the luminescence reaction in virtually all cases.

(10) Discovery of profound structural similarities in the skeleton of key organic components not only of coelenterate luminescence systems, but the close relation of these skeletons to those of Cypridina luciferin (and its counterpart in certain fishes), and of the cephalopod mollusc, Watasenia scintillans, the "firefly aquid", or "hotaru-ika" of Japan.

I. Introduction

The primary purpose of this paper is to fulfill the requirement of a final report to the Office of Naval Research concerning the investigations on Bioluminescence conducted under Contract N00014-67-A-0151-0025, Task Unit No. NR 108-860, during the period 1970-74. It seems appropriate and potentially useful, however, to take the opportunity offered by this occasion to view this research comprehensively, from a mature, in-depth perspective that could be achieved only after years of dedicated effort within the same area. In order to clarify the picture as a whole, with its interlacing details pertaining to specific objectives and pathways of approach, even at the very real risk of producing a document that may be considered "too long", yet one which could not be substantially shortened without ruthless deletion of integral, significant portions of the total panarama, this paper undertakes to include the general background and course of development of the research that has led into particular investigations dealt with during the much more limited period of this contract referred to above. It seems appropriate also, to state at once that, despite the inestimable value of the support received at one time and another from several Branches of the Office of Naval Research, the total work reviewed in this paper could not have been accomplished without support also from other sources, especially the National Science Foundation. There would seem to be no real use in trying to attribute separate aspects of accomplishments to precise support received from the one rather than the other agency, any more than trying to assess the individual efforts of collaborating workers; the total results could not have been achieved without the total support and wholehearted cooperation for progress towards closely related goals.

In point of fact, ONR support of the author's scientific endeavors goes back to 1950, some 25 years ago, when a modest contract supplementing the stipend of a John Simon Guggenheim Memorial Foundation Fellowship helped to provide the means of spending 14 continuous months, on a year's leave of absence, at the University of Utah, for the purpose of completing a treatise, in close collaboration with Professor Henry Eyring, at that time Dean of the Graduate School of the University of Utah, following his number of years in Princeton as Professor of Chemistry. Eyring and the author had already collaborated extensively in Princeton. The project that involved further collaboration at Utah was a natural outgrowth of this earlier work, and it profoundly influenced the course of the author's work thereafter. In the interests of clarity and completeness of this review, it seems desirable to make brief reference to the circumstances pertaining to the author's research on bioluminescence and the factors responsible for its development in the manner described in this review.

II. Significance of bioluminescence

This is one of the first, and in many respects most important, questions that the non-specialist is naturally prompted to inquire about any special line of research. In the present instance, it is an easy question to answer from nearly every point of view except one, namely, the significance of bioluminescence to any of the many different types of organisms, among plants from bacteria to higher fungi, and among animals from protozoa to fishes, that have evolved this property. Apart from fireflies, whose flashes of light can serve as mating signals and thus

promote propagation of the species, and perhaps a few other examples wherein luminescence somehow may aid survival of the individual or the race, the significance of this phenomenon is so obscure that the naturally anthropomorphic interpretations which have been expressed from time to time are apt to appear, on critical examination, little more than childishly naive. This seems true despite the fact that some highly evolved, complex photogenic organs, such as those of euphausiid shrimps, offer fertile ground for speculative imagination, all the more so in view of the possibility that in some instances the light may be emitted as a laser (Bassot, 1966).

A. Basic Research

From the point of view of "pure research", bioluminescence is an example par excellence. It has stimulated the curiosity of intellectuals both great and small, through the ages of recorded time (cf. Harvey, 1954). In our own era we are familiar for the first time not only with light which results primarily through heat, as the light of the sun, or a fire, or an incandescent light bulb, but also with light associated with phenomena of fluorescence and phosphorescence at much lower temperatures than incandescence, e.g., the ubiquitous television screen, fluorescent light tubes, phosphorescent paints and tapes, etc. The experience of witnessing a natural example of "cold light", such as the so-called "phorphorescence of the sea" (in reality due to bioluminescent organisms), or light of more obvious biological sources such as the flash of a firefly, occurring as a physiological process at temperatures of the environment, is nowadays far less shocking than it was in, say, the 17th century, when Robert Boyle (1667, 1672) recorded extensive observations and performed ingeneous experiments on "stinking fish" and "shining fles ", which we now know must have been overgrown with luminous bacteria. Boyle was carrying out "purely" basic research -- uncontaminated by any conscious possibility of a practical application -- but rather with only the incentive of the "wonder and delight" it proffered. Boyle was far ahead of his time, inasmuch as he unwittingly discovered that "air" was essential for the light of shining flesh, that no perceptible heat or stink accompanied this light, and that it could be "vanished" not so easily by water but quite readily by alcoholic beverages ("pure Spirit of wine"), and other chemical agents such as "Oyl of Turpentine", "strong Spirit of Salt", or "weak Spirit of Sal Armoniack" (Boyle, 1667). This was some years prior to the time that bacterial micro-organisms were first beheld by man, through the lenses and eyes of Antonie van Leeuwenhoek (1683), and over a hundred years prior to Lavoisier's and Priestley's (1774-1775) discovery of oxygen, the active component of air eventually found necessary for most types of bioluminescence. Boyle's observations were, in fact, nearly two centuries before luminous bacteria were clearly recognized as such (Heller, 1853).

Boyle was not unique among the illustrious personages who, in the course of history have devoted more than casual inquiry into one or another aspects of bioluminescence; others - to name a few - include, in the first century Pliny the Elder, and in more modern times, Francis Bacon, Descartes, Sir Humphrey Davy, Michael Faraday, Benjamin Franklin, Charles Darwin, Louis Pasteur, and a number of others (cf. Harvey, 1957). All of this merely emphasizes one of the main significances of bioluminescence, namely, a fascinating phenomenon that justifies basic research for its own sake. Apart from the use of certain types of bioluminescent systems as convenient

and occasionally unique biochemical assay methods, due to their extraordinary sensitivity to particular substances, e.g., bacterial luminescence for molecular oxygen, the firefly system for ATP, or the photoprotein aequorin for calcium in biological systems (Table I, from Johnson, Eyring and Stover, 1974), and apart from abortive or purely temporal applications such as the suggested use of luminous bacteria as a source of light in miners' safety lamps (Dubois, 1903), the use of dried, powdered Cypridina by Japanese soldiers (Haneda, 1955), who obtained enough light to read a map in the darkness of a jungle during World War II by moistening with saliva some of the powder held in the palm of a hand, or the use of luminous bacteria as a source of household illumination by Japanese families during "blackouts" (Haneda, 1955), research on bioluminescence has yet to result in practical consequences of widespread, lasting application.

B. Significance to ONR

(1) As basic research

There was a time, between approximately the time of War II and the functioning of the National Science Foundation a few years later, when the U. S. Navy with a wise perspicacity and determined action that might seem over and beyond the call of its duty in safeguarding the best interests of the nation, but with its traditional dedication and efficiency, organized and put into operation the Office of Naval Research, intended as a sort of interim or emergency measure, in recognition of the inestimable importance of maintaining basic research in virtually all fields of science, and in recognition of the undeniable fact, repeatedly demonstrated through the history of modern science, that it is through unrestricted basic research, without primary objective or even casual thought of practical applications, that discoveries resulting in the most revolutionary impact on daily lives, and sometimes misguided means towards wholesale deaths of populations of people, have emerged; the Einstein equation for the quantitative equivalence of matter and energy has been cited ad nauseum, as one example, but this is only one example of many that could be listed in this context, and it is obviously in part because of this circumstance that basic research continues to receive as much support as it does. This is so despite efforts of arm-chair, unfortunately insufficiently enlightened and intellectually misguided, but politically skillful and influential administrators, who arbitrarily undertake (too often with notable success) to compartmentalize research efforts along "mission oriented" lines. What great world-shaking advances in science have arisen in such manner? Even the immensely important inventions, commonly attributed to persistent, inspired efforts of dedicated individuals, e.g., the electric light, the telephone, the electric motor, the airplane, were all preceded by theoretical developments in the physics of electricity, magnetism, and in aerodynamics, Bernoulli's theorem. The role of the Office of Naval Research in fostering and maintaining basic research can properly stand, however unsung, as one of the everlasting monuments to the Navy's service to the nation, and certainly in this instance, to mankind in general.

Thus, the support of basic research on the phenomenon of bioluminescence is appropriate to the traditional policies of the Office of Naval Research. But there are additional reasons, beyond fundamental research as such, that

TABLE I*

Table I A. Luminescence Systems with Properties Potentially Useful in Quantitative or Qualitative Tests for Various Substances and Cells[a]

Luminescence system	Substance	Estimated limit of sensitivity[b]	Author(s)
Bacterial cells	Oxygen	10^{-10} atm in N_2 at 1 atm	K. P. Meyer (1942)
	Air pollution (photochemical oxidants)	0.05 ppm	Serat, Budinger, and Mueller (1965, 1967); Serat (1969); Serat, Kyono, and Mueller (1969)
Bacterial or fungal cells	Rocket fuels (e.g., monomethyl hydrazine; unsymmetrical dimethyl hydrazine)	0.1–1.6 ppm of air	Sie, Thanos, and Jordon (1966)
Bacterial extracts	NADH (DPNH, diphosphopyridine nucleotide)	10^{-7} *M*	Strehler (1953)
		10^{-14} *M*	P. E. Stanley (1971)
	Metabolic intermediates and products (e.g., glucose, malate, ammonia, and prostaglandin hormones)	10^{-12} *M*	Brolin et al. (1971, 1972) Nicholas and Clark (1971)
Firefly extracts	ATP (adenosine triphosphate)	10^{-9} *M*	Strehler and Trotter (1952); McElroy and Green (1956)
	ATP	2×10^{-11} *M*[c]	
	ATP	2×10^{-12} *M*	H. A. Cole, Wimpenny, and Hughes (1967)
	Cyclic AMP	7.2×10^{-9} *M*	R. A. Johnson et al. (1970)
	Cells of bacteria and other microorganisms	100 cells	Levin (1964); Levin, Usdin, and Slonim (1968)
Sea-pansy extracts	DPA (3′,5′-diphosphoadenosine)	10^{-9} *M*	Cormier (1962)
Jellyfish photoprotein aequorin	Calcium	10^{-9} *M*	Shimomura, Johnson, and Saiga (1962a, 1963b)
Odontosyllis extracts	Cyanide	10^{-7} *M*	Shimomura, Johnson, and Saiga (1963a); Shimomura, Beers, and Johnson (1964)
Chaetopterus extracts	Ferrous iron	10^{-10} *M*	Shimomura and Johnson (1966)

[a]Somewhat modified and extended from F. H. Johnson and Shimomura (1972a, p. 322).
[b]Approximate, subject to the sensitivity of the photometric method used.
[c]From descriptive literature on the DuPont 760 Luminescence Biometer; see also the JRB ATP-Photometer.

*Table 2.4, pp. 106-107, in Johnson, Eyring and Stover, 1974.

properly contribute to the Navy's interest in this phenomenon in particular, as follows.

(2) As a strategic problem

It is an open secret that the "phosphorescence" of the sea, for the most part unpredictable as to locality of occurrence and intensity of manifestation, can assume major proportions as a factor in military strategy. It takes practically no first hand experience and only a minimum of imagination to realize that any large "bloom" of luminescent marine animals such as protozoa, jellyfish, ctenophores, and various other types whose light-emitting systems are notoriously sensitive to mechanical stimulation, would be likely to reveal not only a ship plying the surface of the ocean, but also military devices underneath the surface such as mines, submarines, "frogmen", or other objects, either in stationary positions where currents in the water bring luminous organisms into contact with the object, resulting in excitation of luminescence, or mobile objects, not anchored in one position, which lead to the same result by moving through the water and striking these miniature but numerous sources of bright flashes of light. In principle, practical solutions to problems of this kind might result from basic research on the biological phenomenon itself, especially from a full understanding of the factors which control the intensity and duration of the light, and this remains a possibility. The initial support by ONR during the 20 year period under discussion was, in fact, for a project entitled "Factors influencing the intensity of bioluminescence", and the general objective has remained the same throughout, though this has involved excursions, so to speak, into the more purely bio- and physical chemical aspects of various systems, as necessary prerequisites to arriving at definitive solutions concerning mechanisms of rate-controlling reactions in the overall process of light emission, the quantum yields, etc. Before summarizing the contributions that have resulted from either of these somewhat distinct but closely related approaches, it should help clarify the rationale behind the course of research as it has been pursued, to delve at least briefly a little further into the background, with special reference to a third significance of research on bioluminescence, namely, its use as a tool for investigating fundamental reaction rate control in biological systems. It should perhaps be emphasized explicitly, however, that while this is appropriate to the purposes of the present paper, it by no means exhausts the various points of view from which research on bioluminescence seems significant; others obviously include ecology of species and populations of luminous plants and animals; histology, histochemistry, and morphology of photogenic tissues and organs; physiology of stimulation and response; et cetera.

C. Significance as a reaction rate tool

The phenomenon of bioluminescence lends itself to use as a research tool for investigating more general biological phenomena through two distinct approaches, namely (1), under steady-state conditions in living cells, e.g., the uniform glow of light emanating from a population of non-reproducing luminous bacteria, and (2), the extracted, and preferably purified components, usually thought of and sought for as a specific substrate, with the general designation "luciferin", and specific enzyme, similarly referred to by the

general term "luciferase", in vitro. Each approach has its advantages and disadvantages; ideally, it is desirable to use both the in vivo and in vitro systems from the same source, because many parameters of the reaction in living cells not only cannot be controlled at will, but often involve details that can scarcely be known, e.g., absolute concentrations of the reactants. With the in vitro system, if really pure, the concentration of substrate and of enzyme can be readily manipulated and data such as the Michaelis-Menten constant can be obtained with some assurance (cf. Kauzmann, Chase and Brigham, 1949; Shimomura, Johnson and Saiga, 1961). However, the kinetics of light-emission of the system in vitro can be profoundly influenced by impurities of various sorts, including even luciferin itself in a reversibly oxidized state. Moreover, interpretation of the action of various factors that influence the steady-state luminescence in living cells is inevitably subject to the sort of uncertainties that beset all experiments with intact, living cells. Unfortunately, it was not until 1953 that bacterial luminescence in vitro was successfully demonstrated out with partly purified preparations and some additional substances (Strehler, 1953; McElroy et al., 1953). Prior to that time, essentially all experiments with bacterial luminescence in a steady state were limited to comparisons between the intensity of the light under one set of conditions and the intensity under another, such as before and after adding a narcotic, or before and after changing the temperature of the medium. By analogy with partially purified luciferin and luciferase components of a different type of system, viz., that of the ostracod crustacean Cypridina, it was reasonable and logical to assume that the intensity of luminescence in living bacteria at any moment was limited by the activity of an enzyme, i.e., a postulated "bacterial luciferase", and that under conditions of constant intensity over a period of time, the postulated "bacterial luciferin" must be present either in great excess or else present in effectively a constant concentration due to its formation at a constant rate. Although final proof of the mechanism of action had to await extraction and purification of the components of the bacterial system, some interesting and significant results were obtained with the system in vivo, especially in regard to the effects of narcotics, to which the luminescence of bacteria had long been known to be more sensitive than other functions, including the rate of total oxygen consumption (Taylor, 1934). A thorough investigation of the relation between luminescence intensity and rate of respiration of luminous bacteria, in relation to oxygen tension, oxidizable substrate, effects of cyanide and of narcotics, separately and in combination, was contributed by van Schouwenburg (1938), leaving virtually no doubt that the two overall processes, i.e., luminescence and aerobic respiration, of luminous bacteria, are limited by two different, autoxidizable, intracellular enzymes, one being an especially cyanide-sensitive, "heme" catalyst, the other being an especially narcotic-sensitive "luciferase", the latter of which was normally responsible for only a minor fraction of the total oxygen consumption of the cells.

Over a period of time it gradually became apparent that in general the intensity of steady-state bacterial luminescence could be taken as providing an index to the reaction velocity of the underlying catalytic system, and this view was strengthened by experiments even with practically unpurified luciferin and luciferase extracted from Cypridina, whereby it was

shown that, under given favorable conditions, the total light produced was proportional to the amount of luciferin, and the rate of production was proportional to the amount of luciferase, i.e., the intensity at any moment was proportional to the product of (luciferin) X (luciferase). The intensity was found to decrease with time in accordance with a reaction that was first order with respect to substrate concentration (Amberson, 1922). The specific rate of the reaction was thus represented by the slope of a straight line resulting from plotting the logarithm of luminescence intensity against time, and this slope increased with increasing concentration of luciferase. It therefore seemed that the intensity of bioluminescence really provided an instantaneous index to the reaction velocity of the system.

It was this last-mentioned property that provided a basis for some significant advances in the fundamental theory of the biological action of temperature, pressure and narcotics in 1941, through collaborative research of the author with Professor Dugald Brown and Douglas Marsland of New York University. It is interesting to note, in passing, that the only financial support for the author's part of this work was a modest grant of $500. from the American Philosophical Society for research on luminous bacteria, and this fund was practically exhausted before the above-mentioned collaboration was begun. Scientifically, the facts were briefly as follows.

At the time (1941), there was no basis in experiment or theory to suspect a fundamental relation between as least two, possibly three, distinct fields of biological research, namely, pertaining to the influence of temperature, hydrostatic pressure, and narcotics. In fact, only a few years earlier a comprehensive review on the physiological effects of increased hydrostatic pressure (Cattell, 1936), going back to the earliest research in this area by Regnard and Certes in the 1880's, included scarcely any mention of temperature. Various effects were described, on a completely empirical basis, and viewed as a property of the particular tissue or process involved. Thus, increased pressure tended to inhibit some processes, whereas it tended to accelerate or to "stimulate" other processes, while in still others stimulation under moderate increases in pressure was followed, as the amount of pressure was increased, by inhibition at the higher pressures. Both reversible and irreversible effects of pressure were noted, i.e., in some instances complete recovery took place at normal pressure after the material had been subjected to fairly high pressures, up to several hundred atmospheres, although in some instances evidence of destructive effects was found.

At about the same time as Cattell's (1936) review, a comprehensive monograph on "Temperature and Living Matter" (Bělehrádek, 1935) was published, containing virtually no mention of pressure. Attempts to interpret the quantitative effects of temperature on biological processes on a rational, or rather semi-rational, basis in terms of the Arrhenius theory of chemical reaction rates were partly successful, but for the most part only through a limited range of temperature below the normal "optimum" for the process involved. Through some such limited ranges, various processes appeared to conform, as a whole, to the Arrhenius relation, at least to the extent that

plotting the logarithm of the observed rate against the reciprocal of the absolute temperature yielded a straight line, the slope of which was interpreted as indicating the Arrhenius "activation energy" or "μ" value for some particular, slowest, or pace-setting, or "master reaction" that was assumed to limit the rate of the total process. No clear theory was available to account for the changing values of μ as the temperature was increased towards and beyond the "optimum", i.e., the maximum of the process under the conditions involved, except for the notion that, at sufficiently high temperatures, thermal destruction of essential reactants such as enzymes always occurred, characterized by rates that were increasingly fast as the temperature was increased. Although explicit reference was made to occasional observations indicating that the deleterious effects of high temperatures were reversible on cooling, the possibility that such effects might involve an equilibrium reaction that was immediately reversible on cooling, the more completely so the shorter the exposure to the high temperature, was generally not taken into account. The changing, nearly always decreasing, numerical values of μ as the temperature of the experiment was raised towards the optimum, where the observed μ became zero, and beyond the optimum, where the sign of the observed μ was of course reversed, remained a mystery.

At the same period of time, theories of narcosis were woefully inadequate, as far as having a sound basis in theoretical chemistry was concerned. Partial success was achieved a few years later in interpreting the relation between concentration of narcotic and amount of its effect, by using equations derived from adsorption isotherms or the law of mass action, but only at constant temperature. The possibility of a relation between depth of narcosis and hydrostatic pressure was apparently undreamt of, and had not been accidentally discovered. The most widely accepted theory at the time, and seemingly at present also, was not actually a theory but rather a largely empirical correlation, namely, between potency of action and solubility in olive oil, a correlation pointed out independently by Meyer and Overton at the turn of the century. In a few selected instances, changes in potency of action of narcotics qualitatively paralleled changes in solubility in oil with changes in temperature (cf. discussion in Johnson, Eyring and Stover, 1974, p. 522 ff). Thus, higher concentrations of salicylamide, benzamide or monacetin were needed to cause narcosis in frogs at 30° than at 3° C, and the distribution coefficient in olive oil-water decreased at the higher, as compared to the lower of these temperatures. The opposite results, both with respect to potency and distribution coefficient in olive oil-water, were found with ethanol, chloral hydrate, and acetone. Apart from this limited and rather rough agreement of the "lipid soluble theory" of narcotic action with the effects of temperature on partition coefficient of the narcotic between olive oil and water, the role of temperature remained largely a mystery. A more precise theory was arrived at through Warburg's study of the action of narcotics on respiration, cell division, etc. in a variety of types of cells, and especially his "charcoal model" (Warburg, 1921). The model consisted of animal charcoal with the property of catalyzing the oxidation of oxalic acid and certain amino acids in a manner that was subject to inhibition by the presence of narcotics. The amount of inhibition was satisfactorily in accord with mathematical formulations derived from the hypothesis that the narcotic became adsorbed

in a monolayer on the active charcoal, thereby displacing the oxidizable substance. Although the success of this bit of rigorous theory led to some rejoicing among pharmacologists, it was soon recognized (cf. Henderson, 1930), that the theory was not so clearly applicable to cells as it was to charcoal. When the adsorption hypothesis was studied with regard to the action of various concentrations of urethane on bacterial luminescence (Taylor, 1936), there was no evidence of agreement between the theory and the data from experiments; the influence of temperature was not investigated at that time.

It was not until the temperature-pressure-inhibitor parameters of narcotic action on bacterial luminescence was extensively studied by Johnson, Brown, Marsland, and their associates, beginning in 1941, that a rational theory of narcotic action at the molecular level, applicable to the catalytic activity of enzymes in homogeneous solution as well as to complex physiological functions in animals, became possible. The "keys" that suggested a solution to these problems, and at the same time served, in principle, to clarify numerous previously observed relationships that had been practically without a rational interpretation for many years, were found in experiments initially carried out by Brown, Marsland and Johnson in 1941 (Johnson, Brown and Marsland, 1942a,b; Brown, Johnson and Marsland, 1942). The key data are illustrated in figures 1 - 3, based on their collaborative research.

The profound influence of temperature on the net effect of pressure on a given biological process is strikingly apparent in Fig. 1,A, where the changes in steady state luminescence intensity are plotted as per cent with reference to the intensity at normal pressure arbitrarily taken as 100 at each temperature. Obviously, this single over-all process, i.e., luminescence, in a single species of organisms, exhibited essentially all the known effects of increased pressure among various processes, at temperatures that were unspecified. Thus, with rise in pressure, at a relatively low temperature, the intensity of luminescence was progressively decreased; at a relatively high temperature, it was progressively increased, and at an intermediate temperature, increasing pressure had little influence, though there was a small "stimulation" followed by a small inhibition with further rise in pressure,i.e., when pressure was applied at a temperature that was evidently near the "optimum" for the species. The fact that these phenomena did not depend on the exact temperature as such, but on the relation of the temperature of the experiment to the normal optimum for the process in the biological species involved, is made clear by the data in Fig. 1,B, illustrating results which might have been anticipated by those of Fig. 1,A. Figure 1,B shows that a psychrophilic species, Photobacterium phosphoreum, having a relatively low optimum, exhibits an increase in luminescence intensity at a temperature of 25°C, a few degrees above the optimum, whereas the reverse is true for Achromobacter harveyi, having an optimum temperature a few degrees above this same temperature of the experiment, 25°, and finally, that in a species, Achromobacter fisheri, having an optimum temperature close to that of the experiment, there was little effect of increased pressure. These results were quite analogous to those which Brown had already found with reference to muscle tension, and the effects of acclimitization of the animal

to the seasonal temperatures of winter and summer; in fact, it was observations such as these on muscle that led to the investigation of bacterial luminescence, as a different process and one which was easily found among different species with different temperature optima.

The data of Fig. 1 are somewhat more understandable when plotted in the manner of Fig. 2, showing the variation in intensity of luminescence in a single species of bacteria as a function of the reciprocal of the absolute temperature for 3 different hydrostatic pressures. It should be kept in mind that these data represent practically instantaneous changes in steady state intensity with pressure and with temperature, and that they represent changes in the luminescence intensity in a population of non-reproducing cells. Moreover, the changes induced by increased pressure were fully reversible on decompression, even though momentary overshoots of intensity occurred, to an extent and in a manner dependent on both the temperature and amount of pressure. Fig. 2 is plotted in accordance with the Arrhenius equation, conformity to which would be revealed by a straight line, at least for temperatures well below the optimum. Approximate agreement is indeed apparent at each pressure. Apparent also are steeper slopes under increased than under normal pressure. A value of the Arrhenius activation energy μ can be readily estimated from the slope of each of these lines in the range of temperature where the intensity is between some 10 and 20-30% of the maximum. The Arrhenius theory does not contain an explicit expression for the relation between chemical reaction rates and hydrostatic pressure, and therefore lacks an expression for the influence of this factor on activation energies. Moreover, the investigators, along with most physiologists and other specialists in biology at the time were unaware of any theoretical basis for interpreting the action of increased pressure in enormously increasing the intensity of the light at temperatures well above the optimum. In fact, the currently held view was that high pressure in general somehow denatured proteins and enzymes. Inspection of the data in Fig. 2, however, reveals that the increase in luminescence is apparent only in the range of temperatures above the optimum, where the intensity has been reduced by the unfavorably high temperature. Such reductions are familiar characteristics of "temperature-activity curves" practically throughout biology; they had long been thought to be due, in general, to the thermal destruction of a key component of the system. In the present instance, in view of the seeming reversal of a destructive reaction simply by increasing the hydrostatic pressure, the question naturally arose as to whether or not reversal at normal pressure would take place on cooling. Experiments to test this point were specially devised, and it was soon discovered that very brief exposures to even the highest temperatures, at which the light was practically extinguished or greatly dimmed, caused very little damage that was not immediately reversed on cooling quickly. The conclusion followed that the reduction in activity at high temperatures might be due to a reversible thermal denaturation of a limiting enzyme, an interpretation that appeared all the more reasonable in view of evidence that was accumulating at about this time, to the effect that in certain conditions the thermal denaturation of purified proteins, including enzymes which exhibited virtually no activity at the high temperatures, underwent spontaneous reversal on cooling. In fact, it had been suggested (Anson and Mirsky, 1931) that reversible protein denaturation might be a type of controlling reaction in physiological processes. This idea seemed

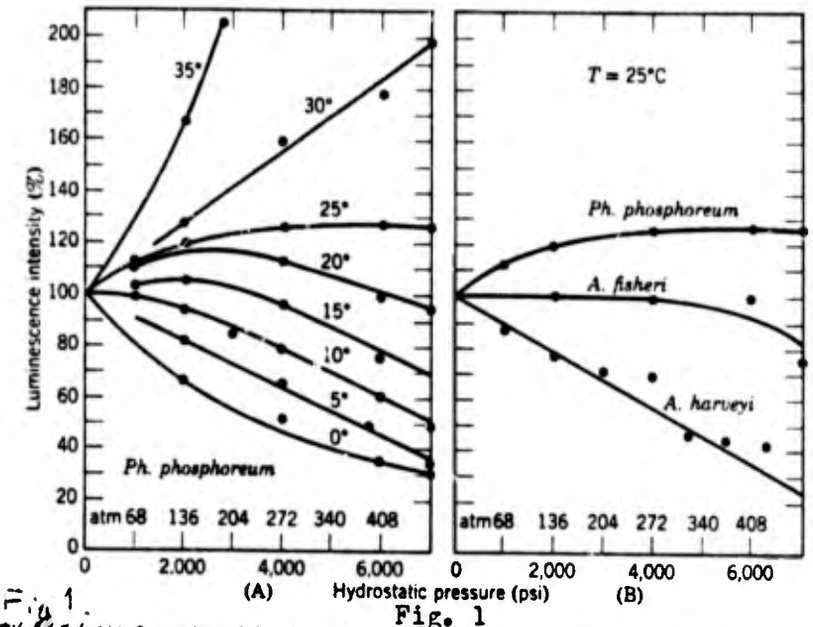


Fig. 1

Fig. 1 (A) Intensity of luminescence of *Photobacterium phosphoreum* as a function of pressure at various temperatures (Brown, Johnson, and Marsland, 1942). The intensity at normal pressure is arbitrarily taken equal to 100 at each temperature in order to show the percentage of change in intensity with change in pressure. (B) Influence of increased pressure on the luminescence of three different species of bacteria at 25°C.

(Fig.4.8, p. 301, in Johnson, Eyring, and Stover, 1974).

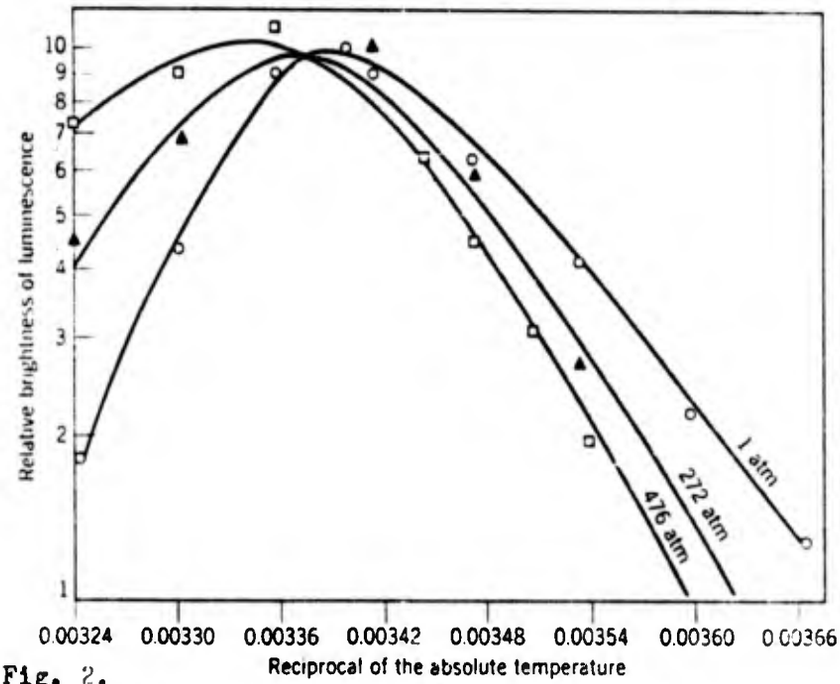


Fig. 2.

Fig. 2. The brightness of luminescence in *Photobacterium phosphoreum* as a function of temperature at three different hydrostatic pressures. The points represent data from experiments by Brown, Johnson, and Marsland (1942). The smooth curves were calculated by Eyring and Magee (1942) in accordance with equation 4.11 as discussed in Chapter 4

(Fig. 1.6, p. 26, in Johnson, Eyring, and Stover, 1974)

to fit the process of bacterial luminescence as far as temperature was concerned, but it left the influence of pressure still a mystery, all the more because, as mentioned above, the effect of increased hydrostatic pressure on proteins was thought to be generally that of causing, not reversing, denaturation.

In any event, the pressure reversal of the thermal diminution of luminescence at high temperatures invited experiments to see if pressure would similarly counteract the diminution brought about by various inhibitory drugs at the normal optimum temperature, where pressure alone had very little effect. The results of an experiment along these lines are illustrated in Fig. 3, showing that the inhibitory action of certain drugs, e.g., chloroform, ethyl carbamate (urethane), alcohol, and ethyl ether, was susceptible of partial to complete reversal under increased pressures of a few thousand psi. In other instances, e.g., with inhibitions caused by p-aminobenzoic acid or sulfanilamide on this system, and likewise with sodium barbital and chloral hydrate, there was no reversal of the inhibition, or even an increase in amount of inhibition, with rise in hydrostatic pressure.

The earlier work by Brown on the pressure-temperature relations of muscle tension, publication of which in detail was delayed for a number of years by various circumstances (Brown, 1957; Brown, Guthe et al., 1958; Guthe, 1957; Guthe and Brown, 1958), had yielded results remarkably like those subsequently found with bacterial luminescence, showing that the latter was not a unique phenomenon, and suggesting that fundamentally similar mechanisms were involved in both instances (cf. Figs. 1, A and 4). The basic similarity is even more impressive when data for the two processes, i.e., muscle tension (M.T.) and luminescence (L.) are plotted in the same graph (Fig. 5) as per cent changes for various amounts of increased pressure.

Qualitatively, the foregoing phenomena could be reasonably explained on the basis of a few simple ideas or assumptions, as follows. The brightness of bacterial luminescence is limited by the activity of an enzyme which for convenience and according to convention we may call bacterial "luciferase," catalyzing the oxidation of an unidentified substrate, a type of "luciferin". Under steady state conditions wherein the light intensity of a population of luminous bacteria remains constant over a period of minutes or longer, it seems justified to conclude that the luciferin is in excess. At a low temperature the light is relatively dim, limited by the rate of the chemical reaction of luciferase with luciferin. With rise in temperature the rate increases in approximate accord with the Arrhenius equation, and by an amount frequently found with specific enzyme reactions as well as more complex biological processes of numerous sorts. In terms of the Arrhenius activation energy, the numerical value of μ amounts to between 10 and 20 kilocalories, computed from the slope of the line at temperatures relatively low in relation to the optimum, i.e., with reference to the temperature of maximum intensity. At temperatures above the optimum, the decrease in intensity with rise in temperature is again approximately in accord with the Arrhenius equation for a reaction that interferes with, rather than giving rise, to luminescence, but with a much higher activation energy, between 50 and 100 kilocalories, such as would be expected in the thermal denaturation of many proteins and enzymes. However, this activation energy could scarcely represent a rate process of destruction, because

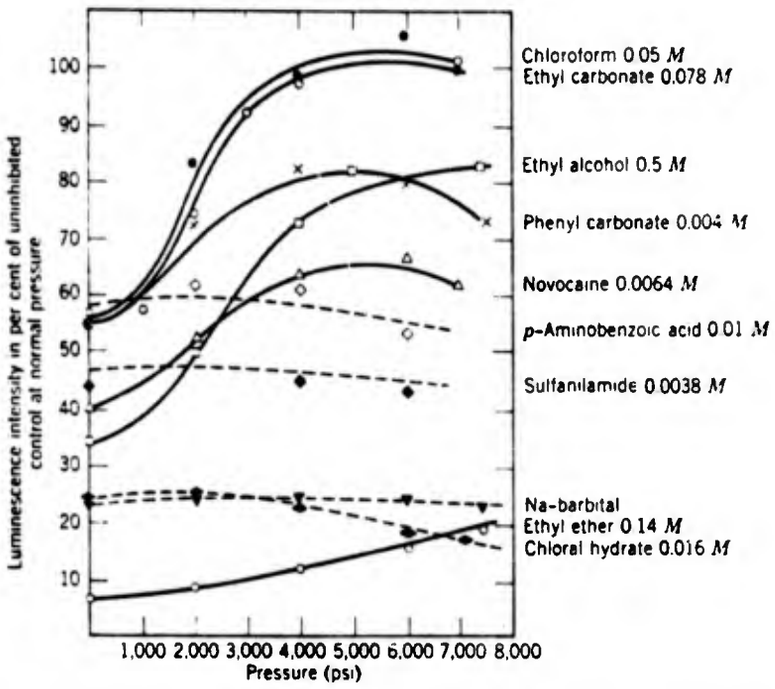


Fig. 3

~~Fig. 177~~ Influence of hydrostatic pressure on the luminescence of *Photobacterium phosphoreum* suspended in phosphate-buffered sodium chloride solution, pH 7.3, at 17 to 18°C, in the presence of various inhibitors as indicated at the right of the figure. (F. H. Johnson, Brown, and Marsland, 1942b). The luminescence of the control, without inhibitor, is taken as 100% at atmospheric pressure. Its intensity varied only slightly with pressure under these conditions (cf. Fig. 4.8A). The broken curves represent inhibitions that are not appreciably influenced by pressure; the solid curves represent inhibitions that are reduced by pressure. (Fig. 5.22, p. 412, in Johnson, Eyring, and Stover, 1874).

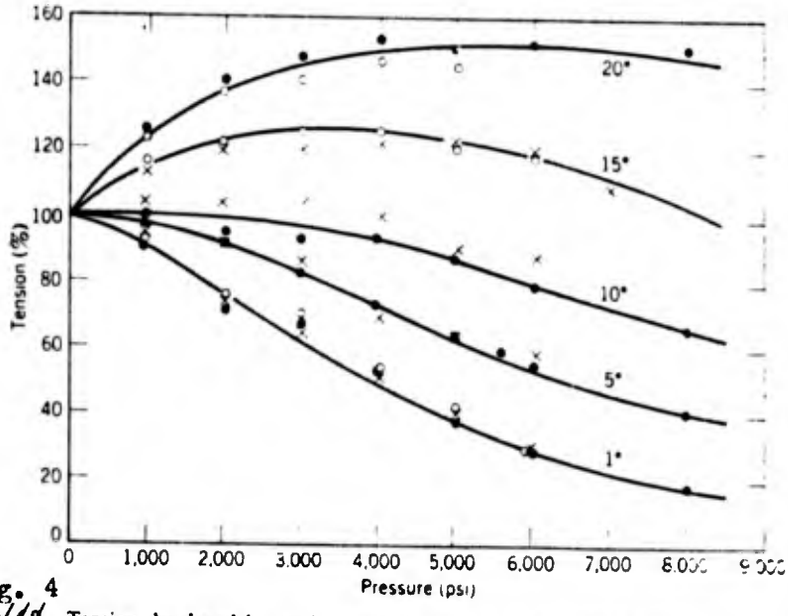


Fig. 4

~~Fig. 178~~ Tension developed by turtle auricle in relation to hydrostatic pressure and temperature. Tension is expressed as a percentage of the control at atmospheric pressure. Data from three experiments by D. E. S. Brown are plotted (by courtesy of Prof. D. E. S. Brown). (Fig. 4.9, p. 302, in Johnson, Eyring, and Stover, 1974).

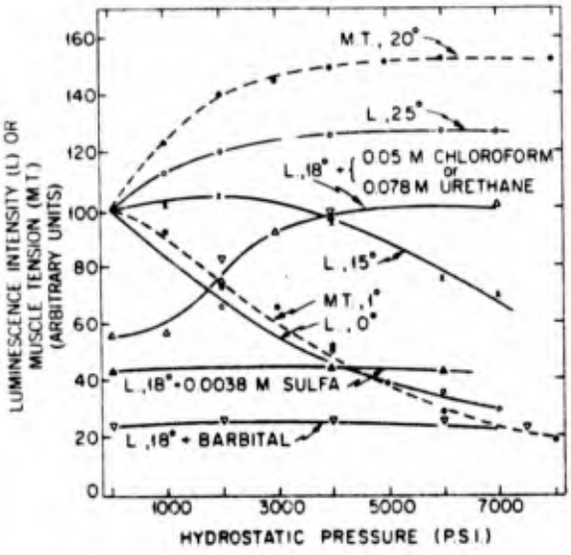


Fig. 5

Fig. 19. Changes in the intensity of luminescence (L) in the living cells of a psychrophilic species of luminous bacteria, *Photobacterium phosphoreum*, suspended in an isotonic buffered salt solution at neutral pH as a function of changes in hydrostatic pressure at various temperatures, with and without added drugs in the final concentrations indicated in the figure (F. H. Johnson, 1971). Data on muscle tension (M. T.) in excised turtle auricle are also included (broken curves) for two temperatures (1 and 20°C) without added drugs. For each curve the intensity of a control at normal pressure was arbitrarily taken as 100 to show clearly the relative changes induced by increased hydrostatic pressure at different temperatures and with added drugs at one temperature: the actual intensity of luminescence or the actual tension in the muscle varied in the usual manner with changes in temperature at normal pressure. The points shown in this figure are averaged data from experiments by Brown, Marsland, and F. H. Johnson, partly in collaborative research, and have been replotted from some of the same data illustrated in Figs. 9.8 (p. 310), 9.9 (p. 311), and 10.21 (p. 408) of F. H. Johnson, Eyring, and Polissar (1954). (Fig. 1.10, p. 32, in Johnson, Eyring and Stover, 1974).

throughout a wide range of temperatures from well below to well above the optimum, the effects on luminescence were found immediately reversible on short exposures to the various temperatures. At the highest range of temperature which the stability of the system under the conditions involved permitted investigating, a rate process of thermal destruction, also with a very high value for the Arrhenius activation energy, was conspicuously evident. The diminution at somewhat less than the highest temperatures becomes readily understandable in terms of mobile equilibrium, by which the native luciferase in its catalytically active state shifts rapidly with rise in temperature into a reversibly denatured form that is catalytically inactive. The μ values computed from the quantitative data would then represent heats of reaction, rather than activation energies.

In sum, at normal pressure, the temperature-activity curve can be accounted for in terms of only two chief reactions involving the same species of molecules: (1) a rate process of an enzyme-catalyzed oxidation leading to the production of light, and (2) a reversible thermal inactivation of the enzyme, becoming apparent as the temperature is raised from some relatively low level to approach, then exceed, the "optimum". The activation energy of the former falls within the range commonly associated with enzyme-controlled processes, whereas the heat of reaction of the latter, several times that of the activation energy, is in the range typical of protein denaturation. It is such that the light-producing result of the first reaction is rendered more and more ineffective by elimination of more and more of the active catalyst as the temperature is raised. The influence of the inactivation, or denaturation, is negligible at low temperatures, but increases from an equilibrium state where only one part in a million of the total enzyme is in a reversibly denatured state, to as much as 1 part in 2, i.e. half of the total enzyme is in a reversibly denatured state near the optimum temperature; with further rise in temperature beyond the optimum, the increase in proportion of enzyme in the denatured state occurs so fast with increase in temperature that 9 out of 10 of the total enzyme molecules are in a reversibly inactivated state at only a few degrees higher than the optimum. This means that under these conditions the over-all speed of the reaction and hence the observed brightness of luminescence is only about one tenth what it should be without the deleterious influence of the denaturation reaction. These conclusions could have been reached on the basis of classical equilibrium theory together with the Arrhenius relation, but prior to the research on pressure-temperature relations of bacterial luminescence the important influence of a reversible, thermal inactivation of a limiting enzyme had not been recognized.

Investigation of biological processes other than bacterial luminescence soon revealed that various phenomena behaved qualitatively in accord with the same general interpretations. The high temperature diminution in over-all rate was found to be often reversible on cooling, and the equation derived in accordance with the hypothesis described above for the temperature activity curve (at normal pressure) proved to be a satisfactory basis in accounting for the data involved. Figures 6-8 are included here to illustrate the more general applicability of the theory. At the same time, it should be noted that the theory, taking into account only the 2 reactions involving the same species

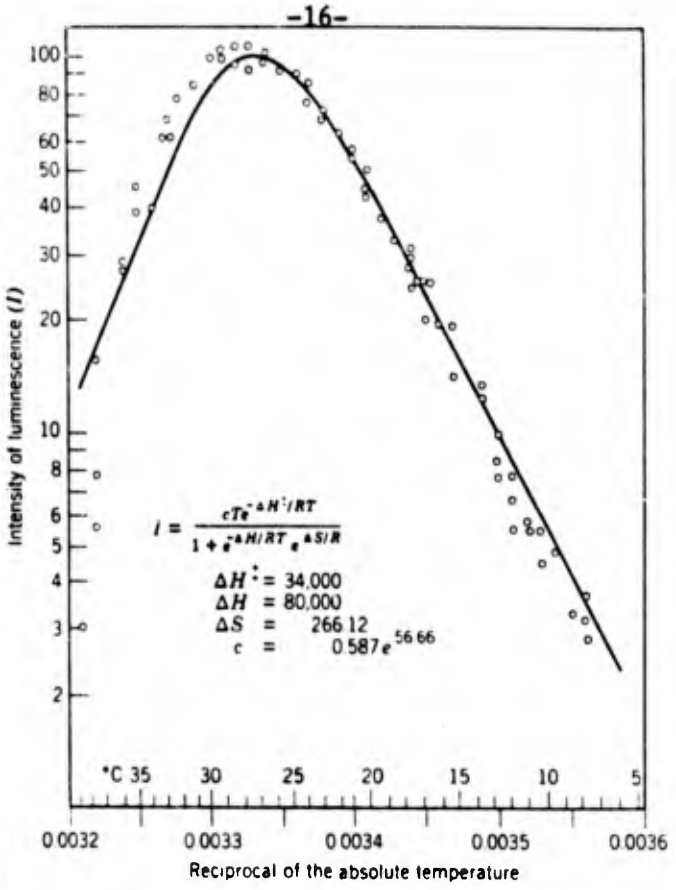


Fig. 6

Fig. 3.20. Influence of temperature on the intensity of luminescence in *Achromobacter fischeri* (F. H. Johnson, Eyring, and Williams, 1942). The smooth curve was calculated in accordance with equation 3.5, with the constants as given in the figure. (Fig. 3.20 p. 190, in Johnson, Eyring and Stover, 1974).

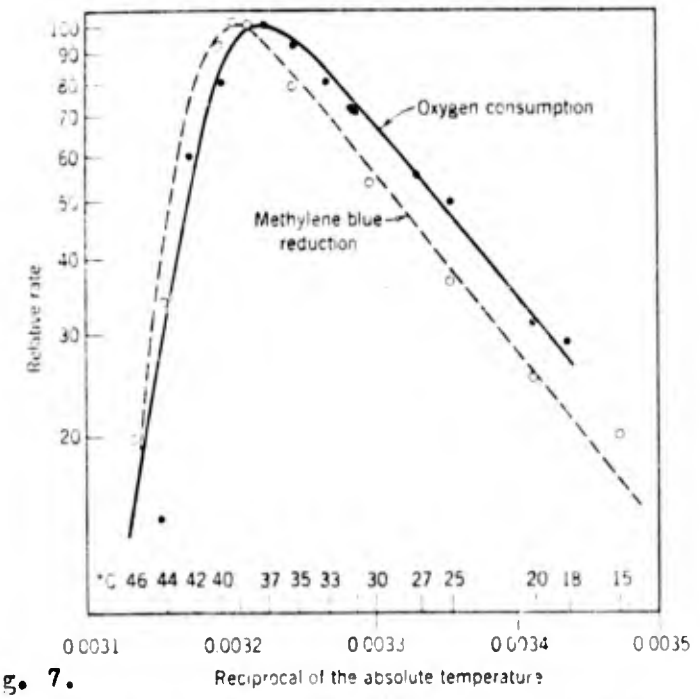


Fig. 7.

Fig. 3.21 Relative rate of oxygen consumption (solid curve, solid circles) and of methylene blue reduction (broken curve, open circles) by *Rhizobium trifolii* (Koffler, Johnson, and Wilson, 1947). The curves were calculated in accordance with equation 3.5, with the following constants: for oxygen consumption, $c = 0.3775e^{21.61}$, $\Delta H^{\ddagger} = 13,400$ cal, $\Delta H_1 = 96,000$ cal, and $\Delta S_1 = 306.14$ eu; for methylene blue reduction, $c = 0.3613e^{21.79}$, $\Delta H^{\ddagger} = 13,600$ cal, $\Delta H_1 = 126,000$ cal, and $\Delta S_1 = 399.72$ eu. (Fig. 3.21, p. 193, in Johnson, Eyring and Stover, 1974)

of molecules, i.e., the enzyme-catalyzed, rate process reaction and the equilibrium reaction between native and reversibly denatured states of the enzyme, is over-simplified, inasmuch as other reactions are not without influence, especially in living cells. Prominent among other reactions that are not specifically included in the temperature-activity equation is the irreversible denaturation of the enzyme that always occurs at temperatures sufficiently above the optimum. Also, it should be noted that the quantitative formulation does not necessarily describe the measured process equally well among different species. For example, the calculated curve for luminescence of A. fischeri (Fig. 6) shows impressive agreement with the data through a wide range of temperatures, but efforts to do likewise with respect to the luminescence of P. phosphoreum were not so successful; i.e., for unknown reasons, no combination of reaction rate and equilibrium constants was found that agreed satisfactorily in a quantitative sense with the observed data. The equation for the temperature-activity curve, based on the hypothesis described above, is included in Fig. 6, along with the constants pertaining to the rate and equilibrium constants of the enzyme-catalyzed reaction and denaturation equilibrium. The constant "c" in this figure has an arbitrary value, chosen to make the curve go through 100 at the maximum, thereby relating all other points to the maximum in convenient terms of per cent. In Fig. 7, the same fundamental equation is applied for calculating the temperature-activity curves for the overall rate of oxygen consumption and, in absence of oxygen, the rate of methylene blue reduction by a non-luminescent species of bacteria. Both processes were shown to undergo a reversible diminution at temperatures above the optimum. Fig. 8 illustrates application of the same equation to the rate of reproduction of bacteria (E. coli) under special conditions whereby plate counts of surface colonies were made over short periods of time (3 to 4 hours) during the early logarithmic growth phase, when the time was sufficiently short and the population of cells sufficiently small (usually of the order of 10^3 to 10^4 cells/ml at most, that the medium remained essentially constant with regard to concentration of oxygen, nutrients, H-ions, by products, etc. The rate of reproduction was constant throughout the period involved, and changed immediately on change in temperature in the manner shown by the points along the calculated curve of Fig. 8.

The data of Fig. 8 are replotted in Fig. 9 along with data available in the previous literature for various species of micro-organisms representing a wide range in normal optimum temperatures, from about 25°C for the psychrophile, Sporotrichum carnis, capable of measurable growth below the freezing point of water, to about 55°C for the thermophile Bacillus circulans, capable of reproducing at temperatures exceeding 60°C. These data have all been computed in terms of per cent, with respect to 100% as the maximum rate. Evidently, the curves drawn by inspection are all fundamentally alike, apart from different temperatures for the optimum, and apart from minor differences in slopes for temperatures below the optimum as well as somewhat greater differences in slopes above the optimum, the latter probably being due in part to differences in experimental conditions whereby different lengths of time were involved in measuring the rates at the higher temperatures, thereby causing greater errors due to irreversible destructive reactions whenever longer periods of time were required. In any event, the basic similarities seem more impressive than the detailed differences. It seems reasonable to expect that with reliable data pertaining to a sufficient number of

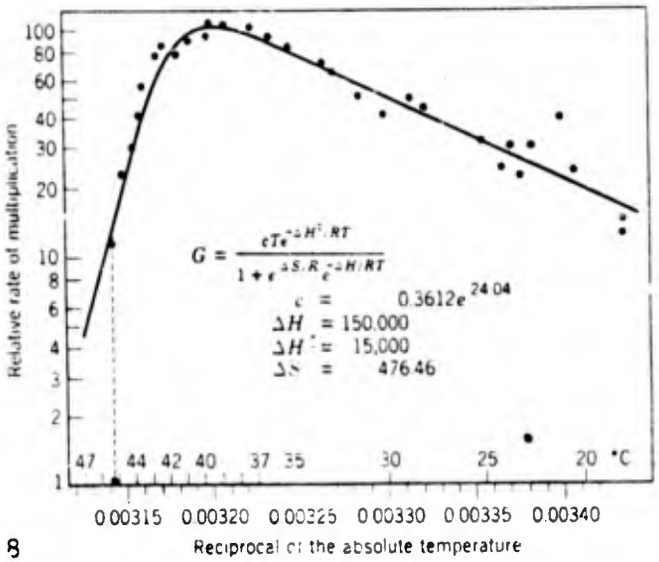


Fig. 8

Fig. 7.7. Relative rate of multiplication (G) of *Escherichia coli* in a simple medium as a function of temperature (F. H. Johnson and Lewin, 1946c). The maximum rate is arbitrarily taken as 100. The points are data from experiments, and the solid curve is calculated in accordance with the equation and constants shown in the figure.
(Fig. 3.25, p. 196, in Johnson, Eyring and Stover, 1974)

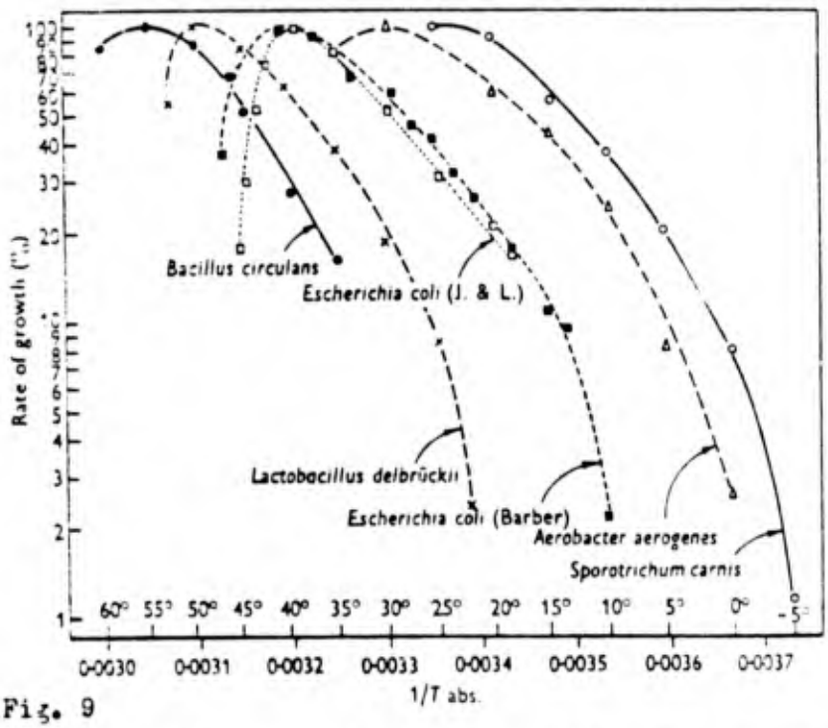


Fig. 9

Fig. 7.7. Influence of temperature on the rates of growth, relative to the observed maximum as 100 in each, of various species of bacteria and mould (F. H. Johnson, 1957b). The data were obtained from the following sources: *Bacillus circulans*, Allen (1953); *Lactobacillus delbrückii*, Slator (1916); *Escherichia coli* (solid squares), Barber (1908); *E. coli* (open squares), F. H. Johnson and Lewin, 1946c; a strain of *Aerobacter aerogenes*, Greene and Jezeski (1954); *Sporotrichum carnis*, Haines (1930).

(Fig. 3.27, p. 199, in Johnson, Eyring and Stover, 1974).

different species, an imperceptible graduation in positions of the optimum temperature would be revealed. Relatively slight variations in the free energy of activation for rates of growth, and in free energy of reaction for denaturation of a limiting enzyme would seem sufficient to displace the observed maximum, i.e., "optimum" in either direction along the temperature scale. The familiar occurrence of "temperature mutants" in many types of biological processes, as well as the fact that reversible shifts in the optimum for a given process can be brought about by changes in the chemical environment of the cells, e.g., by the presence of narcotics or other types of inhibitors (cf. Johnson, Eyring and Stover, 1974), tends to eliminate some of the mystery in which such variations as those illustrated in Fig. 9 appear at first thought to be shrouded. They also emphasize the artificiality of the natural, human tendency to "quantize" the seemingly continuous variables in biology by means of an arbitrary classification, in the present instance represented by the several ranges of temperature commonly referred to as "psychrophilic", "mesophilic", or "thermophilic". The same probably is true in reference to pressure, as witnessed by the current, rather loose use of the term "barophilic", more according to instinct than according to any generally understood, precise definition.

Interpretation of the influence of pressure on bacterial luminescence, and on the effects of narcotics, became possible through active collaboration with Professor Eyring and further application of the theory of absolute reaction rates. The origin and significance of this theory cannot be considered in detail here, beyond the summary statement that the dedicated efforts of many theoretical chemists after the turn of the century and advent of the quantum theory, culminated in the Eyring theory (Eyring, 1935; Glasstone, Laidler and Eyring, 1941). This theory, which has become known as the theory of absolute reaction rates, makes it possible for the first time, in principle, to calculate the specific rate of a chemical reaction from a knowledge of the properties of the reactants and the laws governing their behavior. In a word, the theory states that a chemical reaction of whatever nature involves the formation of an intermediate, unstable compound, the "activated complex" or "transition state" according to a probability given by the "free energy of activation", or exp. $- \Delta G^{\dagger}/RT$, where $\Delta G^{\dagger}$ for all practical purposes behaves in a manner that is entirely analogous to the ΔG of a thermodynamic equilibrium. An expression for the relation between pressure and reaction rates follows by analogy with the equilibrium quantities, i.e., $\Delta G^{\dagger} = \Delta H^{\dagger} - T\Delta S^{\dagger} = \Delta E^{\dagger} - p\Delta V^{\dagger} - T\Delta S^{\dagger}$. Qualitatively, the effects of increased hydrostatic pressure on bacterial luminescence were now understandable simply on the dual basis that first, catalytic activity of the enzyme proceeded with a volume increase of activation, which could be calculated, at least as a first approximation, from the ratio of luminescence intensity at increased as compared to normal pressure, at temperatures well below the optimum. The decreases in intensity under pressure can be taken to mean that a sizable increase in molecular volume occurs on formation of the activated complex. Similarly, the action of pressure in reversing the thermal inactivation of the system at temperatures above the optimum can be assumed to indicate that the reversible denaturation reaction proceeds with an even larger molecular volume increase in the final over that of the initial state. At intermediate temperatures, the two reactions, having opposite effects on the brightness of luminescence, tend to cancel each other, and they so balance

each other at the optimum that practically no effect of moderately increased pressures is observed. A somewhat more sophisticated discussion of these phenomena is given in Johnson, Eyring and Stover (1974).

The theory of absolute reaction rates states that the activated complex in any reaction decomposes with a universal frequency -- the same for all chemical reactions -- given by the expression kT/h, or RT/Nh, where k is the Boltzmann constant =R/N, or the gas constant R over Avogadro's number N, and T is the absolute temperature. A probability factor κ, often equal to unity, is introduced to designate the likelihood that formation of the activated complex will result in reaction, i.e., the activated complex will decompose in the direction of products of the reaction, rather than to a reconstitution of the initial reactants.

The formulation for the temperature-activity curve for bacterial luminescence is a straightforward derivation from the theory briefly outlined above. The same is true with respect to luminescence intensity at different pressures, except that the close fit of the curves in Fig. 2 to the data from experiments required taking into account a temperature dependence of the volume changes of activation and of reaction, in the catalytic activity and reversible denaturation, respectively, of the enzyme. The smooth curves in Fig. 2 were calculated by Eyring and MaGee (1942) in this manner.

The action of inhibitors, as illustrated in Fig. 3, was of especial interest in view of the fact that there was no previous report of an influence of hydrostatic pressure on the effectiveness of a narcotic, nor was there any theory that would in any way anticipate the possibility that such an effect would be found. In line with what has already been discussed, however, such a relationship was understandable on the grounds that the inhibitory action of the narcotics exhibiting such sensitivity to pressure in their action must be related to the reversible thermal denaturation which showed a quantitatively similar sensitivity to increased hydrostatic pressure. Substantiating evidence that such was indeed the case was found in the temperature relationships of the inhibition of luminescence caused by the lipid soluble narcotics, on the one hand, and by the other drugs such as barbital and sulfanilamide on the other hand. The latter appeared to act more in the manner of forming a loose combination with the catalyst, independently of the thermal denaturation equilibrium. Pressure had little influence on the amount of inhibition in this case, but a rise in temperature tended to dissociate the drug-enzyme complex, with the net result that the amount of inhibition decreased with rise in temperature. This type of inhibition was referred to as "Type I", and designated by the symbol Γ_1, typified by the action of sulfanilamide on the intensity of bacterial luminescence, an action that was manifestly different from the action of this drug on growth and reproduction of bacteria, which action was known to be generally reversible by small concentrations of p-aminobenzoic acid, not true of luminescence. With the aid of appropriate formulations, straightforwardly derived, it was not difficult to obtain numerical values for the ratio of inhibitor:enzyme molecules, as well as the heat and entropy, in the equilibrium reaction involved. The ratio turned out to be close to unity, but the fact that the values obtained were not strictly integers showed that the theory was somewhat oversimplified. Examples of the data are illustrated in Figs. 10 - 12.

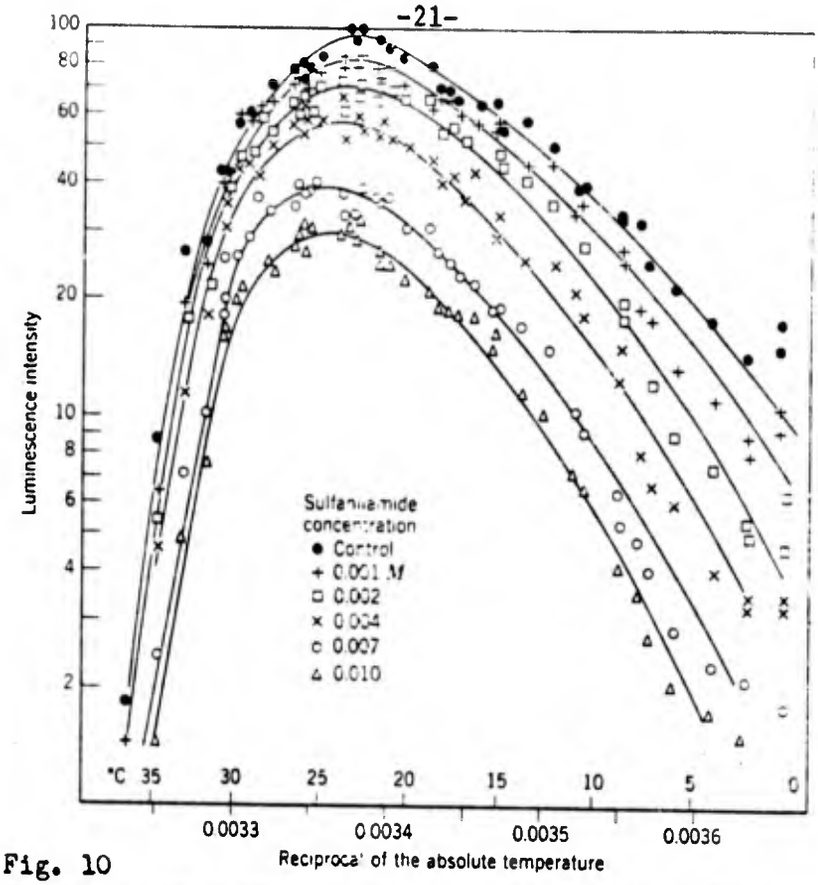


Fig. 10

Fig. 5.6. Influence of temperature on the luminescence intensity of *Photobacterium phosphoreum* suspended in phosphate-buffered sodium chloride solution containing various concentrations of sulfanilamide (F. H. Johnson et al., 1945).

(Fig. 5.6, p.388, in Johnson, Eyring and Stover, 1974)

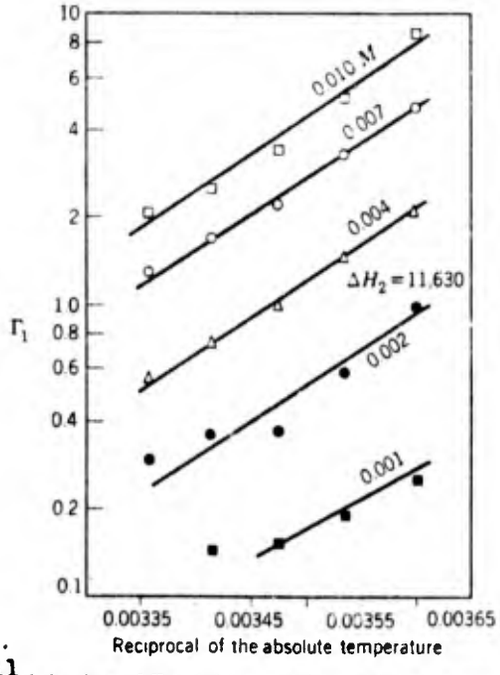


Fig. 11

Fig. 5.8. Analysis of the data of Fig. 5.6 in accordance with equations 5.9 and 5.10 for relation between inhibition and temperature at various concentrations of sulfanilamide (F. Johnson et al., 1945).

(Fig. 5.8, p.390 in Johnson, Eyring and Stover, 1974)

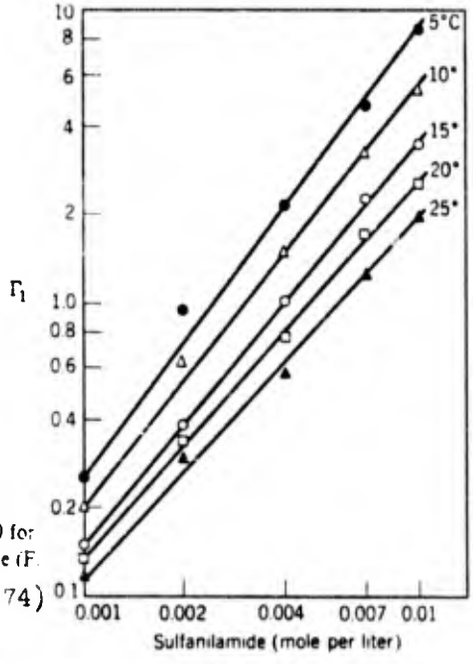


Fig. 12

Fig. 5.7. Analysis of the data of Fig. 5.6 in accordance with equations 5.9 and 5.10 for the relation between inhibition (Γ_1) and the concentration of inhibitor at various temperatures (F. H. Johnson et al., 1945). (Fig. 5.7, p. 389, in Johnson, Eyring and Stover, 1974),

In Fig. 10 it will be noted that there is a slight shift of the "optimum", or more strictly the "maximum", to higher temperatures as the concentration of sulfanilamide is increased; the observed activation energy also appears to increase with concentration of sulfanilamide. All effects produced by this drug as illustrated in Fig. 10 are reversible.

Turning to the lipid-soluble narcotics such as alcohol, chloroform, and including also urethane, the temperature relationships of their action suggest that these agents act in a manner that promotes the reversible thermal denaturation, in a second type of inhibition, Type II, designated also by the symbol Γ_2. Formulations derived on the postulated basis that such drugs act through an equilibrium reaction, characterized by equilibrium constant K_3, with the reversibly denatured form of the enzyme in the equilibrium reaction with constant K_1, seemed largely satisfactory when applied to data from experiments. While it is perhaps easier to picture the different equilibria in terms of K_1 and K_3, assuming that the drug combines only with the final state, i.e., the denatured form, of the thermally inactivated enzyme, it should be pointed out that there is no physical way to distinguish between this mechanism and combination with only the native form in accordance with the product of K_1 and K_3, i.e., by K_1K_3. Data from experiments are illustrated in Figs. 13-15, which make it clear that the temperature relations of the alcohol inhibition are opposite to those of the sulfanilamide inhibition in that alcohol causes a seeming decrease in activation energy of the process of luminescence, along with a decrease in temperature of maximum intensity, i.e., a lowering of the "optimum".

These effects again are nearly all reversible, and they favor the view that alcohol acts by promoting a reversible thermal denaturation of the catalyst. However, the data reveal some seeming complications: first, in Fig. 13, in the presence of alcohol there seems to be a slight "stimulation" of luminescence at low temperatures, although this stimulation is overcome by the inhibitory effects of the higher concentrations of alcohol at the same temperatures; second, with change in temperature there is a pronounced change in slopes of the lines in the analytical plot in Fig. 15 of the data in Fig. 13; and third, the non-integral values of the slopes, indicating ratios of 2.4 to 3.7 of drug to enzyme molecules in the complex involved; and fourth, the tendency for points along the lines in Fig. 14 to fall too high at the higher temperatures.

In regard to the first of the above, the "stimulation" by alcohol in low concentrations at low temperatures has no unique explanation, though it is not an unusual type of biological effect. The second of the seeming complications listed is understandable on the basis that the slopes of the lines in Fig. 15 must represent average values pertaining to more than one equilibrium. This interpretation could also explain how the slopes of the lines change with temperature. Finally, in regard to points tending to fall above the lines at the high temperatures in Fig. 14, such an effect might be expected if alcohol enters into more than one reaction with the enzyme. In fact, a study of the time-course of inhibitions caused by alcohol, urethane, etc. revealed that the initial inhibitions, especially at high concentrations of the drug, or at high temperatures, increased with the kinetics of a first order reaction, thus indicating that the same agents which act in the manner

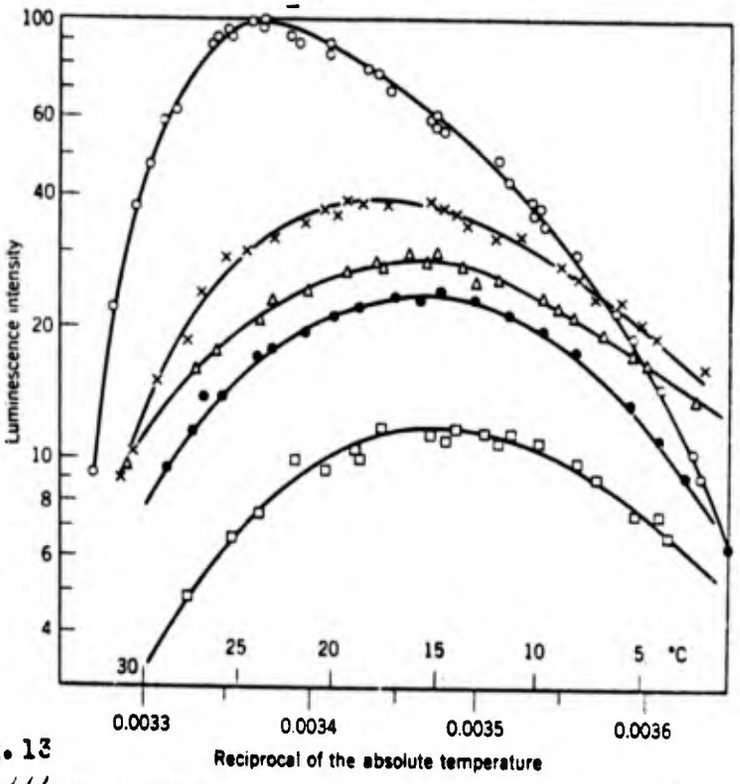


Fig. 13

Fig. 5.24 The influence of temperature on the inhibition of luminescence in *Photobacterium phosphoreum* by various concentrations of ethanol under neutral conditions (F. H. Johnson et al., 1945).

(Fig. 5.24, p. 414, in Johnson, Eyring and Stover, 1974).

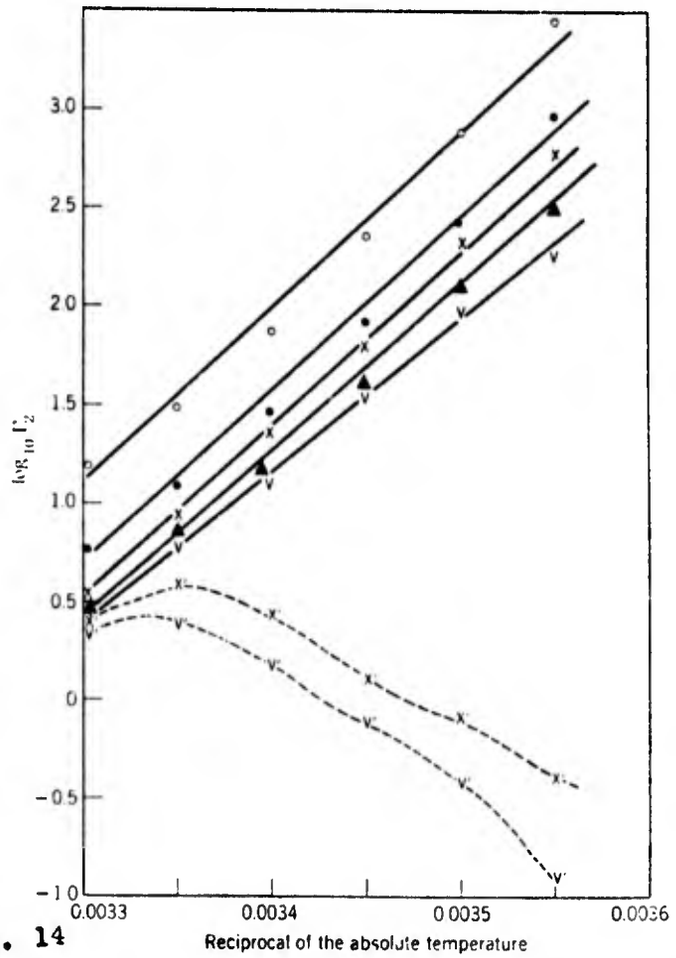


Fig. 14

Fig. 5.26. Analysis of the inhibition of luminescence by ethanol with respect to temperature in accordance with equation 5.16; data from Fig. 5.24. The broken curves indicate the results of using equation 5.9 instead of equation 5.16 in the analysis.

(Fig. 5.26, p. 416, in Johnson, Eyring and Stover, 1974).

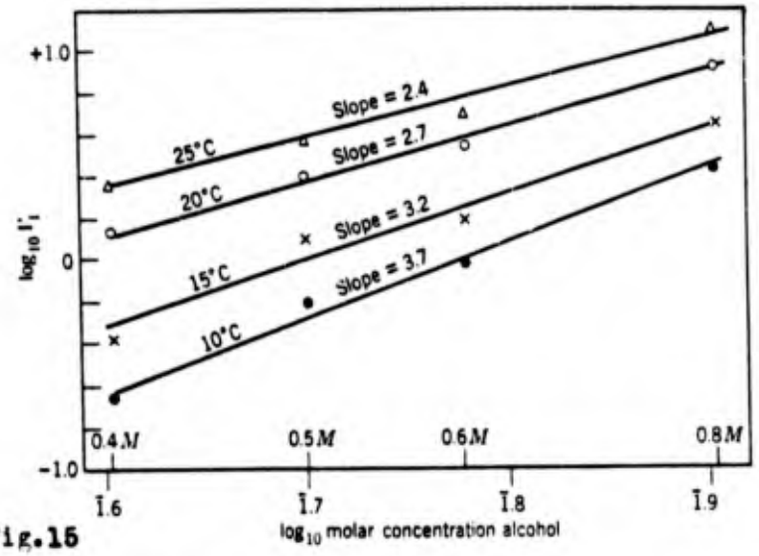


Fig.15

Fig. 5.27/ Analysis of the inhibition of luminescence by ethanol with respect to concentration at various temperatures, in accordance with equation 5.16; data from Fig. 5.24.

(Fig. 5.27, p. 417, in Johnson, Eyring and Stover,1974).

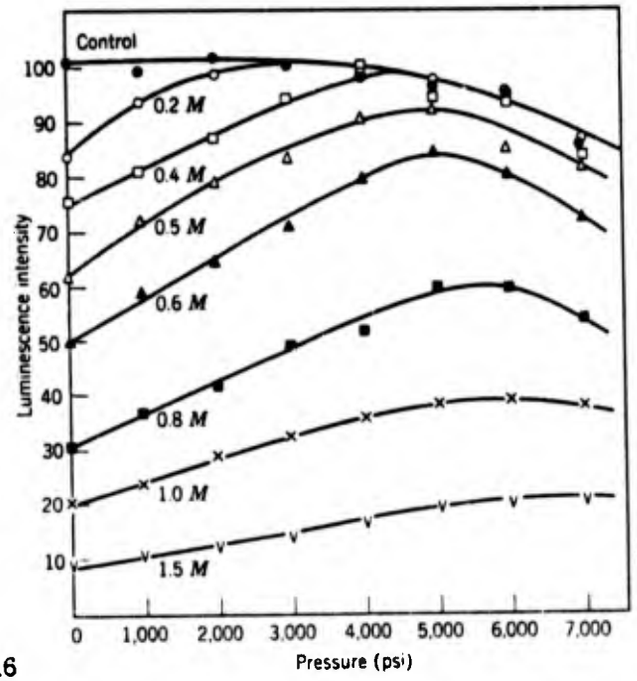


Fig. 16

Fig. 5.32. Luminescence intensity of *Photobacterium phosphoreum* as a function of hydrostatic pressure at 17.5°C, in the presence of various concentrations of ethanol in the phosphate -buffered sodium chloride solution, pH 7, of the suspending medium (F. H. Johnson et al., 1945). (Fig. 5.32, p. 429, in Johnson, Eyring and Stover, 1974

of promoting a reversible denaturation of the enzyme also promote an irreversible destruction. Full details supporting this conclusion may be found in Johnson, Eyring and Stover, 1974, pp. 371-548.

At the stage described so far in development of the theoretical basis for the biological action of temperature, pressure, and narcotics, some rather striking aberrancies in traditional views were apparent. They included the following: (1), increased pressures of a few hundred atmospheres can reverse the thermal denaturation of enzymes, whereas previous evidence was only to the opposite effect, i.e., the destruction, or denaturation of proteins in general under increased pressure; (2), the newly discovered relation between increased pressure and amount of inhibition of bacterial luminescence by narcotics, a finding that did not seem altogether anomalous, inasmuch as there was no previous report of such a phenomenon, (3) a reversible thermal denaturation of enzymes as a controlling reaction in a physiological process, and representing a fundamental factor in temperature-activity curves. Such a concept, however, was not altogether foreign to a few avant-garde biochemists including Anson and Mirsky (1931), as already mentioned, but still it was a concept that, by and large, appeared to be viewed with skepticism if not suspicion. In addition to these seeming anomalies, acceptability of the interpretations described, despite the quantitative, theoretically sound evidence on which they were based, might be added another source of objections, namely, on the grounds that practically all the data pertained to bacterial luminescence and therefore a highly special case, with no obvious bearing on processes in organisms as complex as higher animals including man. Furthermore, the mere fact that much of the data were obtained in regard to a process that was taking place within living cells was sufficient to arouse the determined mistrust of all really hard-core biologists who, since the agnostic vitalists of Louis Pasteur's era, have rejoiced in the complexities of living systems and flaunted their intellectual insecurities in the face of the most substantial, convincing experimental evidence and rigorous conformity to rational theory. In order to eliminate all doubt generated by the notion that bacterial luminescence constituted a source of unique phenomena, rather than a useful tool having unique advantages for investigating fundamental reaction rate controlling mechanisms in living systems, it seemed important to study other types of material than luminous bacteria, and include complex animals as well as non-living systems such as purified proteins in solution.

The influence of increased pressure and small concentrations of alcohol on the thermal denaturation of a highly purified protein, viz. human serum globulin, in homogeneous solution was among the first examples reported in which the rate of denaturation was retarded by increased pressures up to 10,000 lbs psi (Fig. 17). The term "denaturation" is used advisedly here, inasmuch as, in its classical meaning, it refers to the property of solubility, the denatured protein precipitating from aqueous solution. The kinetics here of denaturation indicates that the process is more complicated than that of a first order reaction, the discrepancy possibly being due to heterogeneity of the population of serum globulin molecules which were in fact purified from the pooled blood of a number of different individuals. Whatever the explanation of the kinetics, it is evident (Fig. 17) that: (1), a moderately increased hydrostatic pressure greatly retards the rate of thermal denaturation of this

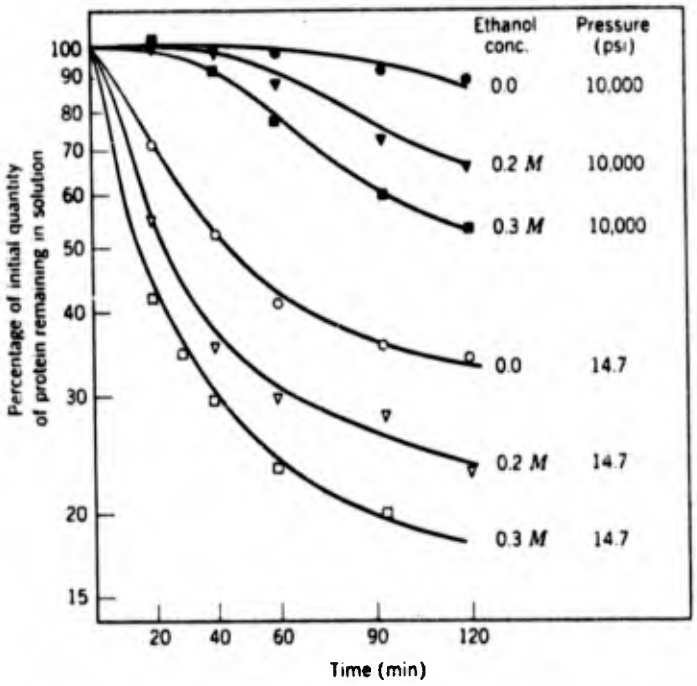


Fig. 17

Fig. 7.45. Acceleration of precipitation of highly purified human-serum globulin by ethanol and retardation of precipitation by 10,000-psi hydrostatic pressure, at 65°C (F. H. Johnson and Campbell, 1946). (Fig. 5.45, p. 450, in Johnson, Eyring and Stover, 1974).

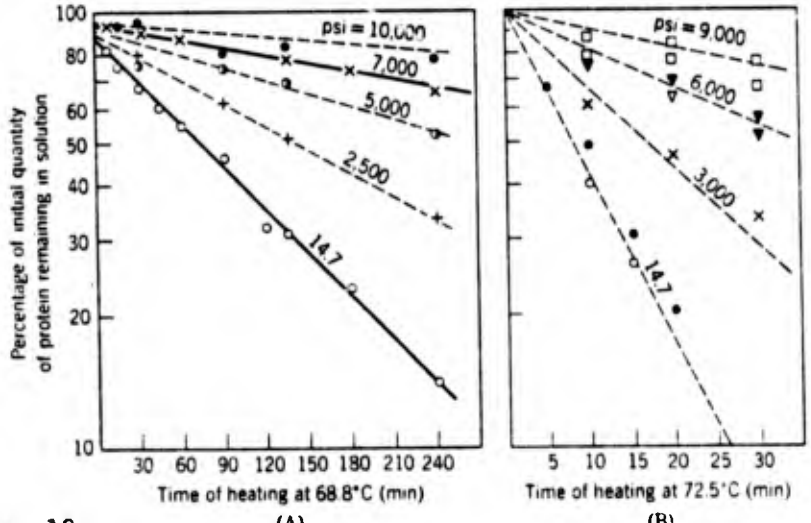


Fig. 18

Fig. 7.47. Influence of increased pressure on the rate of tobacco-mosaic-virus denaturation at 68.8 (A) and at 72.5°C (B), in solution of pH 7.05 (F. H. Johnson, Baylor, and Fraser, 1948). The rates at normal pressure and 7000 psi were measured (solid lines in (A)). and the remainder (broken lines in (A) and (B)) were computed as described in the text. The points represent the percentage of the initial amount of protein remaining in solution, according to Kjeldahl nitrogen determinations of the supernatant after high-speed centrifugation of the specimens. (Fig. 423, p. 423, in Johnson, Eyring and Stover, 1974)

protein, both with and without alcohol, and (2), small concentrations of alcohol accelerate the rate, both at normal and increased hydrostatic pressure.

The thermal denaturation of other proteins, e.g., tobacco mosaic virus (TMV), may take place with the kinetics of a first order reaction, under both normal and increased pressure (Fig. 18). Under such conditions, analysis of the data is simplified, and reliable quantitative predictions are made possible by the theory of absolute reaction rates. In Fig. 18A the slopes of the solid lines, pertaining to atmospheric and 7,000 p.s.i., were used to compute the volume change of activation, and the value obtained was then used to predict the dashed lines for 2,500, 5,000, and 10,000 p.s.i., actually in advance of doing the experiments which yielded the points shown in the figure. In addition, by using a published value for activation energy at normal pressure, together with the same value (close to 100 cc/mole) for volume increase of activation, it was possible to predict with considerable accuracy the rates at a different temperature and for a different set of pressures. The results are illustrated in Fig. 18B, wherein the dashed lines were computed in advance, and the points representing the data from experiments were then added. The variation in specific rate constant with variation in pressure at two temperatures is shown in Fig. 19.

With reference to the action of chemical agents on these processes, the effects of urethane (ethyl carbamate) and ethyl alcohol on bacterial luminescence had already indicated that both of these substances act primarily by promoting a reversible, but in addition an irreversible, denaturation of the limiting enzyme, in a manner that is partially reversible, or retarded, respectively, by increased pressure. It is of interest therefore to find analogous effects of alcohol (Fig. 17) and of urethane (Fig. 20) in the denaturation of pure proteins. The data with respect to the action of urethane in accelerating the thermal denaturation of tobacco mosaic virus show that urethane acts by more than one reaction in this process. This conclusion was reached earlier in regard to living systems (cf. Johnson, et al., 1945).

The possibility that increased pressure could counteract not only the effects of certain narcotics on bacterial luminescence (and the accelerating effects of the same narcotics, in particular alcohol and urethane, on the process of protein denaturation in solution), but could counteract also a typical narcosis in animals was confirmed in a study with tadpoles. Besides being a "classic" animal for research on the action of narcotics, tadpoles constitute a type of animal especially suited for research involving increased pressure, because they are completely aquatic, having no lungs or other bodily spaces unfilled with water. Terrestrial animals that breathe by lungs cannot be subjected to hydrostatic pressure while submerged in an aqueous medium for obvious reasons, including the crushing of their air spaces under high pressures over their entire bodies. The experiments at issue were conducted with small tadpoles of the frog Rana sylvatica and with small larvae of the salamander Amblystoma maculatum. When specimens were placed in 2 to 3 per cent alcohol, or 0.08 M urethane, they underwent full narcosis, with loss of spontaneous movement as well as loss of response to gentle mechanical stimulation. This narcosis was gradually reversible by transferring the animals to drug-free

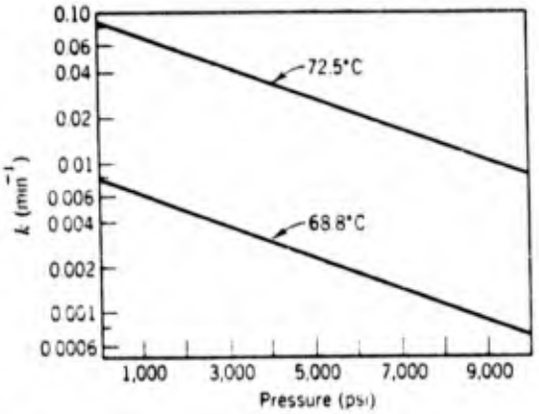


Fig. 19

Fig. 4.24 Relation between first-order rate constant and hydrostatic pressure in the denaturation of tobacco mosaic virus (F. H. Johnson, Baylor, and Fraser, 1948).
(Fig. 4.2--, p. 323, in Johnson, Eyring and Stover, 1974).

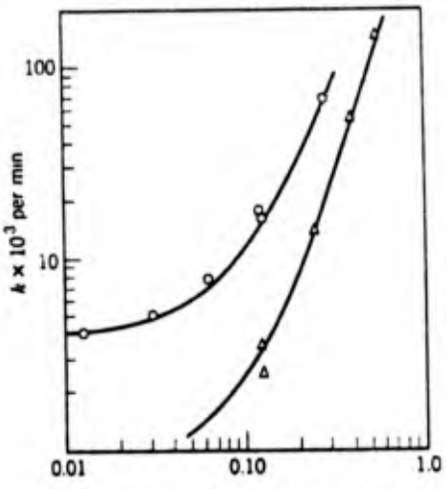


Fig. 20

Fig. 5.44. Influence of urethane on the rate of denaturation of pure tobacco mosaic virus in phosphate buffer, pH 7.0, at 68.8°C, at normal pressure (circles) and under 7000-psi hydrostatic pressure (triangles). The solid curves are calculated according to equation 5.26 (Fraser, Johnson, and Baker, 1949).
(Fig. 5.44, p.4 48, in Johnson, Eyring and Stover,1974).

water. While in the narcotizing solutions of alcohol or urethane, however, the narcosis was immediately reversible by raising the hydrostatic pressure from atmospheric to between 2,000 and 3,000 p.s.i. (Fig. 21).

Thus, in effect the fundamental phenomena discovered first with regard to bacterial luminescence were duplicated in an amphibian animal: the experiments involved can be looked upon as among the most important ones ever carried out in reference to the theory of narcosis. And they could hardly fail to disturb the smug complacency of any die-hard biologist who so enjoys being an intellectual recluse that he automatically refuses to accept a relatively simple theory in even partial explanation of a very complicated phenomenon.

"Warum es einfach machen, wenn es auch so komplizierte geht."

III. Results of ONR-Sponsored Research, 1954-1974

The foregoing account has been intended to outline the background of the research which led up to support by the Office of Naval Research. This seemingly long account might be made briefer, but inevitably at the expense of clarity and documentation. As it stands, it is by no means complete -- a more nearly complete account is available in the recent monograph by Johnson, Eyring and Stover (1974), of how the research on bacterial luminescence ushered in new concepts, which brought together previously unrelated fields of inquiry, viz., the biological action of temperature, pressure and narcotics, on the rational basis provided by the theory of absolute reaction rates, a theory which itself had only a few years earlier crowned the efforts of more than one generation of brilliant theoretical chemists, finally culminating in the complete theory set forth by Henry Eyring in 1935. The 1974 monograph referred to above is based in large part on an earlier one by Johnson, Eyring and Polissar (1954), but with so much revision, updating, and more uniform theoretical approach that it was appropriately given a new title, namely, "The Theory of Rate Processes in Biology and Medicine", rather than the earlier title of "The Kinetic Basis of Molecular Biology". The earlier book could not have been written without the support of ONR, Physiology Branch, which made it possible for the present author to spend full time on the task of completing the manuscript while on leave of absence from Princeton in order to collaborate with Professor Eyring of the University of Utah during the academic year 1950-51 plus the preceding summer and the summer which followed. Academic responsibilities in Princeton were then resumed for a year, until another one-year's leave of absence was devoted to a tour of duty as a staff member of the newly born National Science Foundation, during 1952-53. It was at the end of this absence that laboratory research was taken up again in earnest, this time with support from the Biology Branch of the Office of Naval Research for the investigation of "Factors Which Influence the Intensity of Bioluminescence." Achievements under ONR support, modest in amount but important for what it made possible for the next some 20 years under several different Branches, can perhaps be most clearly and concisely reviewed separately with reference to each type of the number of different types of luminescence

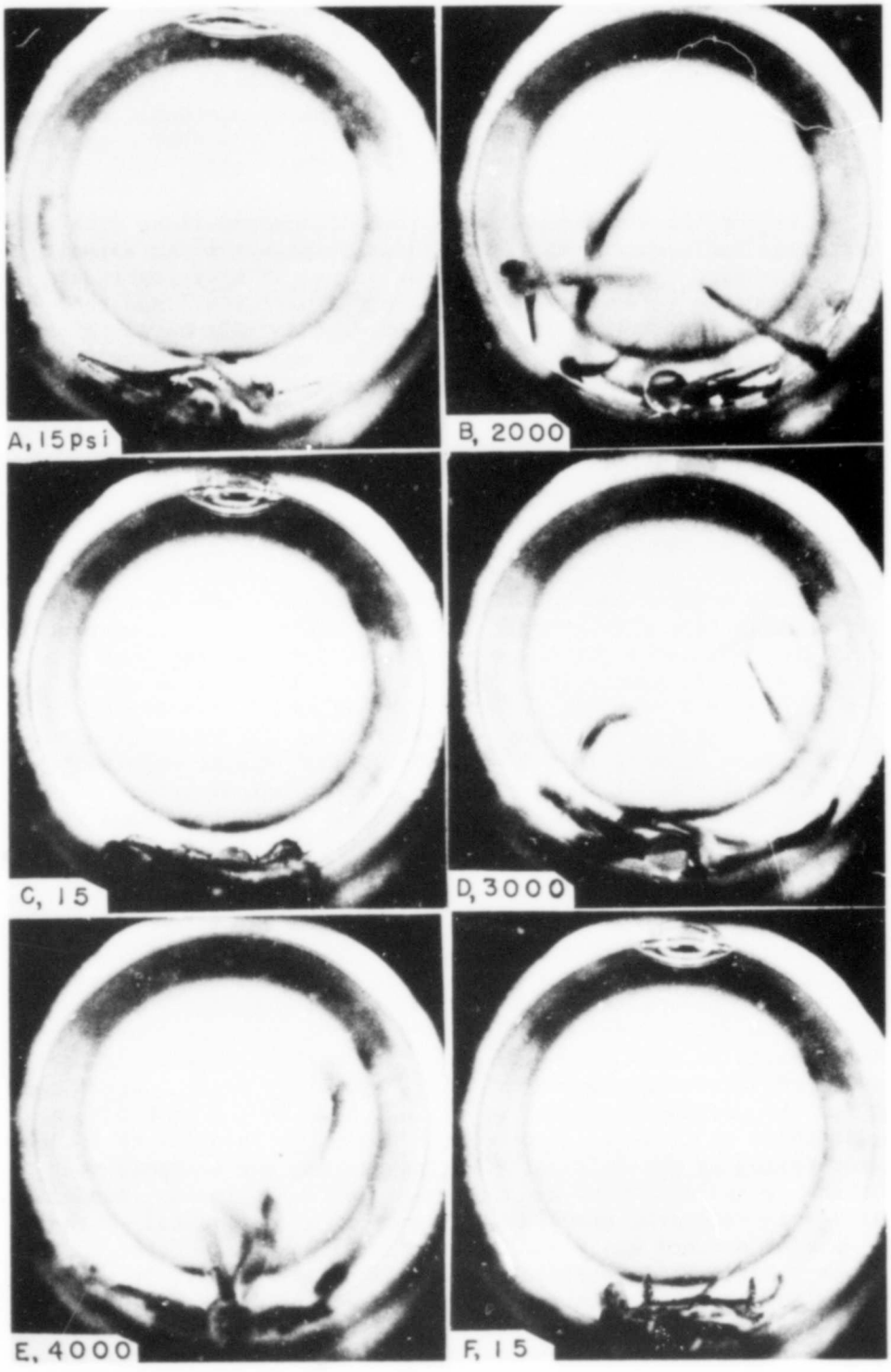


Fig. 21

Activity of *Amblystoma* larvae, in 2.5% ethanol, with successive changes in hydrostatic pressure over a total period of 4 min (F. H. Johnson and Flagler, 1951b). Photographs were made through a window in the pressure chamber. The air bubble visible at the top of the compartment under atmospheric pressure disappears as the pressure is raised and reappears as the pressure is released. The slight increase in the amount of dissolved oxygen that occurred in this manner cannot account for the decreased narcosis, inasmuch as similarly narcotized animals showed no recovery when transferred to a corresponding narcotic solution that had been equilibrated with pure oxygen.

(Fig. 5.67, p. 527, in Johnson, Eyring, and Stover, 1974)

systems which have been included. It should be pointed out that collateral support for studying aspects related to those sponsored by ONR has been received through grants from the National Science Foundation. It seems appropriate from not only a chronological, but from other, more significant points of view, to begin with the bacterial system.

A. Bacterial Luminescence

While "The Kinetic Basis of Molecular Biology" was in Press, the first well-substantiated report of luminescence in cell-free extracts of (luminous) bacteria was published by B. L. Strehler (1953), who noted its dependence on reduced duphosphopyridine nucleotide (DPNH). Very soon thereafter, McElroy and collaborators (McElroy et al., 1953) reported evidence that thus luminescence depended also on the presence of flavine mononucleotide (FMN). In addition, Strehler and his colleague M. J. Cormier discovered that a substance in dried kidney cortex was an exceedingly potent factor in promoting a bright, lasting luminescence of partially purified bacterial "luciferase", provided an adequate amount of DPNH and FMN were also present. The active factor from dried kidney cortex was first referred to as "KCF". It was later identified as palmitic aldehyde (Cormier and Strehler, 1953), and further investigation of the effectiveness of aldehydes revealed that straight chain, unsubstituted aldehydes having more than 7 carbons were effective to varying extents, the longer chain lengths being generally more effective until solubility in water became limiting (Strehler and Cormier, 1954). The function of the aldehyde was a matter of speculation, and remained so for many years, especially because no one succeeded in extracting any compounds of the same type from the cells themselves.

The availability of luminescent extracts offered a means of obtaining further evidence concerning the possible role of cell integrity in the extensive data derived from the research on the influence of pressure, temperature and narcotics as well as other chemical agents on the luminescence of intact cells. With the cooperation of Dr. Strehler, who furnished acetonized powders of the species known as Achromobacter fischeri, some key experiments were carried out in the Princeton laboratories concerning the effects of the above variables on steady-state luminescence of extracts. The enzyme preparation consisted simply of an aqueous extract of the acetonized powders, with particulate matter removed by centrifugation at 70,000 x g for one hour. It was apparent practically at once that the steady state luminescence of extracts behaved in fundamentally the same manner as the steady state luminescence of the living cells in regard to changes in pressure and temperature (Fig. 22). A difference was noted in the time required for the change from one steady state to the other, when pressure was suddenly applied or released, the transition being much faster in the living cells than in the extracts, but otherwise the observed phenomena were very similar, even quantitatively so. Because of various circumstances, a prolonged investigation did not prove possible at that time, but suggestive evidence was obtained that alcohol and urethane acted to inhibit the luminescence of extracts in essentially the same manner as had been found with cells. Unfortunately, the only partially purified enzyme preparations available at the time did not invite a detailed study in effort to find, with assurance, the precise step that was most sensitive

to increased pressure in the chain of reactions leading to light-emission. Even now, this problem remains unsettled. The chief importance of the study of the influence of pressure and temperature on luminescence in extracts was its demonstration that the effects were much like those in the prior study of these factors on the luminescence of living cells, and therefore the effects were not due indirectly to some vague mechanism existing within the complexity of an intact cell, but were on the luminescence system itself.

With particular reference to the action of temperature, pressure and narcotics on bacterial luminescence, the fact that increased pressure would reverse the alcohol or urethane narcosis of tadpoles was not convincing to the minds of unimaginative, over-cautious, intellectually hidebound conservatives that the interpretation regarding the molecular mechanism was correct. Evidence that might be taken as supporting the view of similar basic molecular mechanisms in the limiting reaction of bacterial luminescence and in activity of nerves,however, was found in research by Tasaki and Spyropoulos (1957) on the activity of isolated nerves of the squid (Figs. 24 and 25). Thus, reduction in action potentials due to ethanol was found to be counteracted by increased pressure at 22° (Fig. 23). Moreover, reduction in action potentials by alcohol at 22° was largely counteracted by simply lowering the temperature to 4°, all at normal pressure (Fig. 24).

Finally, in reference to the same phenomenon, the pioneering study that was published in 1951 by Johnson and Flagler on the pressure reversal of alcohol narcosis of tadpoles by increased hydrostatic pressure lay buried in the literature for some 20 years -- sufficiently buried, at any rate, that it failed to prompt any outspoken perturbation in the thoughts of pharmacologists, who continued to dwell on the Meyer-Overton "theory" based on the correlation between narcotic potency and lipid solubility. A renewed interest in the fundamental action of narcotics arose in the 1960's, stimulated perhaps by the theory proposed independently by S. L. Miller and Linus Pauling in 1961, based on the correlation between potency of anesthetics and their hydrate dissociation pressure at 0°C. This correlation, was found to be not as close as the correlation with lipid solubility. Moreover, a more satisfactory theory of the action of anesthetics and narcotics, at the molecular level as well as the level of nerve physiology, has been proposed by Eyring and collaborators (cf. Eyring, Woodbury, and D'Arrigo, 1973; Ueda and Kamaya, 1973; Johnson, Eyring and Stover, 1974).

The same theory provides a basis for understanding the action of increased pressure in reversing the effects of narcotics and anesthetics. Thus, it is of special interest to note that in very recent years the relation between pressure and narcosis, discovered in regard to bacterial luminescence then first demonstrated in animals in tadpoles (Fig. 22), has been confirmed both in aquatic and in air-breathing animals (mice) through studies of the influence of increased helium pressures on the narcotic action of a variety of agents (Lever et al., 1971). Some quantitative data concerning the effects of helium pressure on the nitrogen narcosis of newts are illustrated in Fig. 25.

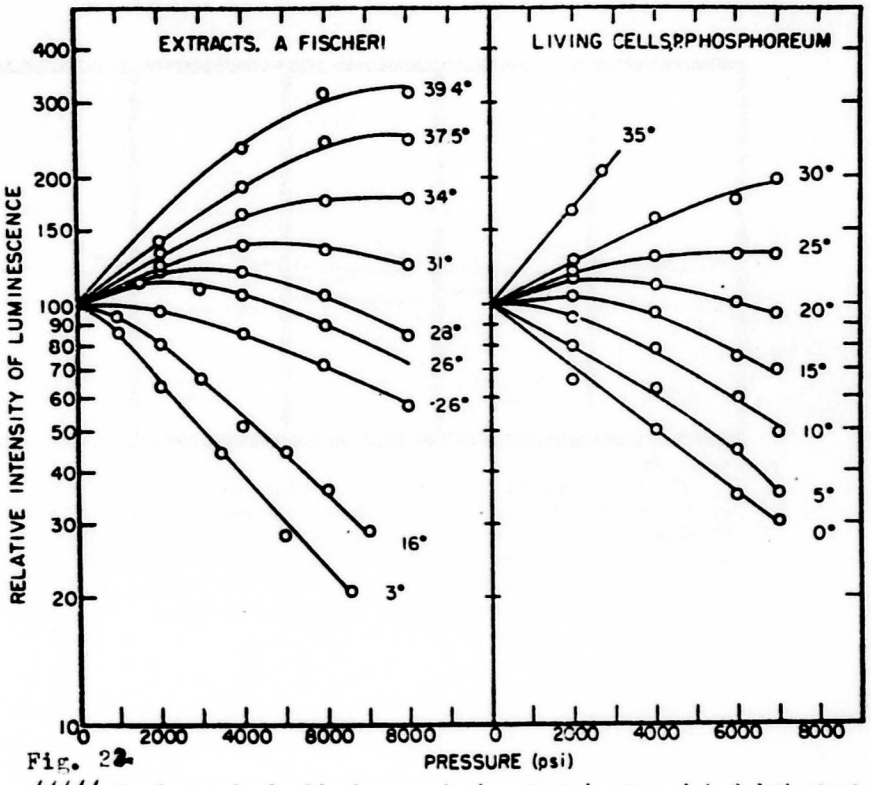


Fig. 2.

Fig. 2—Steady-state levels of luminescence in the saturated system of *A. fischeri* extracts (*left*) and in living cells of *P. phosphoreum* (*right*). The data on the right are replotted from Brown, Johnson, and Marsland, *J. Cellular Comp. Physiol.*, 20, 151-168, 1942. The initial intensity at normal pressure is arbitrarily taken equal to 100 at each temperature, and allowance is made for decay, when significant, in the luminescence of extracts. The lower of the two curves for 26° C. was obtained with an enzyme solution that had stood for several days at room temperature (Fig. 2, p. 610, in Strehler and Johnson, 1954).

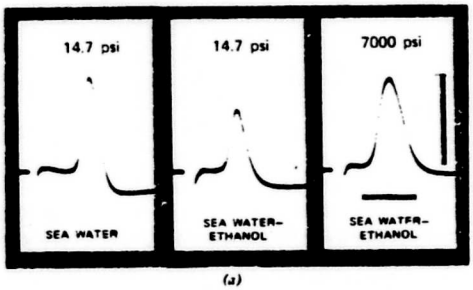


Fig. 23.

(a)

Fig. 23 Records showing the counteraction of ethanol narcosis with high pressure (Spyropoulos, 1957b). A glass-pipette internal electrode was used. The axon was stimulated near its cut end with two steel wires. The glass pipette recorded the conducted response. Ethanol concentration 3%; temperature 22°C. The vertical bar at right subtends 100 mV. Time marking 1 msec. (Fig. 1.11, p. 33, in Johnson, Eyring and Stover, 1974).

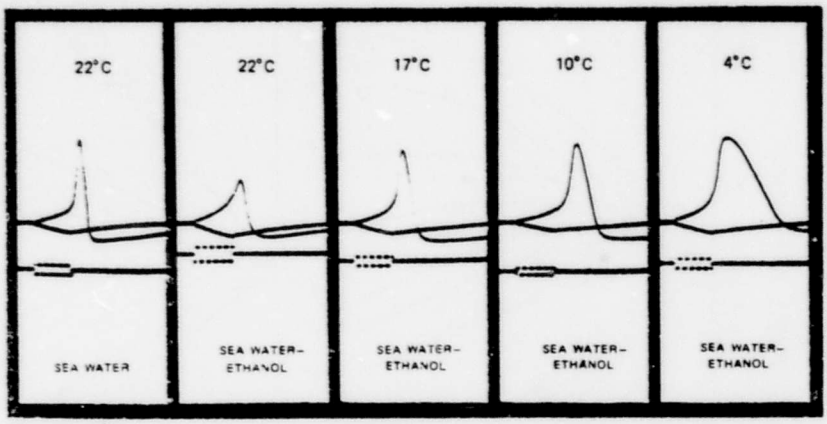


Fig. 24

(b)

Fig. 1.12 Records showing the counteraction of ethanol narcosis with low temperatures (Spyropoulos, 1957b). The amplitude of the response of the giant axon treated with ethanol at 22°C varied with the intensity of the stimulus. At 4°C the fiber fired spontaneously. The record at 4°C was obtained by electrical stimulation with the internal wire electrode. Concentration of ethanol 3%. Time marking 5 kHz. (Fig. 1.12, p. 33, in Johnson, Eyring and Stover, 1974).

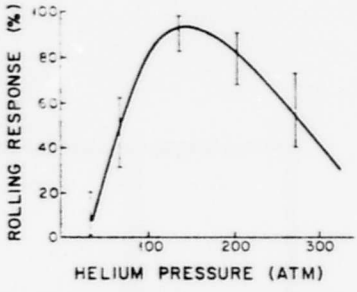


Fig. 25

Fig. 5.68. Rolling response (in terms of percent of normal, i.e., of unanesthetized specimens) in newts as a function of helium pressure in the presence of 34 atm of nitrogen and a partial pressure of 1 atm of oxygen (Lever et al., 1971). Error bars indicate the 95% confidence limits. Ten animals were used in the experiments. The temperature was 20°C, and the rotation speed was 4 rpm. (Fig. 5.68, p. 530, in Johnson, Eyring and Stover, 1974).

Following the demonstration of essentially similar pressure-temperature relations of steady-state luminescence intensity in intact cells and in extracts (Fig. 22), research on the bacterial system became more and more biochemically-oriented. Procedures were developed chiefly by Hastings and his associates and by Cormier and his associates (cf. Chapter 2, in Johnson, Eyring and Stover, 1974) for purification of bacterial luciferase. Evidence was sought and schemes consistent therewith were proposed regarding intermediary reactions involved in the light-emitting process, especially in bacterial extracts. Further study of the bacterial system in Princeton, however, was not undertaken in depth until the early 1970's. In the meantime, the luminescence system of the New Zealand fresh water limpet Latia neritoides had been studied (Shimomura, Johnson and Haneda, 1966) with the result that the luciferin component had been isolated. Shortly thereafter its chemical structure was determined (Shimomura and Johnson, 1968a), followed by total synthesis (Frascheboud et al., 1969). Purification and determination of the properties of Latia luciferase (Shimomura and Johnson, 1968b) and its cofactor requirements were also accomplished. Although the limpet and luminous bacteria are hardly more closely related biologically than that they are both species of living organisms, the luminescence system of the two was found to share certain common properties, including the following: (1), in Latia, the "luciferin" component consisted of a colorless, hydrophobic enol-formate of an aliphatic aldehyde, analogous to the long-known requirement of a colorless, hydrophobic aliphatic aldehyde in the luminescence of bacterial extracts, and (2), the known requirement of a flavin in bacterial luminescence and probably also in the Latia system as judged by a newly discovered (Shimomura and Johnson, 1968) correspondence between the spectral distribution of the bioluminescence reaction and the fluorescence emission of flavin. Further research on these two systems (Shimomura, Johnson and Kohama, 1972) provided evidence that bacterial extracts emitted light through a flavoprotein-catalyzed oxidation of fatty aldehydes with a photon yield of 0.17 ± 0.01 photons per molecule of aldehyde oxidized, independently of chain length from 9 to 14 carbons. The long debated role of the aldehyde in luminescence of bacterial extracts was thus cast into the function of a "luciferin", though more direct evidence of the necessity of such aldehydes in luminescence of bacterial cells remained to be established through a later study in which the aldehydes were isolated from luminous strains, whereas they were scarcely extractable from visually dark mutants (Shimomura, Johnson and Morise, 1974). This is where the problem stands at present.

B. The New Zealand luminescent species: limpet Latia, glowworm Arachnocampa and earthworm Octochaetus

From 1960 to 1963, research on the biochemistry of luminescent systems was carried on in Princeton in collaboration with Dr. O. Shimomura, earlier of Nagoya University, Japan. This collaboration was made possible by grants from the National Science Foundation. Because of visa regulations, Dr. Shimomura returned to Japan in 1963 and spent the ensuing two years at Nagoya, after which he returned to Princeton under the same auspicies as before, and rose to the rank of Senior Research Biochemist , equivalent to the rank of full professor but without guarantee of salary which continued to depend on a grant or other suitable source of support from outside the University. During the

period 1963-1965, limited collaboration between the author and Dr. Shimomura was continued by mail. In 1965, a grant through the U.S.-Japan Cooperative Research Program, by joint action of the U.S. National Science Foundation and the Japan Society for the Promotion of Science, made it possible for full collaboration between the author and Japanese colleagues to take place again. This included field work in New Zealand, laboratory research at the University of Nagoya, and an International Conference on the subject of Bioluminescence. The conference papers were published in 1966 by the Princeton University Press, under the title of "Bioluminescence in Progress", edited by F. H. Johnson and Y. Haneda, with modest financial aid from both the U. S. and Japanese side towards the cost of publication.

Three types of luminescent organisms were collected in New Zealand: the fresh-water limpet Latia neritoides, the larvae of the "New Zealand Glowworm" Arachnocampa luminosa, and the large earthworm Octochaetus multiporus. The limpet was found clinging to rocks in cold mountain streams in the Waitakere range outside of Auckland, the glowworm larvae in caves, tunnels and deep ravines, and the earthworms at various depths in dense clay below the surface soil in southern parts of the North Island. With the aid of graduate students of the University of Auckland, and with the cooperation of several officials of the New Zealand O.S.R.D. (Office of Scientific Research and Development) in Auckland and in Palmerston North, a small stockpile of limpets was gathered and the specimens were preserved with dry ice.

The glowworms were more difficult to come by, and some reluctance was encountered among graduate students who, despite the offer of what seemed to be generous financial rewards, simply did not take to the idea of going into the depths of unexplored caves with slippery floors and deep, dark crevices in an effort to obtain specimens that were mostly out of convenient reach on the ceilings. The author and Dr. Haneda went some distance down a water conduit tunnel, with a kerosene lamp and the assurance of workmen at a local sub-station of the water department that if we were not back by a specified time, they would come dig us out. As it happened, there was no cave-in on this occasion, but only a limited number of specimens were obtained, near the far end of the tunnel. From this, and from a cave near Waipu, north of Auckland, a total of at most a few thousand specimens was obtained and preserved in dry ice.

A few luminescent earthworms were collected at an O.S.R.D. soil laboratory east of Wellington, with the help of Dr. Lee. Most of the specimens were kept alive until used in experiments by the author at the O.S.R.D. labs at Palmerston North; a few others were frozen with dry ice and transported by Dr. Shimomura to Japan, along with frozen specimens of Latia and glowworms.

At Nagoya, isolation of Latia luciferin and partial purification of the luciferase was achieved and the properties of these substances were studied sufficiently to report them at the Luminescence Conference (Shimomura, Johnson and Haneda, 1966a). Some results of the research on the glowworm (Shimomura, Johnson and Haneda, 1966b) and on the earthworm (Johnson, Shimomura and Haneda, 1966) were also reported at the Conference, but because of limitations in amount of specimens as well as the short time available for research on them in Japan, these results must be regarded as largely preliminary.

Subsequent to the Conference, Dr. Shimomura came to Princeton and resumed collaboration with the author. Despite some difficulties, several shipments of specimens of Latia preserved with dry ice were successfully made to Princeton. The supply proved adequate to determine the structural formula (Shimomura and Johnson, 1968a), followed by synthesis (Frascheboud et al., 1969) of Latia luciferin. The enzyme ("luciferase") component was purified and its properties studied, revealing thereby the necessity for organic cofactors (Shimomura and Johnson, 1968b), of as yet unknown identity. Evidence for the flavin nature of the enzyme was found in the research done in conjunction with the bacterial system, already referred to in the preceding section.

C. The Ostracod Crustacean, Cypridina hilgendorfii.

The first noteworthy chemical study of the luminescent system of this tiny, primitive crustacean was done in 1916 at the Misaki Marine Station in Japan by the late Professor E. Newton Harvey of Princeton University. Although he studied many other kinds of luminescent organisms, plants as well as animals, during the next 40 years, when he retired in 1956 and became Henry Fairfield Osborne Professor Emeritus of Biology, Cypridina, or the "Umi-hotaru" ("Sea firefly") as it was known in Japan, remained his favorite above all others, and he maintained this interest to the end of life in 1959. Many kilograms of dried specimens were shipped to Princeton from Japan for this purpose. Luminescent material remained remarkably stable so that when moistened with water it sprang into activity with the emission of a spectacular blue luminescence. Despite dedicated efforts of his own, along with the capable help of several associates, chiefly Doctors Rubert S. Anderson, Aurin M. Chase, Howard Mason, and Fred I. Tsuji, efforts to obtain the essential components of the luminescent system, i.e., the specific substrate Cypridina luciferin and specific enzyme Cypridina luciferase in pure preparations, neither component was ever fully isolated. The most highly purified luciferin preparations had an estimated potency of about 2,000 times that of the starting material.

One of the major difficulties in trying to isolate Cypridina luciferin was its extreme instability in solution in the presence of oxygen. Another difficulty was a tendency to believe the amount of luciferin present in the raw material to be much greater than it actually was, perhaps because the brightness of the light made it easy to overestimate the true amount. This, together with the need to conserve the dried raw material from Japan, may have been largely responsible for the understandable reluctance to "invest" large amounts of material in any one set of experiments. From what we now know, it seems practically impossible that success in isolating the luciferin in a significant amount could ever have been achieved with the amount of starting material used. When a few milligrams of pure, crystalline luciferin were finally obtained by Shimomura et al. in 1957, 500 grams of dry Cypridina powder was used at the start, i.e., about 10 times the quantity ever used by Harvey and his colleagues at one time; the increase in potency was ca. 40,000 times.

The author elected to carry on research on some of the biochemical aspects of luminescence in which Harvey had been most active before he retired in 1956. In the summer of 1957 the author went to Japan for the purpose of experimenting with live specimens of Cypridina on the premise that, with the

aid of suitable precedures, it might be possible to separate and purify the luciferin and luciferase components from, say, luminescent slime secreted by the living specimens, rather than from whole, dried specimens as was customary. The customary procedure is somewhat like trying to purify the thyroid hormone by starting with whole cows rather than their thyroid glands alone. This was before it was known that Shimomura et al. (1957) had just succeeded in crystallizing pure luciferin from the dried specimens. The attempt to isolate the luciferin from live specimens was not successful, but it resulted in improved methods of extraction, using live rather than dried specimens at the start, and simply plunging them in an adequate amount into methanol cooled to around -40° C by dry ice; the yield was several times that of dried material, and the procedure of maintaining solutions free of oxygen was greatly simplified by dropping chunks of dry ice into the solutions.

On this occasion (1957) the author and Dr. Haneda discovered that the excised photogenic organs of a small, medium-depth fish, Parapriacanthus beryciformes could be extracted with boiling and with cold water which on mixing at room temperature gave a bright light, due to the presence of the relatively heat-stable and heat-labile components, luciferin and luciferase, respectively. This became the first well established example of a "luciferin-luciferase reaction" in extracts of a fish. It also became unusually noteworthy in some other respects, as follows.

Throughout his long career of active research, on other things but none so dedicated as on luminescence, Harvey tested at every opportunity the possibility that separate extracts containing, or possibly containing, the luciferin and luciferase components from one type of luminescent organism might react with light emission when mixed with those of another type. Such a "cross reaction" was indeed found to occur in a few instances when, but only when the two were biologically closely related, in a strict sense not really "different types", e.g., different species, or even different genera of fireflies. As a different type, Cypridina extracts were routinely tested for a cross reaction with the other organism at issue. Except for some misleading results in his earliest research, no cross reaction was ever observed with components of biologically only distantly related luminous species. It was astounding, therefore, when it was discovered (Haneda, Johnson and Sie, 1958; Johnson, Haneda and Sie, 1960; Johnson, Sie and Haneda, 1961) that partially purified extracts containing luciferin and others containing luciferase from the fish gave a brightly luminescent cross-reaction with those of Cypridina. When told informally of this, Harvey replied simply "I don't believe it." Confronted later with the same detailed evidence, he seemed convinced that it was so, even though unexpected from the previous, extensive experience of his own.

In due course, crystalline luciferin was prepared from the fish Parapriacanthus and found to be identical to that from Cypridina in its major chemical properties (absorption spectrum, chromatographic behavior, etc.) (Johnson, Sugiyama, Shimomura, Saiga and Haneda, 1961). In the course of dissecting out the internal photogenic organs of several thousand specimens of this fish, two accidental but interesting observations were made: first, the stomachs of a few specimens contained dead Cypridinae which were still

emitting light, and second, the five, fairly large pyloric caeca attached to the stomach were rich in luciferin that was active in light emission with Cypridina luciferase. These observations naturally raised the question as to the origin of the fish's luciferin and luciferase. If these components had been somehow harvested from Cypridina taken in as food, the seeming cross-reaction was not real. In that event, however, the mechanism whereby the usually unstable luciferin became stored and remained active in the pyloric caeca remained unexplained, and likewise the mechanism whereby the luciferase component of ingested Cypridina became localized in the photogenic organs to which it would presumably have to be transported via the circulatory system. On the other hand, it would seem no less amazing to find in two species of animals no more closely related than this primitive crustacean and highly evolved teleost fish, an unusual, specific oxidative enzyme. The problem is not yet finally solved in its entirety. Unequivocal evidence may require raising some of these fish from eggs to adult in known, certain absence of Cypridina, to see if they would still be luminous by virtue of luciferin and luciferase of their own manufacture. In the meantime, some immunological and other differences have been reported for the luciferase components of the two species (Tsuji and Haneda, 1966; cf. also, Haneda, Johnson and Shimomura, 1966).

The luciferase component was purified from dried Cypridina, and its properties determined, by Shimomura, Johnson and Saiga (1961). By mutual agreement between authors, this work was reported in the same issue of the Journal of Cellular and Comparative Physiology with a report by Tsuji and Sowinski (1961). The methods of purification were not the same, but the properties of the luciferase described in these two independent reports were in fair agreement, except for molecular weight, which Tsuji and Sowinski found to be considerably higher than the value of about 60,000 found by Shimomura et al.

The structure of Cypridina luciferin was solved through cooperation of two groups of investigators, one in Nagoya (Kishi, Goto and Hirata) and the other in Princeton (Shimomura and Johnson) (1936). Total synthesis soon followed (Kishi et al., 1966). Thus far, however, synthetic luciferin cannot be produced more conveniently or more economically than natural luciferin. In the foreseeable future this situation may change due to the progressively decreasing population of Cypridina, inasmuch as these organisms have all but disappeared from certain regions of Japan where they once occurred in abundance. The reason seems to be the increasing pollution of the waters.

The availability of pure luciferin and pure luciferase has made it possible to obtain definitive data concerning various important properties of the system and the mechanism of the light emitting reaction. Carbon dioxide was identified as a product of the aerobic oxidation of Cypridina luciferin with Cypridina luciferase (Stone, 1968). The actual light-emitter was identified as the oxyluciferin-luciferase complex, by the precise correspondence between the fluorescence emission spectrum of the complex and the emission spectrum of the bioluminescent reaction itself (Shimomura, Johnson and Masugi, 1969). Both the complex and the product oxyluciferin of the light-emitting reaction catalyzed by luciferase emit a strong blue fluorescence

under ultraviolet light, but only the former has the same spectral distribution as the light produced in the enzyme catalyzed reaction. Luciferin itself is strongly fluorescent in the yellow, whereas two degradation products, namely oxyluciferin and etioluciferin, the latter being a hydrolytic product of the former, are both blue fluorescent. The emission maxima and relative intensities are summarized in Table II.

A study of factors influencing the quantum yield (Table III) indicated that the maximum quantum yield (einsteins per mole of luciferin oxidized) in the luciferase-catalyzed reaction in water amounted to 0.31, which was very slightly less than the yield of 0.33 found under similar conditions except that the solvent was 99% D_2O (Shimomura and Johnson, 1970). In aprotic solvents such as dimethylsulfoxide or diglyme, in the presence of oxygen, Cypridina luciferin is spontaneously luminescent, as are a variety of indol derivatives (Johnson, Stachel, Taylor and Shimomura, 1966; Sugiyama, Akutagawa, Gasha and Saiga, 1966). The mechanism involved in the low quantum yield in aprotic solvents was found to be the relatively low proportion of molecules attaining the excited state.

At least 3 pathways of luciferin degradation can occur, depending upon the solvent; one pathway, catalyzed by luciferase certainly leads to luminescence (Fig. 26 A and Fig. 27 A), but it has not been established whether or not any appreciable light emission results from the other two (Fig. 27, B and C). The pathway diagrammed in Fig. 27 is in accordance with the hypothesis of a dioxytane intermediate, originally proposed by McCapra and Chang (1967, cf. also, McCapra, Chang and Francois, 1968) in connection with a study of the oxidation of an analogue of Cypridina luciferin. More recent considerations, however, have shed some doubt on this otherwise attractive hypothesis.

By a tracer method, using $^{18}O_2$ and $H_2^{18}O$, it has been shown that the oxygens incorporated in the carbon dioxide produced in the luciferase-catalyzed luminescent oxidation comes from molecular oxygen rather than from water, whereas the opposite has been reported for the firefly and sea-pansy (Renilla) systems (DeLuca and Dempsey, 1970; DeLuca et al., 1971). It is possible, however, that the data for the firefly and sea pansy were misinterpreted, because of the influence of factors unknown at the time, namely, concentration of the substrate luciferin as well as pH and buffer system employed, all of which markedly affect the rate of exchange of solvent oxygen and the oxygen in the carbon dioxide product of the reaction (Shimomura and Johnson, 1975).

D. The hydromedusan jellyfish Aequorea

Prior to the initial collaboration between Dr. Shimomura and the author in Princeton, the latter had undertaken to investigate the biochemistry of the luminescent system of Aequorea, a jellyfish which occurs in great abundance every summer in the Pacific Northwest. Previous studies by Harvey and others (cf. Harvey, 1952) had always failed to yield extracts that would give a light emitting reaction in vitro; in fact, as Harvey noted, there appeared to be no means of reversibly inhibiting the reaction. When the photogenic tissue is placed in a solution of saponin or even distilled water alone,

Table II

(Table 1.) Fluorescence emission maximums (nanometers) and intensities (in arbitrary units shown in parentheses) for luciferin and reaction products, at $2 \times 10^{-6}M$ at 25°C, measured, at the indicated wavelength, with an Aminco-Bowman spectrophotofluorometer.

Solvent	Luciferin 430 nm (nm)	Oxyluciferin 360 nm (nm)	Etioluciferin 360 nm (nm)
0.05*M* Sodium phosphate – 0.1*M* NaCl, *p*H 7.4	535 (17)	510 (1)	503 (5)
n-Butanol	535 (18)	477 (103)	470 (180)
0.05*M* Sodium phosphate— 0.1*M* NaCl, *p*H 7.4 with $6 \times 10^{-6}M$ luciferase	535 (18)*	465 (100)†	480 (65)

* In the presence of sodium hydrosulfite to stop the luminescence reaction. (Visual observation indicated similar results with a vacuum.) † The solvent, containing luciferase ($4 \times 10^{-6}M$), was chilled in a cuvette to 0°C, and measurement was made within 10 seconds after oxyluciferin was mixed in, to minimize error due to hydrolysis to etioluciferin.

(Table 1) in Shimomura, Johnson, and Kohama's article in SCIENCE, 13 June 1969, volume 164, pages 1299-1300

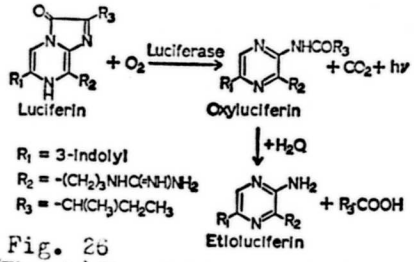


Fig. 26

(Fig. 1.) Degradation of luciferin by luciferase. (From Shimomura et al, Science, 164, p. 1299, 1969)

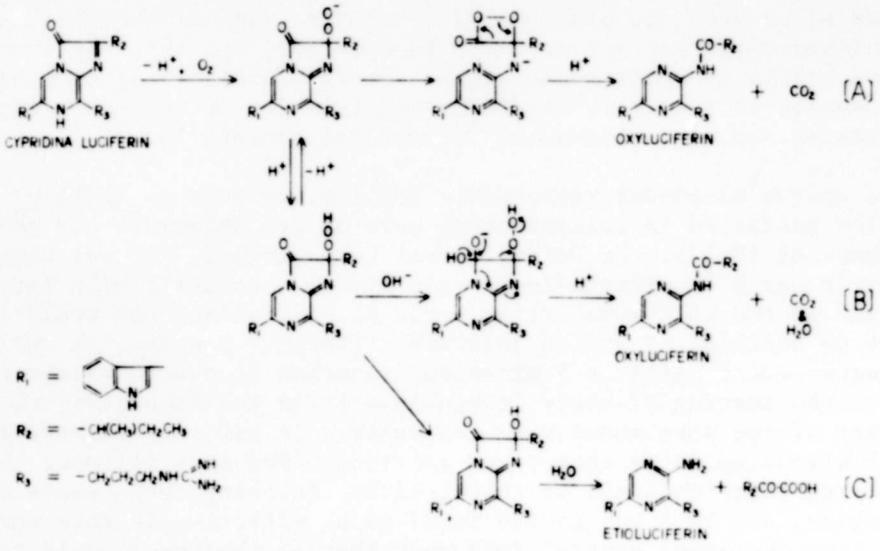


Fig. 27. Oxidative degradation of Cypridina luciferin.

(Fig. 2 p. 342, of Shimomura and Johnson, in Cormier, Hercules and Lee, 1972).

and even when dissolved oxygen has been rigorously removed, a bright flash of light results at once, and that's the end . On the other hand, when such tissues, or whole organisms, are placed in the sun they dry to an extremely thin, gelatinous film which emits light, though rather weakly, when moistened with water. The author was attracted to this chemically unknown system primarily because of the two properties, first, that the system was at least somewhat stable to drying, and second, that it could emit light in virtually the complete absence of oxygen, an unusual property, indeed, among bioluminescent systems. In addition, the luminescence was impressively bright and the potential supply of specimens was practically unlimited.

The author spent the summer of 1959 at the Friday Harbor Laboratories of the University of Washington, on San Juan Island in the Straits of Juan de Fuca, experimenting with Aequorea. Consistent with previously reported results, no method was found that would yield luminescent extracts. On the other hand, it was found possible to reversibly inhibit the light-emitting process by high concentrations (of the order of 6 molar) of urea and certain other agents, including 60 to 70% saturation with $(NH_4)_2SO_4$, and including acid at certain ranges of concentration. Such experiments were carried out with a "brei" obtained by first excising the circumoral ring containing the photogenic tissue, then squeezing a number of such rings through a pocket handerchief. The brei would emit a dim luminescence when left standing for as long as hours, but would give a bright luminescence when poured into distilled water. The initial light could be extinguished at once by adding urea or ammonium sulfate. With the latter, the tissue would then settle out, much as if precipitated in the manner of a protein from solution. The salt solution could be decanted and the residue, conveniently concentrated in this manner, gave a very bright flash of light when distilled water was added. Moreover, this light was green, resembling the light emitted from the intact photogenic organs, in contrast to the distinctly blue light of the slime that can be rubbed off the living specimen. As we now know, the green light must have indicated the presence of the "green fluorescent protein" in close association with the primary light-emitter. The probability, therefore, is that neither component was actually in solution, but that the two were still bound within the photogenic organs (or organelles ?) or else held together somehow in the close proximity required for an intermolecular energy transfer (Morise, Shimomura, Johnson and Winant, 1974).

The active component responsible for luminescence in Aequorea was successfully extracted in collaborative work of Dr. Shimomura and the author in the summer of 1961 at the Friday Harbor Laboratories. It was thought at first that it was a "luciferin-luciferase system" because a mild (acetic) acid extract of the circumoral rings could be neutralized and would then emit light on addition of boiled jellyfish tissues. However, it was found that tap water would elicit a luminescent reaction from such extracts. This led to the testing of every inorganic salt in the laboratory stockroom for activity of the sort shown with tap water. It was soon learned that only calcium or strontium salts showed any activity. The idea followed that the light emitting reaction could be inhibited by chelating these salts during the extraction, and EDTA was indeed found to be effective in this way. The yield was thus increased several fold over that of the acetic acid method.

With the aid of column chromatography on DEAE cellulose a pure protein was finally obtained. It responded with luminescence to the addition of calcium salts in amounts that were equal to or in excess of the moles of EDTA present during isolation. To all appearances, it was a single organic component system whose light was triggered by Ca^{2+}, or to a less extent Sr^{2+}. No enzyme activity seemed to directly involved, and quantitatively the same amount of light was omitted in solutions equilibrated with pure hydrogen, pure oxygen, or air. The protein was named aequorin (Shimomura, Johnson and Saiga, 1962), and later referred to as one of several systems, including those of the paddleworm Chaetopterus and the shrimp Meganyctiphanes as a type of "photoprotein". (Shimomura and Johnson, 1966).

Because of the combined circumstance of the practically ubiquitous presence of calcium (as a constituent of glass, or as a contaminant in "pure" chemicals and in distilled water) and the extraordinary sensitivity of aequorin luminescence to traces of Ca^{2+}, misleading results may be easily obtained with respect to the specificity of the aequorin reaction, unless rigorous precautions are taken to avoid spurious results, e.g., using only acid-washed plastic vessels, pipettes, and specially purified, de-ionized water distilled in a quartz still, etc. Thus some of the results reported by Izutsu et al (1972), to the effect that a variety of metal ions were active in triggering the luminescence reaction, were apparently due to insufficiently controlled sources of error such as those mentioned above. On the other hand, at certain hydrogen ion concentrations and concentrations of divalent cations, a few ions other than Ca^{2+} and Sr^{2+} have been found active (Shimomura and Johnson, 1973). Thus at pH of 6, but only to slight extent at pH of 8, Pb^{2+} and Cd^{2+} showed considerable activity. At pH 6, the rare earths, Y^{3+} and La^{3+} had activities almost as great as Ca^{2+}, but at higher concentrations and pH 8 the rare earths showed quenching activity. The use of aequorin to test for Ca^{2+} in biological systems, as suggested by Shimomura, Johnson and Saiga (1963), can be relied upon for specificity if the pH is regulated to between 7.5 and 8.0, and if rare earth metal ions and Sr^{2+} are absent, Figs. 28, 29 and 30 illustrate examples of the use of aequorin to detect Ca^{2+} in biological systems.

Considerable time and effort were spent investigating the properties of pure aequorin and mechanism of the light emitting reaction (see Shimomura and Johnson, 1969, 1970, 1972, 1973, 1975a, 1975b; Shimomura, Johnson and Morise, 1974; Morise, Shimomura, Johnson and Winant, 1974). The details can be found in the references just cited; it seems appropriate to mention only a few salient aspects here. They may be briefly summarized as follows.

First, the "green fluorescent protein" or "GFP", intimately associated with aequorin in the photogenic organs, was separated and crystallized (Fig. 31). It evidently occurs in the form of polymers of a monomeric form with molecular weight 17,000. When co-adsorbed with aequorin on DEAE-cellulose or DEAE-Sephadex, the energy of excitation, following addition of Ca^{2+}, is transferred by Förster-type mechanism to GFP. which then radiates with an emission spectrum identical to that of the luminescence reaction *in vivo* (Fig. 32). Substantial evidence indicates that the same energy transfer takes place *in vivo*, accounting for the green light observed in the photogenic organs, rather than the blue light of pure aequorin *in vitro*. This noteworthy situation, especially because it is the only example, except for photosynthetic

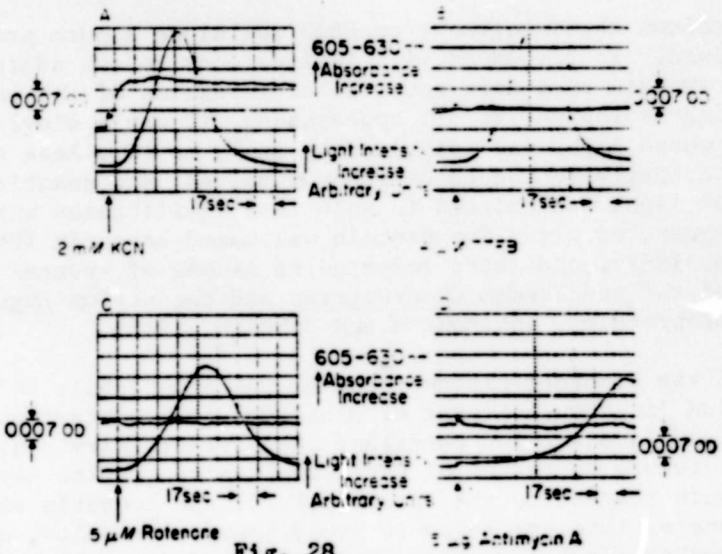


Fig. 28

Fig. 2.13 Changes in calcium concentrations, as indicated by aequorin bioluminescence, accompanying the response of pigeon-heart mitochondria to the addition of (A) cyanide, (B) 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole (TTFB), (C) rotenone, and (D) antimycin A (Chance et al., 1969). (Fig. 215, p. 112, in Johnson, Eyring and Stover, 1974

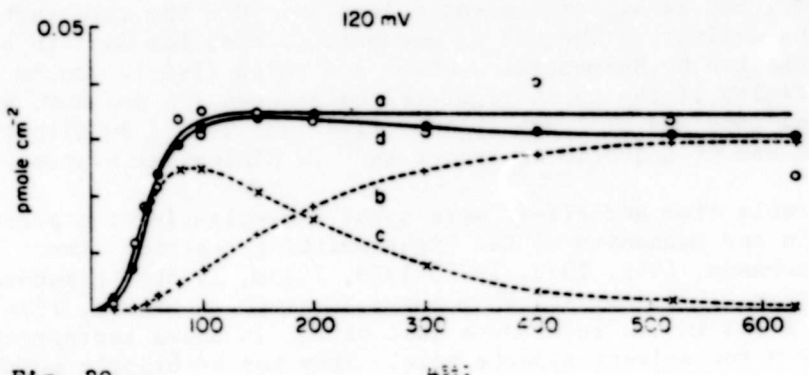


Fig. 29.

Fig. 2.16. Luminescence of aequorin, after injection into the giant axon of *Loligo forbesi*, in response to the entry of Ca^{2+} as a function of pulse duration with 120-mV pulse amplitude (Baker, Hodgkin, and Ridgway, 1971). Curve a (○): observed early Ca^{2+} entry obtained as the difference between the response of aequorin in the presence and in the absence of tetrodotoxin at 22°C, with 112 mM Ca^{2+} in the external solution; curve b (+): Ca^{2+} entry during pulse; curve c (X): Ca^{2+} entry after pulse. Curve d (●): total entry $d = b + c$.

(Fig. 2.16, p. 112, in Johnson, Eyring, and Stover, 1974).

112

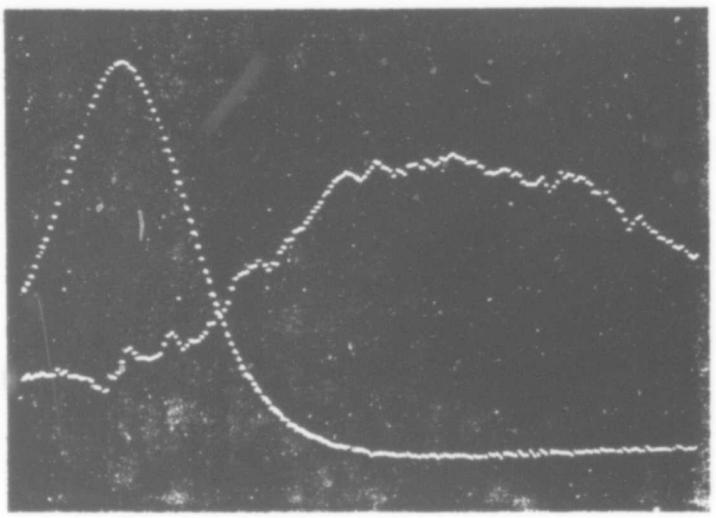


Fig. 30

Fig. 29. Computerized records showing average transients for action potentials (upper trace at left) from a spontaneously rhythmically firing single giant brain cell of the slug *Limax maximus*, together with a simultaneous luminescence response (lower trace) of aequorin previously injected into the cell. The luminescence response was caused by the change in the free calcium concentration within the cell in conjunction with each action potential. The amplitude of the action potentials was about 90 mV. The time interval between adjacent dots was 0.625 msec. (Previously unpublished research record, courtesy of Dr. Joseph Jin Chang, Biology Department, Princeton University, February 1973.) (Fig. 2.17, p. 113, in Johnson, Eyring, and Stover, 1974; see also, Chang, Gelperin and Johnson, 1974).

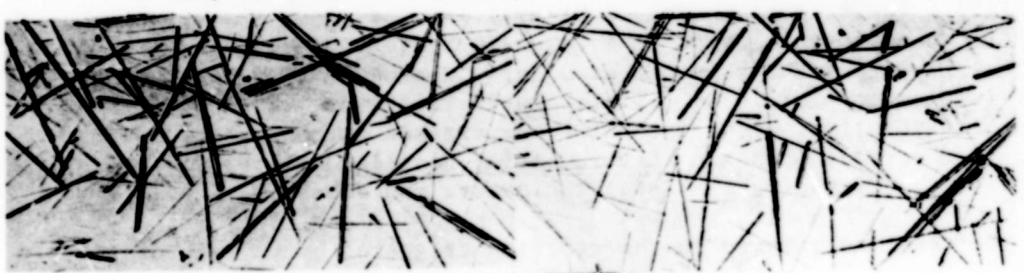


Fig. 31.

FIGURE 3: Photomicrograph of GFPb crystals. (From Morise, Shimomura, Johnson and Winant, 1974, p. 2659)

respectively, of a subunit having a molecular weight of 17,000,

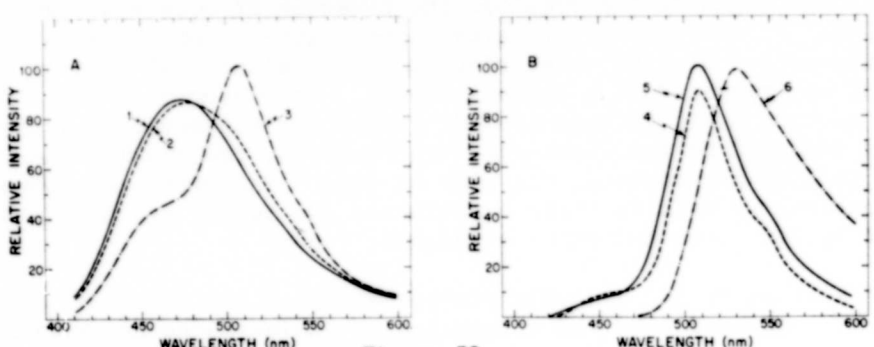


Figure 32.

FIGURE 5: Effects of coadsorption of aequorin and GFP on the emission spectrum when triggered by Ca^{2+}. Each luminescent reaction, except (5), was triggered by addition of 30 μl of 10 mM calcium acetate to the following mixtures: (1) 270 μl of buffer solution containing 11 μg of aequorin, (2) 270 μl of buffer solution containing 11 μg of aequorin plus 46 μg of GFP, (3) 270 μl of buffer solution containing 11 μg of aequorin plus 46 μg of GFP plus 100 μl of finely ground DEAE-cellulose or DEAE-Sephadex suspension (see Materials and Methods), (4) 250 μl of buffer solution containing 11 μg of aequorin plus 460 μg of GFP plus 100 μl of the DEAE-cellulose or DEAE-Sephadex suspension was centrifuged, then the precipitate was resuspended in buffer solution making the total to 270 μl, (5) luminescence of photogenic organs of *Aequorea* (see Materials and Methods), (6) the same as (4) except that GFP was replaced with 50 μg of FMN. The buffer solution used was 10 mM sodium phosphate containing 1 mM EDTA (pH 7.0). All measurements were made with a 10-mm cell, with slit width of 2 mm, at 25°. (From Morise, Shimomura, Johnson, and Winant, 1974, p. 2960).

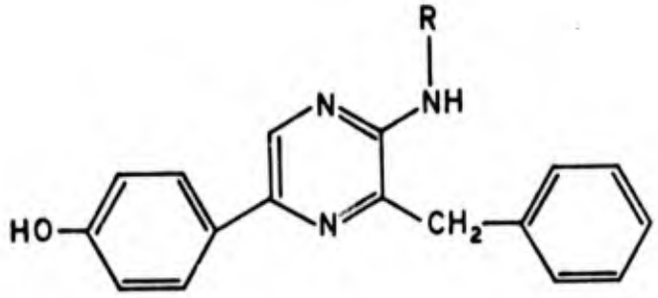
systems, where this sort of energy transfer has been proved to take place in a living organism. It is interesting to note, further, that the energy transfer that occurs from excited aequorin to a fluorescent molecule such as FMN co-adsorbed with aequorin on DEAE-cellulose or DEAE-Sephadex results in an emission spectrum charcteristic of the fluorescent molecule (Fig. 32).

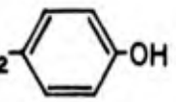
For some time it was thought that the light-emitting reaction of aequorin was irreversible due to destruction of the protein moiety of the system. Normally the reaction is irreversible, but it now seems that this is not due to destruction of the protein but rather to destruction of a much smaller molecule attached to the protein and being the actual light-emitter. Determination of the structure of this group has revealed some surprising similarities in the chemistry of luminescent systems among coelenterates (Fig. 33), and extending in part even to crustaceans, specifically the ostracod Cypridina and to molluscs, specifically the cephalopod, Watasenia and also to fish (Parapriacanthus). Among coelenterates, two types of systems occur, with varying significance from a quantitative point of view: (1), photoprotein type, which can generally be triggered by Ca^{2+}, and (2), a luciferin-luciferase type, Aequorea contains luciferyl sulphate, as in the sea pansy Renilla, but Aequorea lacks luciferase. The functional system in this latter organism is therefore entirely photoprotein. In certain other species, however, the two types of systems appear to be equally important in production of light (Cavernularia), whereas in still others the luciferin-luciferase type apparently predomonates (Renilla) (Table III). The product in the light-emitting reaction, viz., 2-(p-hydroxyphenylacetyl)amino-3-benzyl-5-(p-hydroxyphenyl) pyrazine, is the actual light-emitter, whether it occurs in a Ca^{2+}-triggered photoprotein type of luminescence or in a luciferin-luciferase type. This product is structurally identical among the different classes of coelenterates: Hydrozoa (Aequorea), Anthozoa (Cavernularia, Renilla, Leioptilus) and very likely also the Scyphozoa (Pelagia) (Shimomura and Johnson, 1975b).

The reaction pathway in the reaction triggered by Ca^{2+} is diagrammed in Fig. 34, where YC stands for a yellow compound which has been isolated but which has an as yet unknown structure. When coelenteramide (Fig. 33, Ia) is added to a spent reaction mixture, the protein moiety of spent aequorin acts in the manner of an enzyme and regenerates active, calcium-triggered aequorin. The regeneration requires the presence of molecular oxygen but not calcium. In the absence of calcium the regeneration reaction continues over a long period of time, resulting in a gradual accumulation of aequorin. If calcium is present during regeneration, it is consumed in the light-emitting reaction as fast as it is regenerated. Since the regeneration process is very slow compared to the light-emitting reaction, the result, when Ca^{2+} is present, is a long-sustained, dim glow of light (Shimomura and Johnson, 1975c). The coelenterazine used in these experiments was purely synthetic, kindly furnished by Drs. Goto and Inoue of Nagoya, Japan.

This is where the aequorin problem stood at the termination of the control. The mechanism of the triggering of luminescence by calcium, as well as the structure and precise role of the yellow compound YC remain to be elucidated. Further research will be limited by availability of specimens of Aequorea, for which there are no definite prospects at the moment.

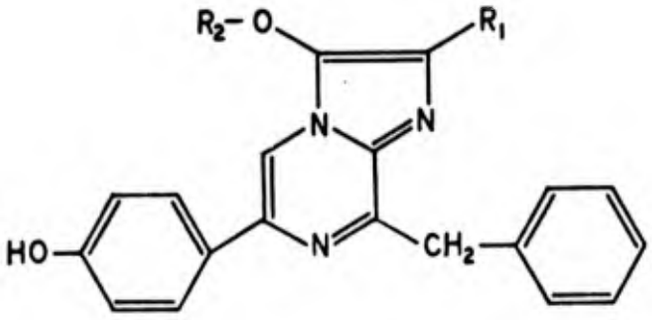
Figure 33

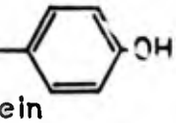


Ia $R = -\overset{O}{\overset{\|}{C}}-CH_2-$ 

Ib $R = -\overset{O}{\overset{\|}{C}}-X$

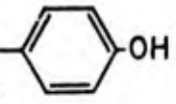
Ic $R = -H$



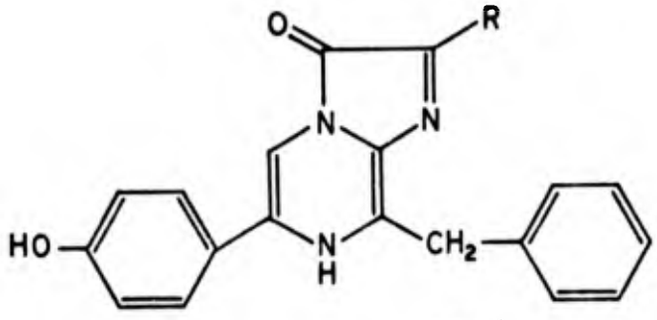
IIa $R_1 = -CH_2-$ 

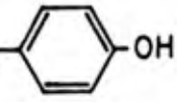
$R_2 = -protein$

IIb $R_1 = -X$
$R_2 = -SO_3^-$

IIc $R_1 = -CH_2-$ 

$R_2 = -SO_3^-$



IIIa $R = -CH_2-$ 

IIIb $R = -X$

Legend:

Ia Coelenteramide (the light-emitter)
 b Oxyluciferin of Cormier
 c."AF-350"

IIa. Chromophore of native aequorin
 b. Renilla luciferyl sulfate (Cormier)
 c. Enol sulfate of coelenterazine (IIIa)

IIIa. Coelenterazine (skeleton resembles Cypridina luciferin)
 b. Renilla luciferin (Cormier)

Fig. 33.

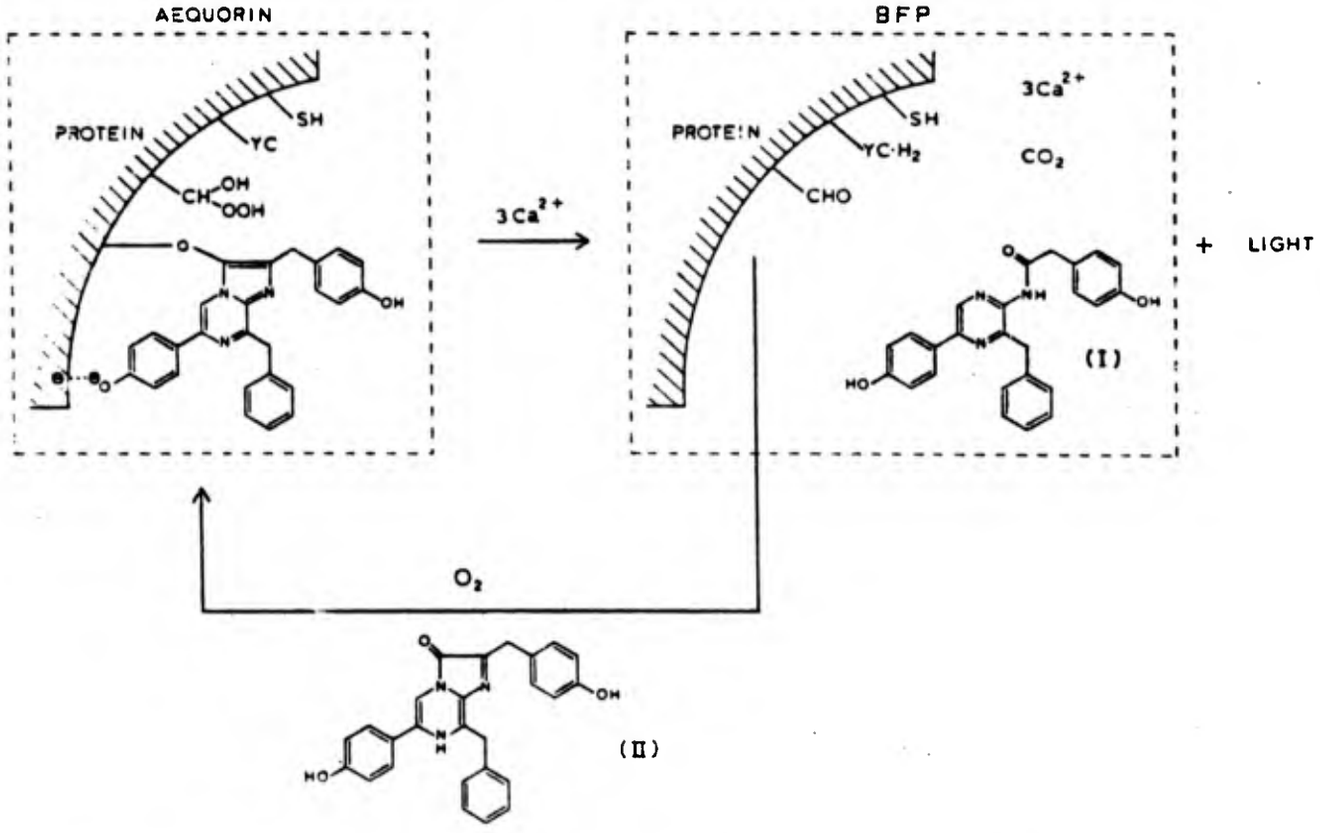


Fig. 34. Luminescence reaction pathway and regeneration of the photoprotein aequorin. (From Shimomura and Johnson, 1975c, Fig. 1).

TABLE III*

Amount of Ca^{2+}-triggered luminescence activity, compound IIc, and compound Ia extractable after in vivo luminescence, in coelenterates.[a]

Species	Body weight (g)	Luminescence in vivo[b] (photons)	Ca^{2+}-triggered luminescence[c] (nmol)	Compound IIc (nmol)	Compound Ia, after in vivo luminescence (nmol)
Aequorea aequorea	0.6[d]	9×10^{13}	0.7	0.03	0.6
Cavernularia obesa	30	3.5×10^{14} [e]	1.5	2	6
Leioptilus (Ptilosarcas gruneyi)	70	f	0.7	2.8	16
Renilla mülleri	6	f	0.14	1	0.5
Renilla kollikeri	5	f	0.07	0.6	0.3
Stylatula-like species (nonluminescent)	5	0	0	0	0
Leptogorgia virgulate (nonluminescent)	10 - 40	0	0	0	0

[a]Data for a single average-sized specimen of each species, in the contracted state.

[b]By stimulation with KCl, without mechanical stimulation.

[c]Luminescence due to photoproteins and luciferin binding proteins are included in these data. Quantum yields of all Ca-triggered luminescence reactions are assumed to be the same as that of aequorin, i.e., 1 nmol emits 1.3×10^{14} photons (2).

[d]This refers only to a thin strip cut off from the margin of umbrella. The weight of a whole body is approximately 50 g.

[e]The sample was pigmented and not transparent, thus a considerable loss of photons due to absorption would be expected.

[f]Data not obtained due to inadequate excitation by KCl.

* ...ata of Shimomura and Johnson, 1975b.

E. The "paddle worm" Chaetopterus

This is a marine annelid worm which lives in self made, parchment-like tubes on the sea floor, often entangled amongst algae and other growth, where they are collectable by SCUBA divers a few feet below the surface. A more or less steady stream of sea water is swept through the tube, bringing in dissolved oxygen, particles of food, and sweeping out wastes. Two types of luminescence are present, both of which can be quite bright when observed in individuals removed from their tubes: one is a luminescent slime secreted from the aliform notopodia near the anterior end, the other is a luminescent flash, not accompanied by noticeable secretion, emitted from the tips of the paired notopodia on each of the segments extending posteriorly throughout the length of the body. This species is one of the outstanding difficulties in accounting for a useful function of the property of luminescence, since the tube in which the worm lives provides a thick, dark shade against the light being easily visible from the outside, at the same time that it offers something of a protective shelter for the animal inside.

Attempts to demonstrate a luciferin-luciferase reaction with hot and cold extracts of the luminescent tissues, and in other ways, by the present author and others met with the usual failure. Information as to the biochemical nature of the system was sought in a study by the author in 1955 at the Hawaii Marine Station, Cocoanut Island, Honolulu, using a small variety of Chaetopterus variopedatus whose tubes had to be hewn from coral rock in which they were embedded. This study was based primarily on experiments with the secreted slime, inasmuch as this was the only way to obtain practically cell free luminous material at that time. A drop or two of such slime could be obtained on repeated occasions from a single individual, simply by mechanically stimulating while holding the specimen just above the mouth of a suitable receptacle.

The light of such slime decayed with a half time of only a couple of minutes at room temperature, but this was not too fast to test the effects of a variety of factors on it. For example, if the freshly secreted slime were added to one or more volumes of ammonium sulfate the light was promptly dimmed, but it would brighten on dilution with distilled or sea water, then decay giving a total light roughly equal to a similar specimen that had not been treated with ammonium sulfate. The addition of factors that favorably influence the luminescence of bacterial extracts, i.e., DPN, FMN and DPNH, separately or in combination, had little effect on the luminescence of the secreted slime, but small concentrations of decaldehyde or 8 volumes of distilled water caused considerable increases in rates of decay of luminescence at the cost of total light produced. The rate of decay of light in slime initially caught in a small volume of 0.4 M urethane did not appear to be greatly affected, though it seemed faster than similar specimens in sea water. On addition of 2 volumes of sea water, however, the brightness quickly increased several fold, then resumed decaying. The kinetics of decay was never more than roughly first order. This fact, plus an inability to obtain identical specimens of slime by the available procedure, makes quantitative data of limited signficance only. However, experiments involving dilution with water, with spent slime, etc. offered no real evidence of dealing with an enzyme substrate system in the usual sense. Reversible

inhibitions of brightness, by urethane, for example, were suggestive of action on an enzyme system, but the fact that the same agents that reduced the instantaneous brightness might cause an acceleration in rate of decay argues against a luciferin-luciferase system as being the basis of this luminescence. It should be mentioned in passing that diisopropylfluorophosphate ("DFP") in final concentrations of 0.0002 to 0.0017 M caused sudden decreases in brightness, followed by sudden increases on addition of an equal volume of sea water. The evidence was not sufficient to conclude that this drug caused a reversible, i.e., by dilution, inhibition of luminescence as was found with bacterial luminescence (Johnson and Plough, 1959). Investigation of the pressure-temperature-inhibitor relations of luminescence in both secreted slime and in electrically stimulated, excised notopodia (Sie, Chang and Johnson, 1958) gave some interesting results without, however, yielding any clear evidence of any direct involvement of luciferin-luciferase system in the process of light production. The same is true of an investigation from the same point of view in regard to the luminescence system of the ctenophore Mnemiopsis, in homogenates ("Squeezates") of whole specimens, and in electrically stimulated, excised portions containing 2 to 4 comb plates from the meridonal canals (Chang and Johnson, 1959; Johnson, Eyring and Chang, 1959). These studies, of course, were prior to the discovery of the photoprotein type of system in Aequorea.

Successful extraction and purification of the Chaetopterus system had to await adequate supplies of suitable raw material and a feasible method of reversibly inhibiting the activity of the luminescence system during the process of extraction and purification. The most likely favorable raw material seemed to consist of the aliform notopodia, and the only sure way to reversibly inhibit the luminescence reaction seemed to be to exclude oxygen. Both aspects guaranteed a troublesome procedure, but the procedure adopted involved them inasmuch as there seemed no alternative at that time.

Specimens of Chaetopterus available from Woods Hole or other East Coast suppliers were quite large and quite expensive. Fortunately, although the West Coast specimens in the Los Angeles area were much smaller, they could be obtained in large quantities at much less expense, and shipped by air to arrive within half a day at Newark Airport. Upwards of 10,000 specimens were obtained in this manner for the study. On arrival they were processed as promptly as possible as follows. Each specimen was removed from its tube, cut open with a pair of scissors. The aliform notopodia were cut off and placed at once in a cellophane bag on dry ice. Several helpers had to be employed for this task in order to complete it in the short time before the specimens went bad. The initial steps in extraction, including first of all homogenizing an aliquot of tissues, were carried out in an oxygen-free controlled atmosphere box. Details of the procedure and requirements for luminescence, which included a peroxide as well as oxygen, are set forth in the paper by Shimomura and Johnson (1966). The originally exceedingly troublesome procedure was later replaced by a somewhat more convenient method, when study of the pure system revealed the requirement of ferrous iron for light emission, and the use of oxine (8-hydroxyquinoline) to reversibly inhibit the luminescence process by chelating the iron. With the aid of precipitation by ammonium sulfate and other conventional methods, the

active material was finally purified and crystallized (Fig. 35, Shimomura and Johnson, 1968). It turned out to be a photoprotein in that the total light was found proportional to the amount of protein involved, and in that no enzyme action appeared to be directly involved in the luminescence reaction itself. However, because of significantly decreasing activity with increasing purification, the need for cofactors in the complete system was suspected. Two were found: (1), a macromolecule resembling a nucleoprotein, and (2), a substance present in impure extracts and also in partially purified preparations of hyaluronidase, with properties of a lipid. Thus, the complete system requires the specific photoprotein, plus ferrous iron, oxygen, 2 organic cofactors, and a hydroperoxide. The kinetics and total lights encountered with varying concentration of the above components were found to be complicated and unsatisfactory for arriving at a clear and convincing reaction scheme, which obviously depends now on future investigations.

F. The deep-sea euphausiid shrimp Meganyctiphanes norvegica.

This species occurs in great abundance in certain parts of the world, living at considerable depths of the sea or at the bottom of fjords, and coming up towards the surface at night. It is known by the common name "krill", a chief source of food for whales in regions where the two occur. All the euphausiid shrimps are luminous, with the single exception of Bentheuphausia. Moreover, the light-producing organs are perhaps the most highly evolved of any type of luminescent animal; they are so complex and precise, in fact, that it is difficult to believe that they do not subserve some important function whatever it may be. The photogenic organ has parts analogous to certain parts of the vertebrate eye, including a large lens with muscles attached in a manner suggestive of providing a means of focusing, a pigment layer at the back, a reflecting layer, and a large number of very precisely formed and oriented "rods" which conceivably take part in converting the bioluminescence into the form of a laser (Bassot, 1966).

Some preliminary studies of this species were made at the Kristineberg's Zoological Station, Fiskebäckill, Sweden, which is located near the mouth of Sweden's only true fjord. Although this fjord abounded with specimens of the luminescent sea pen Pennatula phosphorea (which we were also interested in studying), it was soon evident that the number of specimens of Meganyctiphanes obtainable locally would be far too few to undertake any extensive investigation from a biochemical point of view. Through Dr. Bertil Svedmark, Director of the Kristineberg Laboratory, however, we learned that a much larger quantity of the desired specimens might be obtained at the Norwegian laboratory in Bergen, or at the Scottish Marine Laboratory at Millport, Isle of Cumbrae, near the mouth of the Clyde River. The latter was not far from the International airport at Presswick. Arrangements were made whereby frozen specimens would be sent from Bergen to Presswick, to be picked up there. In the meantime, Dr. Shimomura and the author spent a few days at Millport, practically directly on the way of the return trip to the U.S., and with the very generous cooperation of the Scottish Marine Biological Association staff of the Millport Laboratory, succeeded in collecting some 10 kilograms of Meganyctiphanes, preserved with dry ice almost from the moment of collection.

Specimens were also received as planned from Bergen, Norway, but they were not so suitable for the purposes in view because they had apparently been stored for some while in a freezer, whereas dry ice was required in order to preserve the suspended activity of the luminescence system. Despite some near disasters, due to breakdown of the taxi en route to the airport, and to delayed flight schedules, the Millport specimens were transported successfully to Princeton, where the light-emitting system was extracted and characterized.

As already intimated, this system turned out to be the third example we had found of a photoprotein type (Shimomura and Johnson, 1967). Like all bioluminescence systems, so far as is known, the Meganyctiphanes system involves a specific protein, in this instance apparently in the form of polymers of a monomeric unit of molecular weight about 200,000. The polymer was separated by gel filtration into 2 components, the first with molecular weight of 900,000 and the second of 360,000, the former possibly representing a dimer or trimer of the latter. The quantum yields, i.e.(number of photons emitted)/(number of photoprotein molecules reacted), based on the above molecular weights amounted to 0.55 for the first component and 0.22 for the second (Shimomura and Johnson, 1968).

An utterly amazing aspect of this system is the role of a relatively low molecular weight, diffusible fluorescent molecule separated out during the course of purification. We have referred to this component simply as "the fluorescent molecule", or "F" for short. The luminescence reaction began at once on mixing a solution of "F" with the photoprotein. The evidence is that F is the light-emitter, with a fluorescence emission spectrum corresponding exactly in its peak intensity to that of the bioluminescence maximum, and with a closely similar distribution of intensities as a function of wavelength (Fig. 35). The most extraordinary feature is that the quantum yield of the fluorescent substance is 10 or more, showing that it recycles. Thus, this system appears to be just the opposite to that of an ordinary substrate-enzyme system wherein a protein molecule recycles in the process of catalyzing the chemical alteration of a much smaller, diffusible molecule; in this luminescence system, the smaller, diffusible molecule recycles as the protein decomposes, presumably thereby furnishing the energy for the light that is emitted during the process.

The Meganyctiphanes photoprotein is extraordinarily unstable at ordinary temperatures and even at 0°C. The luminescence reaction is extraordinarily sensitive to pH. In regard to the latter property, in phosphate buffer the maximum initial intensity on mixing photoprotein and fluorescent substance took place at a pH close to 7.6. Under similar conditions but at pH of 7.3 practically no light at all was produced (Shimomura and Johnson, 1967). The mechanism of the luminescence reaction and the chemical structure of the fluorescent substance remain to be elucidated. At the moment, some of the important properties of the system, and a good method of extracting and purifying the essential components have been established. The essential components for light emission include the photoprotein, the fluorescent compound, and oxygen.

G. Odontosyllis enopla

This species of a marine polychaete annelid worm is indigenous to the waters around Bermuda and some other regions of the West Indies. It is said to have been seen by Columbus in the 1490's. If so, he must have been on the look-out an hour or so after sunset during the first few days following a full moon, as that is the only time when the specimens are visible at the surface. They are there in numbers for a period of not more than about an hour on such occasions, although local observers say that a few specimens may appear in early evening throughout the month. Swarming at the surface after each full moon no doubt is related to the breeding habits, but not all species of Odontosyllis seem to share this property of lunar periodicity, e.g., O. phosphorea which was prevalent in the Pacific Northwest some years ago could be found at any time during the month in roughly similar abundance, according to the observations made at Nanaimo, B. C. For unknown reasons, these worms seem now to have disappeared from their former habitat in that region.

To collect specimens in Bermuda, the only feasible method devised so far involves enlisting the cooperation of as many people as possible to go out in small boats at the strategic times, armed with hand nets and flashlights. The female worms appear at the surface and swim around in circles, emitting a luminous blue green slime. The light attracts the males, which are much smaller in size, about 1 cm long by about 1 mm in diameter. It seems, however, that the males cannot tell a female from a flashlight and they will swim just as readily towards the beam of a flashlight (or "torch" as it is called in Bermuda), much to the convenience of the collectors, who quickly scoop them up, hopefully several at a time, and transfer them to a bucket or tub of sea water in the boat. Back at the laboratory the specimens are concentrated on cloth in a large funnel and transferred to be quick-frozen on a layer of cellophane on dry ice. With luck and a large number of cooperating friends, a few grams of frozen worms can thus be obtained in one evening.

With an estimated total of some 50,000 individual specimens, a very small amount of highly purified luciferin was obtained. Some highly purified luciferase was also prepared in a small amount. These preparations were sufficient to determine some of the chief properties of the system, including identification of the light-emitter, which as in various other systems turned out to be the product of the oxidative reaction (Fig. 36). An interesting observation also was that the product of the light emitting reaction catalyzed by Odontosyllis luciferase was the same, as judged by the fluorescence emission spectrum, as the product of oxidation of Odontosyllis luciferin catalyzed by Os_2O_4 plus H_2O_2, which was also accompanied by light emission (Shimomura, Johnson and Saiga, 1963).

Reasonably convincing evidence was obtained that small concentrations of cyanide specifically activated the luminescence reaction in vitro. Dialysis of purified components resulted in loss of activity in a manner that could be restored by adding cyanide in a final concentration of between 10^{-4} and 10^{-6} M (Fig. 37). Efforts to detect cyanide in homogenates of worms

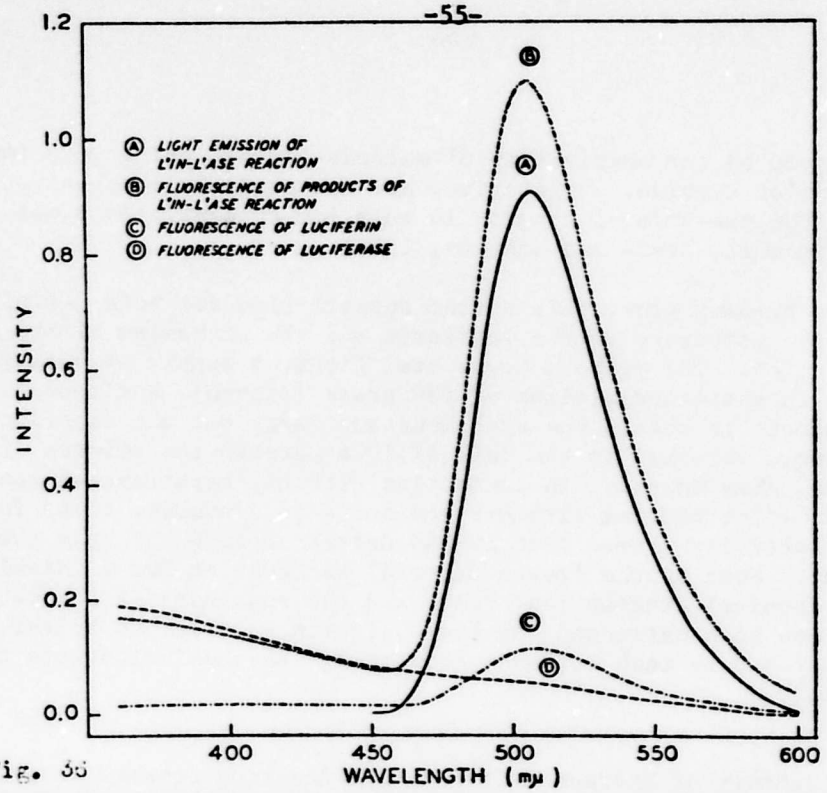


Fig. 36

Fig. 36 Emission spectrum of the luciferin-luciferase reaction, recorded by an Aminco-Bowman spectrophotofluorometer ten minutes after adding 0.2 ml of the standard luciferase solution in 2.5 ml of 0.03 *M* magnesium acetate to 0.2 ml of luciferin solution (A); fluorescence spectrum of the products of the luminescence reaction, 45 minutes after the start (B); control fluorescence spectrum of the luciferin solution (C), and control fluorescence spectrum of the luciferase solution (D). Excitation for the fluorescence spectra was at 350 mμ.

(Fig. 6, p. 284, in Shimomura, Johnson and Saiga, 1963).

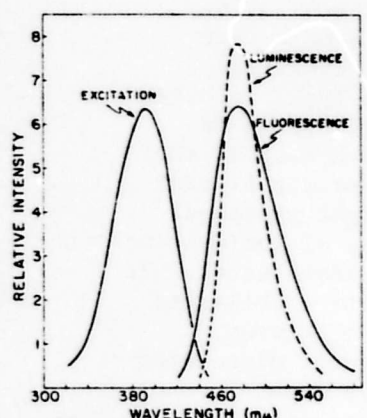


Fig. 35

FIG. 2.—Fluorescence excitation (measured at 475 mμ) and emission spectra (excited at 390 mμ) of the fluorescent substance isolated from *Meganyctiphanes norvegica* (*solid lines*), and emission spectrum of the bioluminescence reaction of this substance with the photoprotein isolated from the same source (*broken line*), measured with an Aminco-Bowman spectrophotofluorometer. Solvent: 0.02 *M* Tris-HCl containing 0.15 *M* NaCl, pH 7.6; temperature: 4°C.

(Fig. 2, p. 476, in Shimomura and Johnson, 1968).

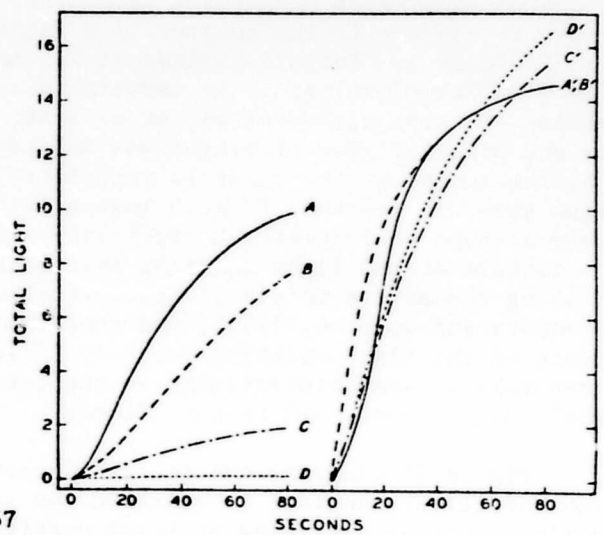


Fig. 37

Fig. 37 Progressive decrease in activity of crude luciferase solution (A) after repeated dialysis and vacuum condensation (B, C and D), and the effects of adding 10^{-3} *M* cyanide at each stage (A', B', C' and D', respectively), as described in the text. (Fig. 2 in Shimomura, Beers and Johnson, 1964; p. 13).

were handicapped by the sensitivity of methods for detecting very low concentrations of cyanide. Suggestive, but not entirely conclusive, evidence for the presence of cyanide in such low concentrations was obtained (Shimomura, Beers and Johnson, 1964).

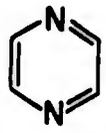
At the present time, this system appears ripe for some definitive research on the structure of the luciferin and the mechanism of the light emitting reaction. The primary needs are, first, a supply of specimens, amounting to an estimated minimum of 500 grams (frozen), and second, financial support to obtain the specimens and carry out the experiments. A few grams were obtained in the fall of 1974 through the efforts of an undergraduate, Alex Knisely, in connection with his departmental senior thesis work. After keeping with dry ice for 4 to 5 months, tests for luminescence activity showed that it had decreased to about half the initial value. Some of the frozen material was sent to Dr. Y. Kishi at Harvard for chemical studies last fall, and the remainder of the material at hand has now been extracted for its luciferin in order to better preserve this component and to seek evidence concerning its chemical nature to the extent possible.

IV. Summary of Extracted Systems and Reaction Schemes as of 1973-74.

Tables IV and V provide a convenient summary of the various types of "luciferin-luciferase" systems and "photoprotein" systems, respectively, as they stood in the period 1973-74. Complete references are available in their source (Johnson, Eyring and Stover, 1974). Since these tables were compiled, notable advances have been made towards elucidation of the luminescent systems of coelenterates, as indicated in the text of the present paper, and in detail in papers by Shimomura and Johnson, 1975. More specifically, it should perhaps be mentioned explicitly here that certain coelenterates apparently have both types of luminescence, one seeming to conform to the concept of a luciferin-luciferase type, referred to in Table IV as "Enzymic Luminescence", and the other referred to in Table V as "Photoprotein". In certain instances, e.g., the hydrozoa, the photoprotein type predominates, or at least it is vastly more significant from the point of view of brightness and total amount of light produced. Among the anthozoa, the relative importance of the two types of luminescence varies with the species; in some instances they seem to be about equal. It should perhaps be pointed out explicitly also that a fantastic similarity in structure of the light-emitting molecules among the coelenterates, including the active moiety of photoproteins, has been recently discovered (Shimomura and Johnson, 1975), and that the same skeleton is found in the product of the light-emitting reaction of luminescent species that are as remotely related biologically as coelenterates, ostracod crustacea, cephalopod molluscs, and teleost fishes.

Figure 38 diagrams the reaction schemes of various systems about which sufficient chemical information was available in 1973-74 to propose a definite scheme including at least partial chemical structure of some of the essential reactants. As in the tables, some updating of the reaction schemes is now desirable, especially in regard to the coelenterate Aequorea, and probably also some modification should be made in the "dioxetane" pathways

represented. Among impressive aspects of this figure, in any event, is the widely distributed essential components containing a pyrazine group



It is perhaps significant that amino-pyrazines, in particular, have apparently never been found in nature except in compounds essential to, or associated with, bioluminescent systems.

TABLE IV*

Enzymic Bioluminescence—Synopsis of Extracted, Cell-Free Luciferin–Luciferase Systems[a]

Type of organism and specific example(s)	Luciferin	Molecular weight	Luciferase	Molecular weight	Additional requirements	Light-emitting molecule(s)	Quantum yield[b]
A. BACTERIA AND FUNGI							
1. SCHIZOMYCETES: various species: *Photobacterium fischeri, Ph. phosphoreum*	R—CHO	—	Pure	79,000	O_2, $FMNH_2$	$FMNH^*$	FMN recycles R—CHO: 0.17
2. BASIDIOMYCETES: various species: *Armillaria, Collybia, Omphalia,* etc.	Pure (?)	—	Partly purified (particulate)		O_2	—	—
B. PROTOZOA							
DINOFLAGELLATE: *Gonyaulax*[c]							
Soluble system	Highly purified	—	Highly purified	35,000	O_2, salts	—	—
Particulate system	Highly purified	—	Highly purified	—	O_2, H^+	—	—
C. COELENTERATES							
ANTHOZOA:							
PENNATULACEA:							
Cavernularia[c]	Partly purified	—	Partly purified	—	O_2, DPA[e]		
Renilla[c]	Pure	—	Pure	12,000[d]	O_2, DPA[e]	Oxyluciferin	
Leiopitilus[c]	Partly purified	—	Partly purified	—	O_2, DPA[e]	—	—
Pennatula[c]	Partly purified	—	Partly purified	—	O_2, DPA[e]	—	—
D. ANNELID WORMS							
1. POLYCHAETE: *Odontosyllis* ("Bermuda fireworm")	Highly purified	—	Highly purified[f]	—	O_2, CN^-	Oxyluciferin	—
2. OLIGOCHAETE: *Diplocardia* (earthworm)	Partly purified	—	Partly purified	—	H_2O_2	—	—
E. MOLLUSKS							
1. GASTROPOD: *Latia* (limpet)	pure	236	Highly purified	173,000	O_2, purple protein	Flavin	0.012 (8°C)
2. CEPHALOPOD: *Watasenia* ("firefly squid")	—[g]	—	—[g]	—	O_2, ATP, Mg^{2+}	—	—
F. CRUSTACEA							
1. OSTRACOD: *Cypridina*[h]	Pure	405	Pure	53,000	O_2	Oxyluciferin–L-ase Complex	.3(25°C)
2. DECAPOD: *Hoplophorus*	Nearly pure[f]	—	Highly purified[f]	150,000	O_2	—	—
G. INSECTS							
COLEOPTERA: Lampyridae and other families; various species: *Photinus, Photuris,* and other fireflies	Pure	280	Pure	100,000[i]	O_2, ATP, Mg^{2+}	Oxyluciferin	0.9 (pH 7.8)
H. HEMICHORDATES							
Balanoglossus ("acorn worm")	Partly purified	—	Partly purified (peroxidase)	—	H_2O_2	—	—
I. VERTEBRATES[h] (TELEOST FISHES)							
1. PEMPHERID: *Parapriacanthus*	pure (same as F.1)	405	Partly purified,	—	O_2	Presumably same as F.1	Presumably same as F.1
2. APOGONID: *Apogon*	Partly purified	—	Partly purified[f]	—	O_2	Presumably same as F.1	Presumably same as F.1
3. BATRACHOID: *Porichthys*	Partly purified	—	Partly purified[f]	—	O_2	Presumably same as F.1	Presumably same as F.1

[a]Somewhat modified and updated from F.H. Johnson and Shimomura (1972a, pp. 302–303).
[b]Photons emitted per molecules reacted.
[c]Luciferin and luciferase interchangeable in luminescence reaction.
[d]Monomer. "Native" *Renilla* luciferase may have a molecular weight of 34,000.
[e]3′, 5′-Diphosphoadenosine (DPA) and a sulfokinase are normally needed in order to form active luciferin from luciferyl-sulfate.
[f]Only extremely small quantities have become available due to lack of raw material.
[g]Unpublished data from experiments by O. Shimomura at Uozu, Japan, 1970. Cell-free, active solutions were obtained, but separate solutions of luciferin and luciferase were not obtained in these experiments.
[h]Luciferin and luciferase components from all these sources react interchangebly, giving a luminescence reaction.
[i]Molecular weight of monomer 50,000.
[j]Luciferases of *Apogon* and *Cypridina* are immunologically distinguishable.

* Table 2.2, pp. 84 & 85, in Johnson, Eyring and Stover,(1974).

TABLE V*

Synopsis of Extracted, Cell-Free Photoprotein Systems

Type of organism and specific example(s)	Photoprotein	Molecular weight	Additional requirements	Light-emitting molecule	Quantum yield[a]
A. COELENTERATES					
1. HYDROZOA:					
Aequorea (medusae)	Pure protein (aequorin)	20,000–31,000[b,c]	Ca^{2+}	Blue-fluorescent protein product with 3 Ca^{2+}	0.23 (25°C)
Obelia, etc. (hydroids)	Partly purified	~20,000(?)[b]	Ca^{2+}	—	—
2. SCYPHOZOA:					
Pelagia (medusae)	Partly purified	~20,000(?)[b]	Ca^{2+}	—	—
B. CTENOPHORES					
Mnemiopsis	Partly purified	~20,000(?)[b]	Ca^{2+}	—	—
C. ANNELID WORMS					
Chaetopterus	Pure proteins, in an amorphous and in a crystalline form	120,000 (amorphous) 180,000 (crystalline)	O_2, Fe^{2+}, hydroperoxide, and two organic cofactors	—	0.009 (amorphous) 0.014 (crystalline) (25°C)
D. MOLLUSKS					
PELECYPOD: *Pholas*	Highly purified	47,500	A second protein from *Pholas*; or $Fe^{2+} + H_2O_2$; or Fe^{2+} + complexing agents (PO_4^{3-}, CN^-, or EDTA)	—	—
E. CRUSTACEA					
EUPHAUSIID SHRIMP: *Meganyctiphanes*	Highly purified: monomer and dimer or trimer	360,000 (monomer) 900,000 (dimer or trimer)	Separated fluorescent substance "F"	"F" (recycles)	Protein: 0.22 0.55 "F" > 10

[a] Photons emitted per molecules reacted.

[b] The value of approximately 20,000 is based on gel-filtration data from studies by several authors. Most of the available evidence favors a value of 31,000 for aequorin (see Kohama, Shimomura, and Johnson, 1971).

[c] The molecular weight of the light-emitting moiety is 277; the molecular weight of the whole functional group in spent aequorin is 411 (Shimomura and Johnson, 1973d).

*(Table 2.3, p. 89, in Johnson, Eyring and Stover, 1974).

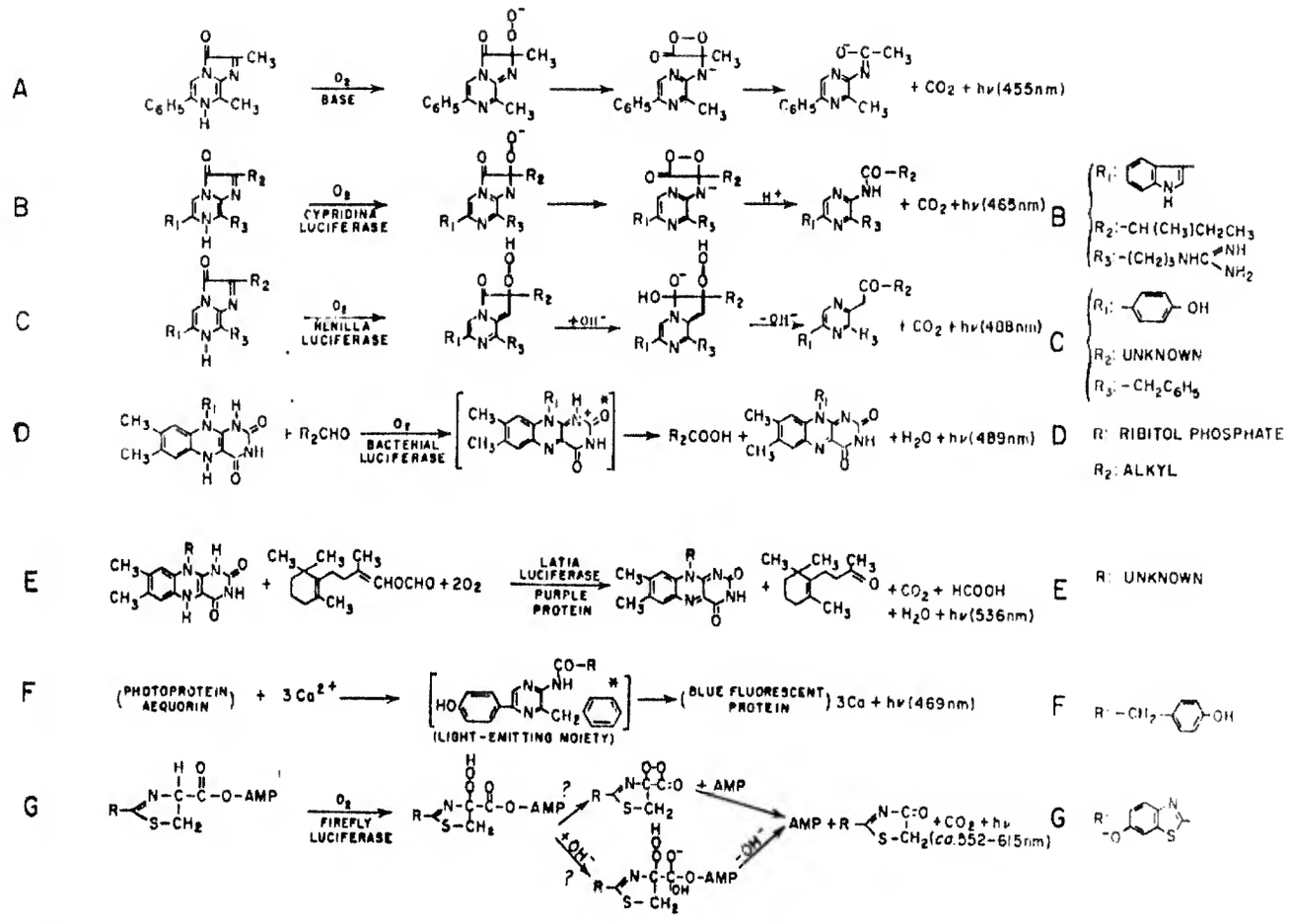


Fig. 38

Fig. 4A7. Reaction mechanisms according to the evidence and theories discussed in the text. (A) an analog of *Cypridina* luciferin; (B) *Cypridina* luciferin oxidation catalyzed by *Cypridina* luciferase; (C) *Renilla* luciferin (proposed partial structure) oxidized with *Renilla* luciferase; (D) the bacterial system. The proposed partial structure of *Renilla* luciferin (C) is shown as revised by Hori and Cormier (1973), as mentioned in the footnote on page 67.

(E) the limpet *Latia*; (F) the photoprotein aequorin; (G) the firefly system, with alternative hypotheses indicated by the alternative pathways, with the two different structures, the upper formula having a dioxetane ring, and the lower one without the formation of this type of ring. The product shown in G has been synthesized by Suzuki and Goto (1971) and established as the light emitter. Evidence to be published by Shimomura, Johnson and Morise in 1974 indicates that in F, Ca^{2+} reacts simultaneously with an initial (native) chromophore having an imidazopyrazine skeleton like that of *Cypridina* luciferin and with a yellow compound and a hydroperoxide group bound tightly and closely adjacent in aequorin, and that the final products remarkably resemble those of the luminescent oxidation of *Cypridina* luciferin.

(Fig. 2.12, p. 103, in Johnson, Eyring and Stover, 1974).

V. REFERENCES, Part I.

The following list contains only the references cited from publications independent of those based on research aided by the office of Naval Research with F. H. Johnson as Principal Investigator.

Amberson, W. R., 1922. Kinetics of the bioluminescent reaction in Cypridina. Jour. Gen. Physiol., 4: 517-534, 535-558.

Anson, M. L., and A. E. Mirsky, 1931. Protein coagulation and its reversal. J. Gen. Physiol., 14: 725-732.

Baker, P. F., A. L. Hodgkin, and E. B. Ridgway, 1971. The early phase of calcium entry in giant axons of Loligo. J. Physiol., 214: 33-34.

Bassot, J.-M., 1966. On the comparative morphology of some luminous organs. In, F. H. Johnson and Y. Haneda (eds.) Bioluminescence in Progress, pp. 557-610, Princeton Univ. Press, Princeton, N.J.

Bělehrádek, J., 1935. Temperature and Living Matter. Gebrüder Borntraeger, Berlin.

Boyle, R., 1667. Concerning the relation between light and air (in shining wood and fish). Phil. Trans. Roy. Soc. (London), 2(31): 581-600.

Boyle, R., 1672. Some observations about shining fles, made by the Honorable Robert Boyle; Feb. 15, 1671-72 and by way of letter addressed to the publishers and presented to the R. Society, Phil. Trans. Roy. Soc. (London), 7: 5108-5116.

Brown, D. E. S., 1957. Temperature-pressure relation in muscular contraction. In Influence of Temperature on Biological Systems, F. H. Johnson, ed., pp. 83-110. American Physiological Society, Washington, D.C.

Brown, D. E., F. H. Johnson, and D. A. Marsland, 1942. The pressure-temperature relations of bacterial luminescence. J. Cell. Comp. Physiol., 20: 151-168.

Brown, D. E. S., K. F. Guthe, H. C. Lawler, and M. P. Carpenter, 1958. The pressure, temperature, and ion relations of myosin and ATP-ase. J. Cell. Comp. Physiol. 52: 59-77.

Cattell, McK., 1936. The physiological effects of pressure. Biol. Rev. Proc. Camb. Phil. Soc., 11: 441-476.

Chance, B., A. Azzi, I. Y. Lee, C. P. Lee, and L. Mela, 1969. The nature of the respiratory chain: location of energy conservation sites, the high energy store, electron transfer-linked conformation changes, and the "closedness" of submitochondrial vesicles. In Mitochondria Structure and Function, Fifthe Symposium of the Federation of European Biochemical Societies, L. Ernster and Z. Drahota, eds., Vol. 17, pp. 233-273. Academic Press, New York.

Cormier, M. J., and B. L. Strehler, 1953. The identification of KCF: requirement of long-chain aldehydes for bacterial extract luminescence. J. Am. Chem. Soc., 75: 4864-4865.

DeLuca, M., and M. E. Dempsey, 1970. Mechanism of oxidation in firefly luminescence. Biochem. Biophys. Res. Commun., 40: 117-122.

DeLuca, M., and M. E. Dempsey, 1973. Mechanism of bioluminescence and chemiluminescence elucidated by use of oxygen-18. In Chemiluminescence and Bioluminescence, M. J. Cormier, D. M. Hercules, and J. Lee, eds., pp. 345-355. Plenum Press, New York.

DeLuca, M., M. E. Dempsey, K. Hori, J. E. Wampler, and M. J. Cormier, 1971. Mechanism of oxidative carbon dioxide production during Renilla reniformis bioluminescence. Proc. Natl. Acad. Sci. U.S., 68: 1658-1670.

Dubois, R., 1903. Sur une"lampe vivante de sùreté."C. R. Acad. Sci., 136: 1493-1494.

Eyring, H., 1935. The activated complex in chemical reactions. J. Chem. Phys. 3: 107-115.

Eyring, H., and J. L. Magee, 1942. Application of the theory of absolute reaction rates to bacterial luminescence. J. Cell. Comp. Physiol., 20: 169-177.

Eyring, H., J. W. Woodbury, and J. S. D'Arrigo, 1973. A molecular mechanism of general anesthesia. J. Anesthesiol., 38: 415-424.

Glasstone, S., K. J. Laidler, and H. Eyring, 1941. The Theory of Rate Processes. McGraw-Hill, New York.

Guthe K. F., 1957. Myosin ATP-ase activity in relation to temperature and pressure. In Influence of Temperature on Biological Systems, F. H. Johnson, ed., pp. 71-82. American Physiological Society, Washington D. C.

Guthe, K. F., and D. E. S. Brown, 1958. Reversible denaturation in the myosin adenosine triphosphatase system. J. Cell Comp. Physiol., 52: 79-87.

Harvey, E. N., 1952. Bioluminescence. Academic Press, New York.

Harvey, E. N., 1957. A History of Luminescence. Amer. Philosophical Society, Philadelphia.

Haneda, Y., 1955. Luminous Organisms of Japan and the Far East. In, F. H. Johnson (ed.), The Luminescence of Biological Systems. Amer. Assoc. Adv. Sci. Washington D.C.

Heller, J. F., 1853. Ueber das Leuchten im Pflanzen - und Tierreiche. Arch. Chem. Mikr., 6: 241-251.

Henderson, V. E., 1930. The present status of the theories of narcosis. Physiol. Rev., 10: 171-220.

Inoue, S., S. Gugiura, H. Kakoi, and K. Hasizume, T. Goto and H. Iio, 1975. Squid bioluminescence II. Isolation from Watasenia scintillans and synthesis of 2-(p-hydroxybenzyl)-6-(p-hydroxyphenol)-3,7-d

Johnson, F. H., D. E. Brown, and D. A. Marsland, 1942a. A basic mechanism in the biological effects of temperature, pressure and narcotics. Science, 95: 200-203.

Johnson, F. H., D. E. Brown, and D. A. Marsland, 1942b. Pressure reversal of the action of certain narcotics. J. Cell. Comp. Physiol., 20: 269-276.

Johnson, F. H., H. Eyring, and Stover, B. J. 1974. The Theory of Rate Processes in Biology and Medicine. John Wily and Sons, Inc. New York.

Johnson, F. H., H. Eyring, R. Steblay, H. Chaplin, C. Huber, and G. Gherardi, 1945. The nature and control of reactions in bioluminescence. J. Gen. Physiol., 28: 463-537.

Kauzmann, W., A. M. Chase, and E. H. Brigham, 1949. Studies on cell enzyme systems. III. Effects of temperature on the constants in the Michaelis-Menten relation for the luciferin-luciferase system Arch. Biochem. 24: 281-288.

Kishi, Y., T. Goto, S. Inoue, S. Sugiura, and H. Kishimoto, 1966. Cypridina bioluminescence. III. Total synthesis of Cypridina luciferin. Tetrahedron Letters, No. 29, 3445-3450.

Lee, J. 1974. Bioluminescence. Photochem. Photobiol. 20:535-537.

Lever, M. J., K. W. Miller, W. D. M. Paton, and E. B. Smith, 1971. Pressure reversal of anaesthesia. Nature, 231: 368-371.

McCapra, F., and Y. C. Chang, 1967. The chemiluminescence of a Cypridina luciferin analogue. Chem. Commun., 1011-1012.

McCapra, F., Y. C. Chang, and V. P. Francois, 1968. The chemiluminescence of a firefly luciferin analogue. Chem. Commun. pp. 22-23.

McElroy, W. D., J. W. Hastings, V. Sonnenfeld, and J. Coulombre, 1953. The requirement of riboflavine phosphate for bacterial luminescence. Science, 118: 385-386.

Shimomura, O., S. Inoue, and T. Goto, 1975. The light-emitter in bioluminescence of the sea cactus Cavernularia obsesa. Chemistry Letters, (to be published).

Strehler, B. L., 1953. Luminescence in cell-free extracts of luminous bacteria and its activation by DPN. J. Am. Chem. Soc., 75: 1264.

Strehler, B. L., and M. J. Cormier, 1954. Isolation, identification and function of long-chain fatty aldehydes affecting the bacterial luciferin-luciferase reaction. J. Biol. Chem. 211: 213-225.

Sugiyama, N., M. Akutagawa, T. Gasha, and Y. Saiga, 1966. Chemiluminescence of indole derivatives. In Bioluminescence in Progress, F. H. Johnson and Y. Haneda, eds. pp. 83-87. Princeton University Press, Princeton, N.J.

Tasaki, I., and C. S. Spyropoulos, 1957. Influence of changes in temperature and pressure on the nerve fiber. In Influence of Temperature on Biological Systems, F. H. Johnson, ed., pp. 201-220, American Physiological Society, Washington, D.C.

Taylor, G. W., 1934. The effect of narcotics on respiration and luminescence in bacteria, with special reference to the relation between the two processes. J. Cell Comp. Physiol., 4: 329-355.

Taylor, G. W., 1936. The effect of ethyl urethane on bacterial respiration and luminescence. Jour. Cell. Comp. Physiol., 7: 409-415.

Tsuji, F. I., and Y. Haneda, 1966. Chemistry of the luciferases of Cypridina hilgendorfii and Apogon illioti. In Bioluminescence in Progress, F. H. Johnson and Y. Haneda, eds., pp. 137-149. Princeton University Press, Princeton, N.J.

Tsuji, F. I., and R. Sowinski, 1961. Purification and molecular weight of Cypridina luciferase. J. Cell. Comp. Physiol., 58: 125-129.

Turro, N. J., P. Lechtken, N. E. Schore, G. Schuster, H.-C. Steinmetzer, and A. Yekta, 1974. Accounts Chem. Res. 7: 97-105.

Ueda, I., and H. Kamaya, 1973. Kinetic and thermodynamic aspects of the mechanism of general anesthesia in a model system of firefly luminescence in vitro. Anesthesiology, 38: 425-436.

van Schouwenburg , K. L. On Respiration and Light Emission in Bacteria. Thesis, Delft.

Warburg, O., 1921. Physikalische Chemi der Zellatmung. Biochem. Zeitschr., 119: 134-166.

V. REFERENCES, Part II

The following publications by F. H. Johnson and collaborators were based on work aided in part by the Office of Naval Research, 1950-1974.

Chang, J. J. and F. H. Johnson, 1959. The influence of pressure, temperature and urethane on the luminescent flask of mnemiopsis leidyi. Biol. Bul., 116: 1-14.

Chang, J. J., A. Gelperin and F. H. Johnson, 1974. Intracellularly injected aequorin detects transmembrane calcium flux during action potentials in an identified neuron from the terrestrial slug Limax maximus. Brain Research, 77: 431-442.

Eyring, H., and F. H. Johnson, 1957. The critical complex theory of biogenesis. In Influence of Temperature on Biological Systems, F. H. Johnson, ed., pp. 1-8. American Physiological Society, Washington, D. C.

Eyring, H., and F. H. Johnson, 1971. The elastomeric rack in biology. Proc. Natl. Acad. Sci. U.S., 68: 2341-2344.

Fracheboud, M. G., O. Shimomura, R. K. Hill, and F. H. Johnson, 1969. Synthesis of Latia luciferin. Tetrahedron Letters, No. 45, pp. 3951-3952.

Haneda, Y., and F. H. Johnson, 1958. The luciferin-luciferase reaction in a fish, Parapriacanthus beryciformes, of newly discovered luminescence. Proc. Natl. Acad. Sci. U.S., 44: 127-129.

Haneda, Y., and F. H. Johnson, 1962. The photogenic organs of Parapriacanthus beryciformes Franz and other fish with the indirect type of luminescent system. J. Morphol., 110: 187-198.

Haneda, Y., F. H. Johnson, and O. Shimomura, 1966. The origin of luciferin in the luminous ducts of Parapriacanthus ransonneti, Pempheris Klunzingeri, and Apogon Illioti. In Bioluminescence in Progress. F. H. Johnson and Y. Haneda, eds., pp. 533-545. Princeton University Press, Princeton, N.J.

Haneda, Y., F. H. Johnson, and H.-C. Sie, 1958. Luciferin and luciferase extracts of a fish, Apogon marginatus, and their luminescent cross-reactions with those of a crustacean, Cypridina hilgendorfii. Biol. Bull. (Woods Hole), 115:336.

Haneda, Y., F. H. Johnson, Y. Masuda, Y. Saiga, O. Shimomura, H.-C. Sie, N. Sugiyama, and I. Takatsuki, 1961. Crystalline luciferin from live Cypridina. J. Cell. Comp. Physiol., 57:55-62.

Johnson, F. H., ed. 1955. The Luminescence of Biological Systems. American Association for the Advancement of Science, Washington, D. C.

Johnson, F. H., ed., 1957a. Influence of Temperature on Biological Systems. American Physiological Society, Washington, D.C.

Johnson, F. H., 1957b. The action of pressure and temperature. In Symp. Soc. Gen. Microbiol., 7: 134-167.

Johnson, F. H., 1966. Introduction. In Bioluminescence in Progress, F. H. Johnson and Y. Haneda, eds., pp. 3-21. Princeton University Press, Princeton, N.J.

Johnson, F. H., 1967. Bioluminescence. In Comprehensive Biochemistry, Vol. 27, M. Florkin and E. H. Stotz, eds., pp. 79-136. Elsevier, Amsterdam-London-New York.

Johnson, F. H., 1970. The bioluminescent protein "aequorin". Naval Res. Rev., 23(2): 16-23.

Johnson, F. H., 1971. Hydrostatic pressure reversal of alcohol narcosis in aquatic animals. In Chemical Dynamics, J. Hirschfelder and D. Henderson, eds., pp. 647-659. Wiley, New York.

Johnson, F. H., and H. Eyring, 1970. The kinetic basis of pressure effects in biology and chemistry. In High Pressure Effects on Cellular Processes, A. M. Zimmerman, ed., pp. 1-44. Academic Press, New York.

Johnson, F. H., and E. A. Flagler, 1951a. Hydrostatic pressure reversal of narcosis in tadpoles. Science, 112: 91-92.

Johnson, F. H., and E. A. Flagler, 1951b. Activity of narcotized amphibian larvae under hydrostatic pressure. J. Cell. Comp. Physiol., 37: 15-25.

Johnson, F. H., and Y. Haneda, eds., 1966. Bioluminescence in Progress. Princeton University Press, Princeton, N.J.

Johnson, F. H., O. Shimomura, 1968. The chemistry of luminescence in coelenterates. In Chemical, Zoology, M. Florkin and B.T. Scheer, eds., Vol. 2, pp. 233-261. Academic Press, New York.

Johnson, F. H., and O. Shimomura, 1972a. Enzymic and nonenzymic bioluminescence. In Photophysiology, A.C. Giese, ed, Vol. 7 pp. 275-334. Academic Press, New York.

Johnson, F. H., and O. Shimomura, 1972b. Preparation and use of aequorin for rapid microdetermination of Ca^{2+} in biological systems. Nature New Biol., 237: 287-288.

Johnson, F. H., H. Eyring, and J. J. Chang, 1959. Reaction rate control of light emission in bioluminescent system. Faraday Society Discussions No. 27, pp. 191-199.

Johnson, F. H., H. Eyring, and W. Kearns, 1943. A quantitative theory of synergism and antagonism among diverse inhibitors, with special reference to sulfanilamide and urethane. Arch. Biochem. 3: 1-31.

Johnson, F. H., H. Eyring, and M. J. Polissar, 1954. The Kinetic Basis of Molecular Biology. Wiley, New York.

Johnson, F. H., Eyring, H., and Stover, B. J. 1974. The Theory of Rate Process in Biology and Medicine. John Wiley and Sons, Inc., New York.

Johnson, F. H., Y. Haneda, and H.-C. Sie, 1960. An interphylum luciferin-luciferase reaction. Science, 132:422.

Johnson, F. H., O. Shimomura, and Y. Haneda, 1966. A note on the large luminescent earthworm, Octochaetus multiporus, of New Zealand. In Bioluminescence in Progress, F. H. Johnson and Y. Haneda, eds., pp. 385-389. Princeton University Press, Princeton, N.J.

Johnson, F. H., O. Shimomura, and Y. Saiga, 1961. Luminescence potency of the Cypridina System. Science, 134: 1755-1756.

Johnson, F. H., O. Shimomura, and Y. Saiga, 1962. Action of cyanide on Cypridina luciferin. J.Cell.Comp.Physiol. 59:265-272.

Johnson, F. H., H.-C. Sie, and Y. Haneda, 1961. The luciferin-luciferase reaction. In Light and Life, W. D. McElroy and B. Glass, eds., pp. 206-218. The Johns Hopkins Press, Baltimore,Md.

Johnson, F. H., O. Shimomura, Y. Saiga, L. C. Gershman, G. T. Reynolds, and J. R. Waters, 1962. Quantum efficiency of Cypridina luminescence, with a note on that of Aequorea. J. Cell. Comp. Physiol. 60: 85-104.

Johnson, F. H., H.-D. Stachel, O. Shimomura, and Y. Haneda, 1966. Partial purification of the luminescence system of the deep-sea shrimp, Hoplophorus gracilorotris. In Bioluminescence in Progress, F. H. Johnson and Y. Haneda, eds., pp. 523-532. Princeton University Press, Princeton, N.J.

Johnson, F. H., H.-D. Stachel, E. C. Taylor, and O. Shimomura, 1966. Chemiluminescence and fluorescence of Cypridina luciferin and of some new indole compounds in dimethyl sulfoxide. In Bioluminescence in Progress, F. H. Johnson and Y. Haneda, eds., pp. 67-82. Princeton University Press, Princeton, N.J.

Johnson, F. H., N. Sugiyama, O. Shimomura, Y. Saiga, and Y. Haneda, 1961. Crystalline luciferin from a luminescent fish, Parapriacanthus beryciformes. Proc. Natl. Acad. Sci. U.S., 47: 486-489.

Kishi, Y., T. Goto, Y. Hirata, O. Shimomura, and F. H. Johnson, 1966. The structure of Cypridina luciferin. In Bioluminescence in Progress, F. H. Johnson and Y. Haneda, eds., pp. 89-113. Princeton University Press, Princeton, N.J.

Kohama, T., O. Shimomura, and F. H. Johnson, 1971. The molecular weight of the photoprotein aequorin. Biochemistry. 10: 4149-4152.

Morise, H., O. Shimomura, F. H. Johnson, and J. Winant, 1974. Intermolecular energy transfer in the bioluminescence system of Aequorea. Biochemistry, 13: 2656-2662.

Shimomura, O., and F. H. Johnson, 1966. Partial purification and properties of the Chaetopterus luminescence system. In Bioluminescence in Progress, F. H. Johnson and Y. Haneda, eds., pp. 495-521. Princeton University Press, Princeton, N.J.

Shimomura, O., and F. H. Johnson, 1967. Extraction, purification, and properties of the bioluminescence system of the euphausiid shrimp Meganyctiphanes norvegica. Biochemistry, 6: 2293-2306.

Shimomura, O., and F. H. Johnson, 1968a. The structure of Latia luciferin, Biochemistry, 7: 1734-1738.

Shimomura, O., and F. H. Johnson, 1968b. Purification and properties of the luciferase and of a protein cofactor in the bioluminescence system of Latia neritoides, Biochemistry, 7: 2574-2580.

Shimomura, O., and F. H. Johnson, 1968c. Light-emitting molecule in a new photoprotein type of luminescence system from the euphausiid shrimp Meganyctiphanes norvegica. Proc. Natl. Acad. Sci. U.S., 59: 475-477.

Shimomura, O., and F. H. Johnson, 1968d. Chaetopterus photoprotein: crystallization and cofactor requirements for bioluminescence. Science, 159: 1239-1240.

Shimomura, O., and F. H. Johnson, 1969. Properties of the bioluminescent protein aequorin. Biochemistry, 8: 3991-3997.

Shimomura, O., and F. H. Johnson, 1970a. Mechanisms in the quantum yield of Cypridina bioluminescence. Photochem. Photobiol., 12: 291-295.

Shimomura, O., and F. H. Johnson, 1970b. Calcium binding, quantum yield, and emitting molecule in aequorin bioluminescence. Nature, 227: 1356-1357.

Shimomura, O., and F. H. Johnson, 1971. Mechanism of the luminescent oxidation of Cypridina luciferin. Biochem. Biophys. Res. Commun. 44: 340-346.

Shimomura, O., and F. H. Johnson, 1972. Structure of the light-emitting moiety of aequorin. Biochemistry, 11: 1602-1608.

Shimomura, O., and F. H. Johnson, 1973a. Chemical nature of the light emitter in bioluminescence of aequorin. Tetrahedron Letters, No. 31, 2963-2966.

Shimomura, O., and F. H. Johnson, 1973b. Exchange of oxygen between solvent H_2O and the CO_2 produced in Cypridina bioluminescence. Biochem. Biophys. Res. Commun. 51:558-563.

Shimomura, O., and F. H. J hnson, 1973c. Further data on the specificity of aequorin luminescence to calcium. Biochem. Biophys. Res. Commun. 53: 490-494.

Shimomura, O., and F. H. Johnson, 1973d. Mechanism of the luminescent oxidation of Cypridina luciferin. In Chemiluminescence and Bioluminescence, M. J. Cormier, D. M. Hercules, and J. Lee, eds., pp. 337-344. Plenum Press, New York.

Shimomura, O., and F. H. Johnson, 1975a. Influence of buffer system and pH on the amount of oxygen exchanged between solvent H_2O and the CO_2 produced in the aerobic oxidation of Cypridina luciferin catalyzed by Cypridina luciferase. Anal. Biochem. In press.

Shimomura, O., and F. H. Johnson, 1975b. The chemical nature of bioluminescence systems in coelenterates. Proc. Nat. Acad. Sci. U.S. in press.

Shimomura, O., and F. H. Johnson, 1975c. Regeneration of the photoprotein aequorin. (MS submitted for publication, 1975).

Shimomura, O., J. R. Beers, and F. H. Johnson, 1964. The cyanide activation of Odontosyllis luminescence. J. Cell. Comp. Physiol., 64: 15-22.

Shimomura, O., F. H. Johnson, and Y. Haneda, 1966. Isolation of the luciferin of the New Zealand freshwater limpet, Latia neritoides Gray. In Bioluminescence in Progress, F. H. JOhnson and Y. Haneda, eds., pp. 391-404. Princeton University Press, Princeton, N.J.

Shimomura, O., F. H. Johnson, and Y. Kohama, 1972. Reactions involved in bioluminescence systems of limpet (Latia nertoides) and luminous bacteria. Proc. Natl. Acad. Sci. U.S., 69: 2086-2089.

Shimomura, O., F. H. Johnson, and T. Masugi, 1969. Cypridina bioluminescence: light-emitting oxyluciferin-luciferase complex. Science, 164: 1299-1300.

Shimomura, O., F. H. Johnson, and H. Morise, 1974. Mechanism of the luminescent intramolecular reaction of aequorin. Biochemistry, 13: 3278-3286.

Shimomura, O., F. H. Johnson, and H. Morise, 1975. The aldehyde content of luminous bacteria and of an "aldehydeless" dark mutant. Proc. Nat. Acad. Sci. U.S., 71: 4666-4669.

Shimomura, O., F. H. Johnson, and Y. Saiga, 1961. Purification and properties of Cypridina luciferase. J. Cell. Comp. Physiol., 58: 113-123.

Shimomura, O., F. H. Johnson, and Y. Saiga, 1962a. Extraction, purification and properties of aequorin, a bioluminescent protein from the luminous hydromedusan Aequorea. J. Cell. Comp. Physiol., 59: 223-239.

Shimomura, O., F. H. Johnson, and Y. Saiga, 1962b. Action of cyanide on Cypridina luciferin. J. Cell. Comp. Physiol. 59: 265-272.

Shimomura, O., F. H. Johnson, and Y. Saiga, 1963a. Partial purification and properties of the Odontosyllis luminescence system. J. Cell. Comp. Physiol., 61: 275-292.

Shimomura, O., F. H. Johnson, and Y. Saiga, 1963b. Further data on the bioluminescent protein, aequorin. J. Cell. Comp. Physiol., 62: 1-8.

Shimomura, O., F. H. Johnson, and Y. Saiga, 1963c. Extraction and properties of halistaurin, a bioluminescent protein from the hydromedusan Halistaura., J. Cell. Comp. Physiol., 62: 9-16.

Shimomura, O., F. H. Johnson, and Y. Saiga, 1963d. Micro-determination of calcium by aequorin luminescence. Science, 140: 1139-1140.

Sie, H.-C., J. J. Chang and F. H. Johnson, 1959. Pressure-temperature-inhibition relations in the luminescence of Chaetopterus variopedatus and its luminescent secretion. Jour. Cell. Comp. Physiol., 52: 195-226.

Stone, H., 1968. The enzyme-catalyzed oxidation of Cypridina luciferin. Biochem. Biophys. Res. Commun., 31: 386-391.

Strehler, B. L., and F. H. Johnson, 1954. The temperature-pressure-inhibitor relations of bacterial luminescence in vitro. Proc. Natl. Acad. Sci. U.S., 40: 606-617.