AN ABSTRACT OF THE THESIS OF

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Title: STUDIES ON THE OCCURRENCE, PHYSIOLOGY, AND ECOLOGY OF BIOLUMINESCENCE IN DINOFLAGELLATES

Abstract approved: Redacted for Privacy
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To provide further information on the occurrence and geographical variations of bioluminescent capabilities of marine dinoflagellates, forty species, representing twelve genera, of dinoflagellates from Yaquina Bay, Oregon, were examined for bioluminescence as single cell isolates. Seventeen species from the genera *Ceratium* (1 sp.), *Gonyaulax* (3 sp.), and *Peridinium* (13 sp.) were found to be bioluminescent. *Ceratium fusus* was the only member of the genus found to emit light; *G. triacantha* was found to be non-bioluminescent.

The total photon emission of each luminescent species is reported. Values ranged from 1.05×10^{10} photons per *P. depressum* to 2.1×10^{7} photons per *G. digitale*. As a taxon, the genus *Peridinium* emitted more light by an order of magnitude than did *Ceratium* or *Gonyaulax*. Comparisons with previous reports are made.

Photoinhibition of the mechanical receptor mechanism is largely responsible for orders of magnitude diel variations of stimulable bioluminescence in the auxotrophic dinoflagellates. The mechanically stimulable bioluminescence of members of the Gonyaulax catenella group can be photoinhibited completely by exposure to as little as 10^{13} quanta/cm^2 delivered as a pulse of width between 0.1 and 10 seconds. There is an initial time lag of one minute, followed by a first order decay to approximately one percent of the bioluminescence of unexposed controls. The half time of this decay is only 50 seconds.

Action spectra for photoinhibition in Gonyaulax catenella, G. acatenlla, and G. tamarensis revealed a single absorption band with a maximum at 562 nm. Photoinhibition appears to raise the threshold of sensitivity of the shear receptor mechanism. Chemically stimulable bioluminescence is unaffected by these brief exposures to light.

Grazing experiments were conducted with three calanoid copepods and three species of bioluminescent dinoflagellates, using procedures which yielded samples of cultures with high and low capacities for mechanically stimulable bioluminescence. In all cases the ingestion rates were lower for the high bioluminescent capacity samples than for the samples having a reduced bioluminescent capacity. These results indicate that dinoflagellate bioluminescence has survival value as a defense against copepod grazing. Of several

possible mechanisms, we propose that the flash is a visual, protean display which startles or confuses the copepod sufficiently to allow the dinoflagellate to escape. The net evolutionary value is that predation would be reduced on a dinoflagellate population as a whole.

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by

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STUDIES ON THE OCCURRENCE, PHYSIOLOGY AND ECOLOGY OF BIOLUMINESCENCE IN DINOFLAGELLATES

I. INTRODUCTION

General

Since the earliest of times men have been fascinated and mystified by the phenomenon of bioluminescence in the oceans, either in the wakes of vessels and fish, or in the surf zone and on sandy beaches. E. Newton Harvey (1957) has reviewed historical accounts of bioluminescence and attributed the earliest documentations to Artistotle and Pliny the Elder. Contemporary man is no less dependent upon his eyes for the majority of his sensory input than were the ancients, and although he is aided by a myriad of artificial illuminating devices he still occasionally finds himself in the dark of night, and perhaps more than ever before is entranced by the gleams, glimmers and glows of the more simple life. It is his nature to classify and describe phenomena, and to determine their causes, mechanisms, and functions. Although scientific investigation may at times detract from the intrinsic beauty of bioluminescent displays, such as is depicted by M. C. Escher in "Luminous Sea," it certainly provides the interested observer with ample opportunity to satisfy his curiosity and more fully appreciate the scintillations of these

delicate organisms.

We now know that the liminescence known by Pliny and Escher is produced by millions of single celled organisms, called dinoflagellates by the protozoologist and peridineae by the algologist, in response to turbulent stimulation. There have been a fair number of investigations with respect to the occurrence of species which possess the capacity for bioluminescence, details of the physiological and biochemical processes required for its maintenance and production, and the selective advantage(s) conferred upon the organisms as a result of their ability. Work in this field has been reviewed by Loeblich (1966). Harvey (1952) has given a more complete presentation of the more classical studies.

With the development of sensitive submarine photometers, a great number of synoptic measurements of stimulable bioluminescence were made, primarily in the North Atlantic, as summarized by Kelly (1968). Bioluminescence was, for a while, treated nearly as an independent property with little biological basis. Attempts at correlating the stimulated bioluminescence with various hydrological parameters such as temperature, salinity, water mass, etc., were unsuccessful. While it was universally accepted that dinoflagellates were the primary causitive agents, it was not until Sweeney (1963) isolated and tested individual species for bioluminescence that it became clear that the distribution could only be explained in terms of

the occurrence and bioluminescent characteristics of certain species.

Kelly and Katona (1966), Kelly (1968) and Tett (1971) extended the examination of individual species and obtained excellent correlations of *in-situ* luminescence (flashes per unit time in response to an arbitrary stimulus) with the abundance of species known to have bioluminescent capabilities. The species which have been examined for luminescence were summarized by Tett; a corrected and up-dated listing of bioluminescent species is given in Appendix A.

These individual tests reveal that geographical and possibly seasonal variations in the capability of species for bioluminescence exist, and that there is a range of three orders of magnitude in the amount of light emitted depending on the species. Swift *et al.* (1970) have demonstrated the existence of physiological clones of *Pyrocystis* in terms of the total photon emission. Further testing and determination of the photon yields of various species are required to assess the extent of seasonal, geographical and physiological variations in bioluminescent species.

Extensive investigation into the biochemistry of dinoflagellate bioluminescence has been made in the past decade. The system is comprised of an enzyme (dinoflagellate luciferase), a small molecular weight substrate (dinoflagellate luciferin), and molecular oxygen. The major components were initially isolated in soluble form in low yield (Bode and Hastings, 1963), but it was later discovered that the

in-vivo system consists of 0.5μ particles (scintillons) which can be isolated from homogenated cells and studied independently. Flash kinetics which closely approximate the normal *in-vivo* flash are obtained when the pH of the suspensions of scintillons is rapidly lowered from 8.0 to about 5.7.

Recent work has resulted in a model of scintillon control of dinoflagellate bioluminescence through pH dependent changes of activities of the high molecular weight luciferase and association between luciferin and a luciferin binding protein, all of which are in close proximity within a membrane bound structure. A decrease in the pH within the vesical, resulting from a mechanically initiated membrane excitatory event involving calcium ions, induces conformational changes in both proteins, activating the luciferase and releasing the luciferin which is immediately oxidized to yield the excited state. This is supported by pH profiles of the necessary reactions, and the ability of several workers to recharge scintillons by incubating them at pH 8.0 with pure luciferin (Fuller *et al.*, 1972; Fogel and Hastings, 1972). The mechanism whereby mechanical stimulation of the cell triggers the internal pH change is not known, but apparently results in a release of calcium ions which then affect the membrane bound structure, or scintillon (Hamman and Seliger, 1972).

It is likely that this mechanism holds for the majority of the

bioluminescent dinoflagellates, since all species which have been examined show identical emission spectra, indicating that the excited state is the same, all require mechanical stimulation, which can be overcome with some degree by lowering the pH of the culture or the addition of various cations, and purified luciferin and luciferase cross-react between species (Hamman and Seliger, 1972).

The majority of the biochemical work has been done with _Gonyaulax polyedra_, and probably applies for all species. It is very clear, however, that _Gonyaulax polyedra_ cannot be regarded as a general case with regard to other physiological aspects of bioluminescence. _Gonyaulax polyedra_ possess pronounced endogenous circadian rhythms in many responses including cell division, photosynthetic capacity, and bioluminescence capacity. Biggley _et al._ (1969) showed that endogenous rhythms are not universal in the dinoflagellates, and that exogenous photoperiod controlled responses resulted in constant scotophase stimulated luminescence in _G. polyedra_, _P. bahamense_, and _P. lunula_. These three species also showed variations of sensitivity to mechanical stimulation in response to ambient light levels which were not endogenous.

In situ studies have shown large diel variations in the stimulable bioluminescence, which have been attributed to variations in cellular luciferin concentrations (Bode _et al._, 1963), and light induced changes in the sensitivity of the cells to stimulation of the type demonstrated

by Biggley *et al*. Yentsch *et al*. (1964) developed a qualitative model to explain the vertical distribution of near-surface bioluminescence based on the data of Sweeney and Hastings (1959) for two responses of *G. polyedra* to light. It is clear that the model was a bit premature, since it has been shown that species vary in their response to ambient illumination. Intuition and observations indicate that light does not play a significant role in regulating the bioluminescence in heterotrophic, non-pigmented species, which constituted the majority of the species found to be bioluminescent by Kelly.

There has been very little work on the ecological and evolutionary significance of dinoflagellate bioluminescence. Harvey (1952) considered it to be fortuitous, and as in the bacteria, a by-product of cellular metabolism. Kelly (1968), and Tett (1969) have discussed the reasons why an adaptive value might exist for the dionflagellates possessing bioluminescence capability. Earlier, Burkenroad (1943) proposed that the flash might have value in removing predators of dionflagellates, since the flash emitted upon capture of a dinoflagellate would alert higher order carnivores to the presence of the predator. Esaias (1970) found that copepods consume samples having reduced bioluminescent capacity, and proposed that the bioluminescence serves as a defense against nocturnal grazing by light-sensitive zooplankton.

Objectives

It was clear that work in the past had suffered from a lack of knowledge of the occurrence and variability of bioluminescence in dinoflagellates, from a physical, geographical, and physiological standpoint. Therefore, the first objective was to determine which dinoflagellates in this area were luminescent, and their total photon emission.

Secondly, attempts would be made to culture representative species to enable laboratory investigations into their nutrition physiology, and bioluminescence. Lack of cultured material, especially heterotrophic forms, has been a major block in the field. The bioluminescent characteristics of the cultured organisms would be investigated in the manner of Biggley *et al*. (1969) with regard to the emission spectra, total photons emitted over photoperiods, presence of endogenous rhythms, stimulability, etc.

The fourth part of the proposed study involved measuring bioluminescence *in-situ* along with dinoflagellate species and abundances, and with a relevant knowledge of the responses of the organisms present, possibly develop a quantitative model of the distribution of bioluminescence. It was soon apparent that this section was entirely too optimistic, first in terms of resources and time available, lack of success in culturing heterotrophic species, and also theoretically

with respect to stimulation mechanisms and the state of the art in phytoplankton systems dynamics. Since it was felt that anything less than the entire program would merely repeat the work of Kelly (1968) and Tett (1969), further attention was directed to the photo-inhibition of stimulability.

Finally, studies on the ecological relationships of dinoflagellate bioluminescence and zooplankton grazing would be continued and extended to other species of dinoflagellates and zooplankton.

This thesis consists of three sections, each of which constitutes a more or less final manuscript which has been or shortly will be submitted for publication.

II. BIOLUMINESCENT DINOFLAGELLATES FROM YAQUINA BAY, OREGON, AND THEIR TOTAL PHOTON EMISSION[1]

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ABSTRACT

Forty species, representing twelve genera, of dinoflagellates from Yaquina Bay, Oregon, were tested for luminescence capability as single cell isolates. Seventeen species from the genera *Ceratium* (1 sp.), *Gonyaulax* (3 sp.) and *Peridinium* (13 sp.) were found to be bioluminescent. The total photon emission of each luminescent species is reported. Values ranged from $1.05 \pm 0.4 \times 10^{10}$ photons per *P. depressum* to $2.1 \pm 1.1 \times 10^{7}$ photons per *G. digitale*. *Ceratium fusus* was the only member of the genus found to emit light; *G. triacantha* was found to be non-luminescent.

INTRODUCTION

Beginning with Baker's observation in 1753 of the luminescence of *Noctiluca*, there have been many reports of luminescence in dinoflagellates. Refined isolation, culture, and detection techniques have permitted accurate determinations of luminescent species to be made in the past decade. Many of the more common dinoflagellates have been examined for luminescent capability at La Jolla, California (Sweeney, 1963), at Woods Hole, Massachusetts (Kelly and Katona, 1966; Kelly, 1968), and Millport, England (Tett, 1971) in a very definitive manner. Still, the luminescent capability of many dinoflagellates is unknown or questionable due to lack of testing, taxonomic problems, or dubious reporting. Geographical and possibly seasonal variations are apparent in the literature.

We have examined and tested many of the common dinoflagellates from Yaquina Bay, Oregon, in order to increase the number of species and geographical areas examined. We have reported the number of photons emitted for each luminescent species. Seliger *et al.* (1969) found that the photon yield was a constant species characteristic of the three species they examined, suggesting the possibility that photon yields may have taxonomic value.

MATERIALS AND METHODS

Dinoflagellates were obtained in water samples or net collections at various points within two miles of the open ocean. Yaquina Bay has a relatively large tidal prism in relation to its area, resulting in an exchange of about 72% of the total volume each tidal cycle (Goodwin et al., 1970). Therefore, the planktonic population of the area sampled reflects the composition of the nearby neritic environment.

The samples were placed in a constant temperature room at 16 ± .5° C, and individuals isolated within 36 hours with a mouth controlled micropipette under a dissecting microscope. Single cells were washed 2-3 times in Millipore® filtered sea water obtained at the time of collection, and placed in 0.5 ml of filtered sea water in selected 13 mm o.d. test tubes. The samples and isolates remained under constant illumination (ca. 500 ft.-c., cool white fluorescent) in the environator 4-6 hours after isolation, and were checked for motility prior to being placed in darkness. The samples were kept in complete darkness for 3 - 6 hours before testing.

The tubes were placed singly before a 1P21 photomultiplier photometer and stimulated by forcefully injecting 0.1 ml of 0.05 N acetic acid. This procedure gave both mechanical and chemical stimulation. The initial calibration of this photometer is described by Esaias et al. (in preparation). The geometry used with the 13 mm

tubes and single cells were calibrated using the known chemically stimulable luminescence of Gonyaulax catenella. One hundred single cell isolates of an early log phase culture were prepared and assayed according to the above procedure. The standard deviation of the values was 14.5% of the mean, slightly higher than what had been found with assays using 3 ml samples of cultures of about 1000 cells per ml. This is attributed to a combination of variations of the photon yield per cell and geometry changes in the 13 mm o.d. test tubes. The overall accuracy is estimated to be within $\pm$ 20%. The minimum detectable luminescence, limited by the photomultiplier gain and geometry, was on the order of 2×10^4 photons (ca. 10^{-5} uw/cm^2). These values assume that the emission spectra of the dinoflagellates tested is identical to that of G. catenella.

After stimulation the samples were covered and refrigerated until identified, usually within one week. Cells stained with Trypan Blue were placed on a standard slide in water or in glycerin gelatin for examination and identification. Recovery of the isolates approached 90%. Limited samples are available for taxonomic examination.

Only cells which were motile before being placed in darkness were tested. Valid tests required recovery and identification of the organism. On many occasions flashes occurred when a tube containing the cell was handled. These results are omitted from the

quantum yield computations.

Unialgal cultures of several photosynthetic species were developed using the micropipette washing technique. Single cells were inoculated into 3 - 5 ml of enriched sea water medium (Table 1), prepared with sea water obtained at the time of collection and sterilized by Millipore^{R} filtration. Initial inoculations were made into 1/10 strength medium; after these cultures reached sufficient concentrations the medium strength was gradually increased. Members of the genus *Ceratium* required 1/10 medium at all times; higher concentrations resulted in poor growth and large numbers of aberrant cells with supernumerary horns.

All cultures were grown in LD 12:12 photoperiods at 16 ± .5° C. Assays of total stimulable luminescence were performed in the fifth hour of darkness by chemical stimulation. Cultures of species reported here as non-luminescent were examined many times during the growth phase and photoperiod.

RESULTS

A total of 40 species, representing 12 genera were examined for luminescence. Seventeen species, from three genera, showed repeated positive responses (Table 2) while the remainder showed no detectable luminescence in the testing procedure used (Table 3).

Results of tests on species examined by others confirmed their

TABLE 1. Growth Medium

$NaNO_3$	150 mg
$NaH_2PO_4 \cdot H_2O$	10 mg
$NaHCO_3$	200 mg
thiourea	1 mg
ferric sequestrene*	10 mg
$CuSO_4 \cdot 5H_2O$	196 ug
$ZnSO_4 \cdot 7H_2O$	44 ug
$CoCl_2 \cdot 6H_2O$	22 ug
$MnCl_2 \cdot 4H_2O$	360 ug
$NaMoO_4 \cdot 2H_2O$	30 ug
Thiamine HCl	200 ug
Biotin	1 ug
B_{12}	1 ug
added aseptically as stock solutions to:	
Sea water S = 28‰	1000 ml

* Sodium iron salt of ethylenediaminetetraacetic acid 13% w/w Fe.

TABLE 2. Dinoflagellates found to be bioluminescent

Genus and Species	No. luminescent / No. tested	photons/cell x 10^{-8}	Previous reports*
Ceratium fusus	23/27	8.7 ± 2.9	1, 3, 5(4)
Gonyaulax catenella	38/42	0.46 ± .12	1
G. digitale	3/5	0.21 ± .11	2, 3
G. spinifera	7/7	0.40 ± .17	3
Peridinium ceraseus	3/5	4.8 ± 3.0	--
P. claudicans	2/5	8.3	2, 3 (1)
P. conicum	3/3	34. ± 7.9	1, 2, 3, 4
P. crassipes	5/5	87. ± 31	--
P. depressum	24/27	105. ± 39	1, 3, 4
P. granii	4/5	8.1 ± 2.0	2
P. leonis	3/3	33. ± 16	2, 3, 4
P. mite	2/2	38.	(3)
P. ovatum	3/4	10.4 ± 2.8	4
P. pellucidem	4/4	2.0 ± 0.6	(3)
P. pentagonum	35/39	51. ± 9.0	1, 3
P. steinii	3/3	1.4 ± 0.27	4
P. subinerme	10/12	15. ± 6.0	3

* Negative reports (Non-luminescent) are shown by parentheses.

1. Sweeney, 1963; 2. Kelly and Katona, 1966; 3. Kelly, 1968; 4. Tett, 1971; 5. Nordli, 1957; 6. Hamman and Seliger, 1972.

TABLE 3. Dinoflagellates not found to be bioluminescent

Genus and Species	No. tested	Date cultured	Previous reports*
Ceratium bucephalum	7	--	--
C. dens	4	1/69	(1)
C. furca	10	--	(1, 4)
C. gibberum	5	--	--
C. lineatum	20	1/69, 3/71, 9/71	(3, 5)
C. minutum	17	3/71	--
C. pentagonum	3	3/71	--
C. tripos	15	1/69, 9/71	(2345)
C. contortum	8	--	--
Dinophysis acuminata	4	1/69	(4)
D. caudata	6	1/69	(1)
D. fortii	4	--	(1)
D. ovum	1	--	--
D. tripos	5	--	(1)
Glenodinium lenticula	3	--	(3, 4)
Gonyaulax triacantha	3	--	--
Noctiluca miliaris	11	9/71	1, 4 (1)
Peridinium excentricum	8	--	--
P. monacanthus	3	--	--
Podolampas palmites	4	--	--
Polykrikos schwarzii	7	--	4, 6
Protoceratium reticulatum	2	--	--
Pyrophacus horologum?	1	--	--

* See Table 2

reports with the following exceptions:

Peridinium pellucidem and *P. mite* were reported to be non-bioluminescent by Kelly (1968) while we found them consistently luminescent with a fairly high photon yield. Kelly also reported variable luminescence in *P. claudicans*, and Sweeney (1963) reported it as non-luminescent. We found this species to be variable within a single collection.

Polykrikos schwarzii has been found to be luminescent at Millport (Tett, 1971) and in the Chesapeake Bay (Hamman and Seliger, 1972), but we found no luminescence of seven individuals in April of 1972.

Ceratium fusus was luminescent whenever it was found, and we succeeded in culturing this organism on two occasions. It has been reported as luminescent at La Jolla and Woods Hole, but not luminescent at Millport.

Noctiluca miliaris was found to be non-luminescent whenever it occurred. Sweeney has discussed geographical variations in this taxon.

Three species unexpectedly showed no indications of luminescence. Three pigmented and motile individuals of *G. triacantha* showed no indications of luminescence. This is the first instance of a member of the genus *Gonyaulax* failing to show luminescence. Attempts to culture the organism failed. *P. excentricum* and

P. monacanthus were the only members of their genus to give negative results.

We also tested various unarmored dinoflagellates of the genera *Gymnodinium* and *Gyrodinium* as they appeared in our collections. None were luminescent and none were identified to the species level due to their fragility and tendency to undergo morphological changes in the test procedure.

The quantum yields of luminescence varied over four orders of magnitude for the species examined. The brightest species was *Peridinium depressum*, emitting an average of $1.05 \pm .39 \times 10^{10}$ and a maximum of 2.3×10^{10} photons. This species is comparable to members of the genus *Pyrocystis* in photon yield. *P. pseudonoctiluca* has been found to emit 1.1×10^{11} photons per cell in culture (Swift *et al.*, 1970). As a taxon, the genus *Gonyaulax* was found to have the smallest photon yield.

The large variations in photon yield within species indicates that these values have limited taxonomic value. These variations are larger than what have been found in cultures of photosynthetic species (Biggley *et al.*, 1969) and are not wholly accountable by the higher intrinsic variation in the single cell technique. We isolated several clones of photosynthetic species and determined the total stimulable luminescence during scotophase by the chemical stimulation technique (Table 4). While significant variations between clones exist, as

TABLE 4. Variations of Stimulable Luminescence in Dinoflagellate Clones Isolated from Yaquina Bay, Oregon.

Genus, Species, Clone	Date Isolated	Quanta Emitted Per Cell
Ceratium fusus (1)	9/20/71	$4.46 \pm .92 \times 10^{8}$
C. fusus (2)	3/14/72	$6.2 \pm 1.1 \times 10^{8}$
Gonyaulax catenella (1)	1/29/69	$6.2 \pm .59 \times 10^{7}$
G. catenella (2)	1/29/69	$6.1 \pm .63 \times 10^{7}$
G. catenella (3)	1/29/69	$5.8 \pm .51 \times 10^{7}$
G. catenella (4)	10/10/70	$3.1 \pm .40 \times 10^{7}$
G. catenella (5)	10/10/70	$3.8 \pm .32 \times 10^{7}$
G. catenella (6)	5/05/71	$4.9 \pm .50 \times 10^{7}$
G. catenella (7)	5/05/71	$3.6 \pm .40 \times 10^{7}$
G. catenella (8)	5/22/71	$2.9 \pm .33 \times 10^{7}$

has been found in the genus Pyrocystis (Swift *et al.*, 1970), they do not fully account for the variability of natural populations of heterotrophic species. We could find no seasonally dependent variation in the photon yields of the species which were present year-round (*C. fusus*, *G. catenella*, *P. depressum*, *P. pentagonum*) and must attribute some of the variation to short term physiological variations within the populations. Tett (1971) found a seasonal component in the number of flashes per luminescent dinoflagellate on a population level in addition to short-term variations. The large range of photon yields found here, coupled with a changing species composition, could account for the observed variations although some question exists as to the intercomparison of quantum yields and flash rates under an arbitrary stimulation which previous authors have used.

Models have recently been developed to explain the mechanical and chemical triggering of luminescent flashes in dinoflagellates on a scintillon level (Fogel and Hastings, 1972; Hamman and Seliger, 1972). We have used a combination of mechanical and chemical stimulation in the tests reported here. In all instances and for all species, the injection of acid produced an initial, turbulence-induced flash, which was followed by a slower pH-induced emission which reached a peak in 2-4 seconds and was completed in 10 seconds. In no instances was further flashing observed, indicating that the reported photon yields represent the total stimulable capacity of the

cell. These responses are in agreement with the proposed models, and indicate that there is a common flash triggering mechanism in the dinoflagellates.

We thank Mr. Harold O'Connors for assistance in collecting the dinoflagellates. This work was supported by a grant from the National Science Foundation No. GB 21430.

III. ACTION SPECTRUM FOR PHOTOINHIBITION OF MECHANICALLY STIMULABLE BIOLUMINESCENCE IN THE MARINE DINOFLAGELLATES *GONYAULAX CATENELLA*, *G. ACATENELLA*, and *G. TAMARENSIS*[1]

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ABSTRACT

The mechanically stimulable bioluminescence of members of the *Gonyaulax catenella* group can be photoinhibited completely by exposure to as little as 10^{13} quanta/cm^2 delivered during scotophase as a pulse of width between 0.1 and 10 seconds. There is an initial time lag of 1 minute followed by a first order decay to approximately one percent of the dark unirradiated control. The decay rate exhibits spectral intensity dependence. The half time of the decay is about 50 seconds. Action spectra for all three species revealed a single absorption band with a maximum at 562 nm. Photoinhibition appears to raise the threshold of sensitivity of the shear receptor mechanism in the dinoflagellates. Chemically stimulable bioluminescence is unaffected by these brief exposures.

MATERIALS AND METHODS

A. Dinoflagellates

Origins and culture conditions of the unialgal clones used in this study are given in Table 1. All cultures were used in log phases of growth except where noted. Cell concentrations were determined by counting three replicate samples in a Sedgewick-Rafter chamber.

B. Luminescence Assays

Details of the luminescence assay procedure have been given by Biggley *et al.* (1969). Briefly, 3 ml samples were taken during photophase from cultures stirred gently to insure uniform mixing, and were then dispensed into 16 mm o. d. test tubes. At appropriate times a single tube was placed in a fixed position in front of a photomultiplier and the bioluminescence capacity measured. Mechanically stimulated luminescence (MSL) was measured by stirring with a motor-driven paper clip for 30 sec. Chemically stimulated luminescence (CSL) was measured by rapidly injecting 0.5 ml of either 0.05 *N* acetic acid or 2.4 *M* calcium chloride. The anode of the photomultiplier was coupled to a D. C. amplifier and the output was recorded on either a Brush Mark 220 or Sanborn Model 320 high speed chart recorder.

TABLE 1. Origins and Culture Conditions of Dinoflagellates

Dinoflagellate	Source	Temperature	Medium‡	Generation Time (days)
Gonyaulax catenella Whedon & Kofoid	Yaquina Bay, Ore. 1969, 1970, 1971	16 ± .5	1, 2	3.1
G. acatenella Whedon & Kofoid	Texas A & M*	16 ± .5	1, 2	3.1
G. tamarensis Lebour	Texas A & M*	16 ± .5	2 (1/2)	4.5
G. monilata Howell	Texas A & M*	30	2 (1/2)	10
G. polyedra Stein	La Jolla, Calif.+	25	1	4.0
Ceratium fusus (Ehrenberg) Dujardin	Yaquina Bay, Ore. 1969, 1970, 1971	16 ± .5	2 (1/10)	3.5
Peridinium pentagonum Gran	Yaquina Bay, Ore. 1970, 1971	16 ± .5	filtered sea water	(not in culture)

All cultures maintained under cool-white fluorescent lights, 500 ft-c; LD 12:12 photoperiod.

* Donated by Dr. Emery Sutton.

+ Isolated by B. M. Sweeney, 1952;

‡ Medium 1 given by Swift and Taylor (1967); Medium 2 given by Esaias and Curl (1972b) (Section II) parentheses give concentrations of media in sea water, S ‰ = 28.0

C. Calibration of Photometers

The electrometer amplifiers and calibration of the assay geometries used at the Johns Hopkins University have been described previously (Biggley *et al.*, 1969; Seliger *et al.*, 1969). An instrument with the same capability was built at Oregon State University and calibrated in terms of quanta $volt^{-1}$ using the scotophase total stimulable luminescence (TSL) of the dinoflagellates *G. polyedra* and *G. catenella*.

As a check on the systematic variations in the calibration using the dinoflagellates which may have resulted from different culturing techniques between the two laboratories, we also determined the quantum yield per organism using the chemiluminescence of Luminol in both DMSO and aqueous solution according to the procedure given by Lee *et al.* (1965) and Lee and Seliger (1972). Luminol chemiluminescence reactions were carried out in selected 13 mm test tubes placed at 0.5 m from the detector, which was masked by a high transmittance (60%) double Fabrey-Perot interference filter (peak 477 nm, HW 11.5 nm, Thin Film Products, Waltham, Mass.). This geometry gave a sufficiently parallel light beam to preserve the transmittance characteristics of the filter. Dinoflagellates were stimulated with acetic acid in the same geometry, and the total light quantum emitted per organizm determined by this procedure was used

to calibrate the more efficient normal assay geometry. Using twice-recrystallized Luminol (Seo and Kuwana, 1965) we obtained calibration factors by the four procedures which had a coefficent of variation of 9.2% and a range of ± 15% of the mean.

D. Sources of Light and Procedures for Photoinhibition Exposures

1. Continuous Assay Procedure

The source was a parallel-focused concentrated zirconium arc, adapted with a camera shutter, 10-cycle light chopper, 2 cm thick infrared absorbing water cell, and filter holder. Narrow-band interference filters with half-widths of 10-15 nm were used to isolate various spectral regions. The narrow-band parallel beams were measured with a calibrated linear thermopile and reduced to numbers of quanta per square centimeter per second. The horizontal beam was reflected vertically with a 90° prism. Flat-bottomed vials containing 3 ml samples of cultures prepared as described above were placed directly on the face of the prism and irradiated from below.

Following a brief exposure a 6 mm stirring bar was added gently to the sample vial which was then placed on a small magnetic stirrer. Stimulated bioluminescence was viewed from above by a mu-metal shielded 1 P21 electron multiplier phototube. The stirrer was activated for 9 sec with a period of 30 seconds by a cycle timer. The

stirrer speed was adjusted to produce gentle stimulation of the cells such that the light emitted during the 9 sec interval was a small fraction of total MSL. Emitted quanta were measured for 20 cycles in irradiated samples as well as in non-irradiated control samples. This procedure enabled us to record the relative decrease in mechanically stimulable luminescence following exposure of single samples at each intensity, wavelength band, and exposure time from which a first order rate constant can be calculated.

2. The Single Point Procedure

Studies done at Oregon State used a Bausch and Lomb High Intensity Monochromator (33-86-25) with grating (33-86-03), tungsten source (33-86-39) and achromatic condenser lens (33-86-53) to provide a uniform beam of 10 nm half width. A Corning filter (3-74) was used to eliminate higher order spectra. Intensities were varied stepwise by inserting neutral density filters between the source and monochromator. Beam energies were monitored with a calibrated Eppley thermopile and Keithley 150A Microvolt-ammeter with the Brush recorder, and reduced to quanta per square centimeter per second.

Culture samples (3 ml in 16 mm test tubes) were irradiated from the side in a square glass holder containing immersion oil to minimize refraction and reflection errors. The samples were then

inserted in the normal assay geometry and assayed for MSL and CSL at appropriate times. The time course of the decrease of MSL was determined from a minimum of four samples at each intensity. Decay rate (slope of MSL versus time) versus exposure relationships were developed with a minimum of four points including zero from 400 to 725 nm in 25 nm increments. Exposure times were always less than 10, and usually less than 1 second.

Extensive masking of the source and samples was required in both procedures to eliminate the effects of stray light due to the extreme light sensitivity of the dinoflagellates. Cell concentrations were on the order of 1000/ml, and no corrections were made for self-absorption. Experiments at Oregon State were carried out in the temperature controlled growth chamber at 16 ± .5°. At the Johns Hopkins University cultures were grown at 18° and exposed and assayed at room temperature (20°).

E. Emission and Absorbancy Spectra

The emission spectrum of *G. catenella* bioluminescence was determined according to previous methods (Seliger *et al.*, 1969). Absorbance spectra of cell suspensions were determined in a Bausch and Lomb 505 recording spectrophotometer by the opal glass technique (Shibata *et al.*, 1954). Cells were concentrated by gentle filtration on nylon screening and methyl cellulose added to the suspension to retard

settling.

RESULTS

A. Emission Spectrum

The bioluminescence emission spectrum of G. catenella is shown in Figure 1. The peak wave length lies at 477 nm and identical to that determined by Seliger et al. (1969) for three other species -- Goyaulax polyedra, Pyrodinium bahamense, and Pyrocystis lunula -- and most probably all bioluminescent dinoflagellates. The half-band-width is slightly smaller than that found for G. polyedra and may reflect some self-absorbance. The emission spectra of G. acatenella and G. tamarensis were not determined and were assumed to be identical to Figure 1 for quantum yield measurements.

B. Stimulable Luminescence

The bioluminescence capacity as determined by MSL and CSL assay of G. catenella through one light-dark photoperiod is shown in Figure 2. This curve is characteristic of all eight clones isolated over a three year period from Yaquina Bay, Oregon, and of G. acatenella and G. tamarensis as well. There are, however, statistically significant differences in the quantum yield depending on the clone and species (Table 2). All cultures were internally consistent

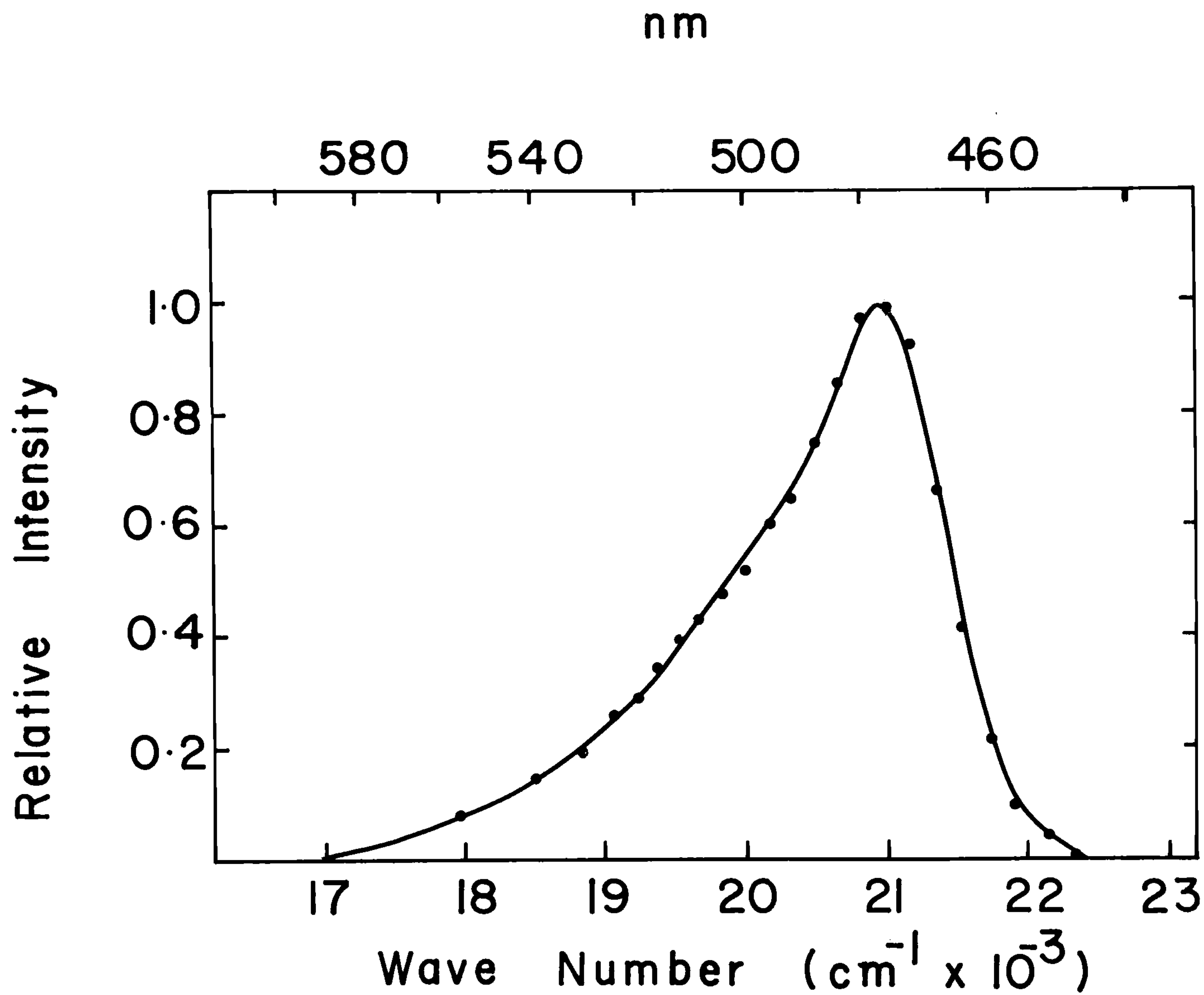

Figure 1. Bioluminescence emission spectrum of Gonyaulax catenella. Relative photons per second per unit wavenumber as a function of wavenumber.

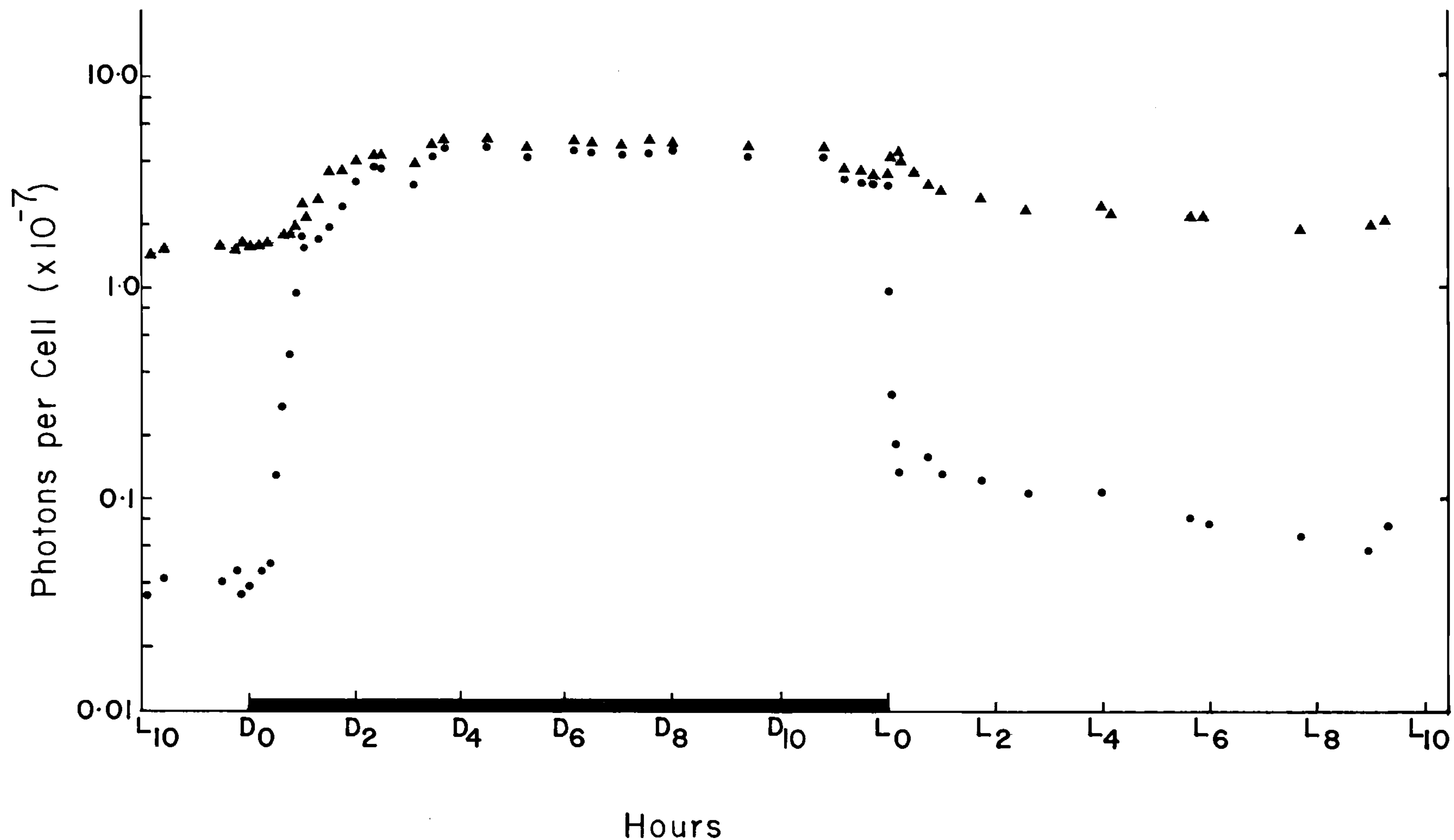

Figure 2. MSL and CSL of Gonyaulax catenella for one LD 12:12 photoperiod. ●, MSL, 30 sec stirring. ▲ CSL assayed with acetic acid following stirring. Each point represents a separate sample.

TABLE 2. Total quanta per organism of dinoflagellate bioluminescence, LD 12:12 photoperiod

Organism	Photophase		Scotophase	
	CSL*	MSL‡	CSL*	MSL‡
	$\times 10^{-7}$		$\times 10^{-7}$	
G. catenella	1.5 ± .09	0.043 ± .007	6.2 ± .5	2.0 - 5.9[+]
G. acatenella	2.2 ± .12	0.050 ± .008	9.1 ± .8	3.1 - 8.8[+]
G. tamarensis	1.0 ± .08	0.025 ± .008	3.6 ± .4	1.5 - 3.5[+]
G. monilata	2.8 ± .19	0.11 ± .03	10.2 ± .8	5.5 ± 0.7
Ceratium fusus	23.0 ± 5.0	0.97 ± .1	53 ± 10	15.3 - 47[+]
Peridinium pentagonum	488 ± 92	390 ± 91	500 ± 90	420 ± 80

* Acetic acid injection, final pH = 5.7.

‡ 30 second stirring.

+ ranges of values due to variations in MSL with culture age. Other data give mean ± 1 s.d.

with constant MSL and CSL yields from D_2 through D_{12}. CSL yields were consistently higher than MSL yields. Under the regular LD 12:12 photoperiod the decline of MSL following L_0 was extremely rapid in the catenella group members, having half times of approximately 50 seconds.

Scotophase: photophase ratios of MSL for the catenella group were on the order of 100 for all isolates and cultures in log phase growth. Scotophase:Photophase ratios of CSL were much lower, of the order of 3, and were independent of the chemical used provided concentrations were optimal. The MSL:CSL ratio during scotophase decreased with culture age in the catenella group from unity two days after inoculation (200 cells/ml) to about 0.3 in stationary phase cultures (ca. 20,000/ml) as the result of a decrease in MSL/cell. The CSL remained constant until senescence. The decrease in MSL/ cell is accompanied by an increase in the percentage of rugose and malformed cells in a linear fashion (Figure 3). We have also observed this effect with cultures of Ceratium fusus. Dupuy (1968) recorded increases in rugose cells in aged cultures of a catenella group species, and several authors have noted malformed cells and supernumerary horns in older cultures of Ceratium (Barker, 1925; Nordli, 1957; Steidinger and Williams, 1970).

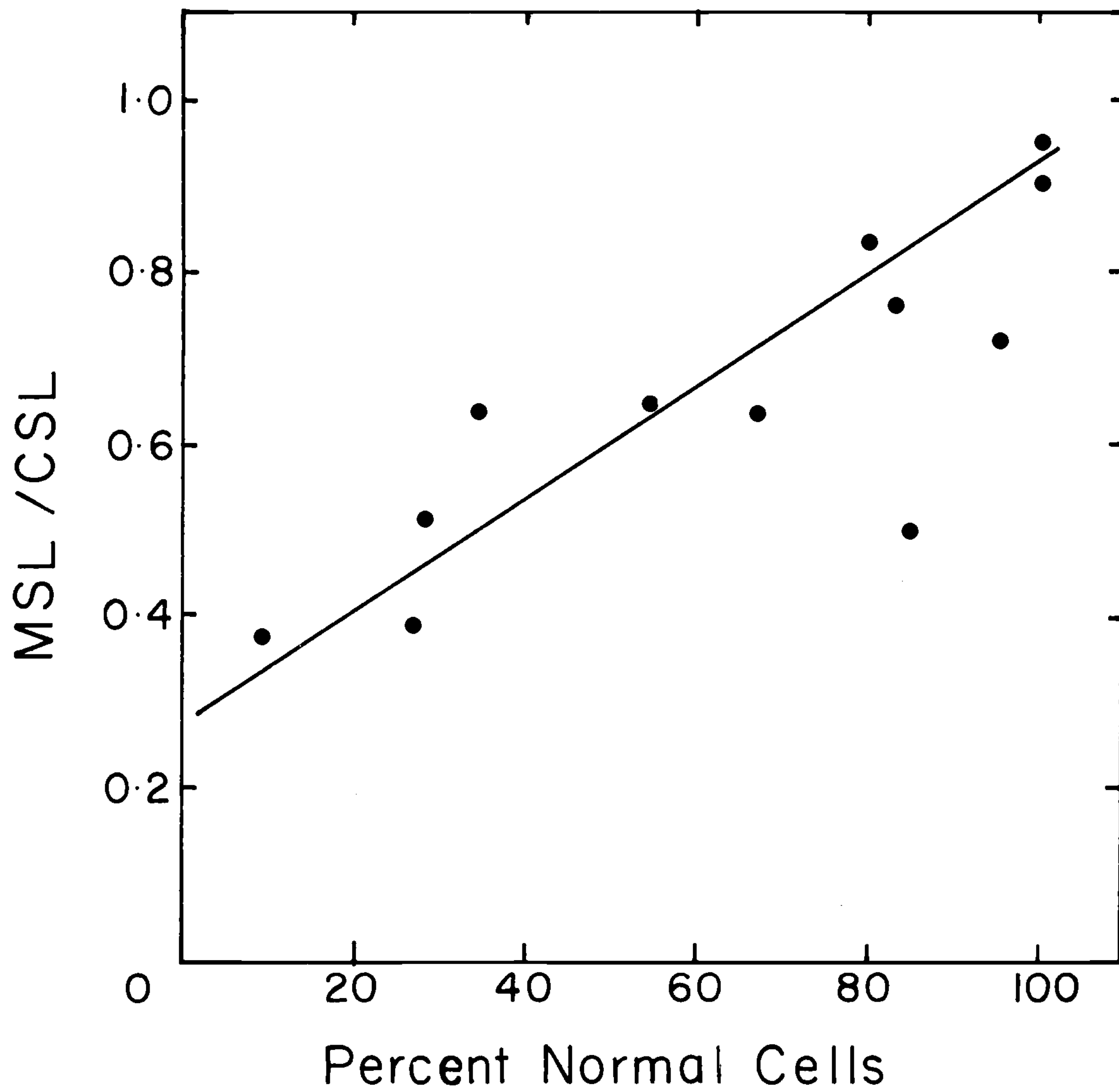

Figure 3. MSL/CSL ratio as a function of the percent of normal cells as opposed to rugose cells encountered in random fields of a Sedgewick-Rafter counting chamber.

C. Kinetics of Elicited Bioluminescence

A detailed study of emission kinetics as a function of degree of stimulation must await a refinement of mechanical stimulation techniques in order to quantify the shear stress experienced per cell (Hamman and Seliger, 1972).

The following qualitative observations are useful in an understanding of the process of photoinhibition. During scotophase the peak flash height resulting from stirring stimulation of the *catenella* species becomes apparent at $D_{+10'}$, increases rapidly to about D_4, and then more slowly until D_{12} ($D_{12}=L_o$). This is very similar to the response observed for *G. polyedra* (Biggley *et al.*, 1969) although a constant increase in the flash height was not observed in the latter species.

During scotophase, injection of acetic acid at optimum concentrations resulted in a rapid flash due entirely to injection turbulence (verified by injecting filtered medium as a control), followed by a slower emission which reaches a peak in 3 - 4 seconds. Light emission is essentially complete within 15 seconds. Injection of calcium chloride at optimum concentration resulted in a single rapid flash which was indistinguishable from the mechanically-induced flash. During photophase or upon photoinhibition during scotophase the mechanically-induced rapid flash was entirely absent for both the

catenella group and for G. polyedra, but not for P. bahamense. Under these conditions acetic acid stimulation yielded only the "slow" emission. Calcium stimulation, however, yielded the normal rapid flash. The flash kinetics upon calcium stimulation were optimal and independent of Ca^{++} concentrations above a threshold value. Increasing acid concentrations above an optimum final pH value of 5.4 gave more rapid rates of emissions but with decreased value of CSL.

Values of MSL during scotophase, photophase, and periods of photoinhibition were very dependent upon the degree of turbulence (shear force) experienced by the cells. As was also found by Hamman and Seliger (1972), chemical stimulation subsequent to mechanical stimulation of photoinhibited cells resulted in additional light emission. Extremely vigorous shaking by hand after stirring resulted in further light emission. The vigorous shaking decreased the additional luminescence resulting from subsequent chemical stimulation to nearly zero. This observation has also been made on stationary phase cultures which exhibit low MSL:CSL ratios during normal scotophase. Although some damage occurs during this vigorous shaking, these observations indicate that the entire luminescence capacity of the cell remains available. The aging and photoinhibition processes presumably raise the threshold of shear force required to trigger the bioluminescent system.

D. Photoinhibition of MSL

Exposure of scotophase cells of the catenella group to short pulses of low-intensity light resulted in a rapid and reversible decrease in the gently MSL after a short but reproducible lag (1 min), while the CSL and MSL (maximum shear) yields per cell remained constant (Figure 4). The relative decrease in gently MSL resulting from inhibition during scotophase is identical to the observed for TML following L_0 at photophase intensities, and is similar to that observed by Kelly and Katona (1966) and Kelly (1968) on unidentified natural populations.

The rate constants of photinhibition as a function of total exposure (quanta per square centimeter for various exposure times) for several representative wavelengths are shown in Figure 5, demonstrating the linearity of the effect at low fluxes and the resulting range of reciprocity of intensity and exposure time.

The slopes of the decay rate constant vs. exposure determined by the single point technique were plotted as a function of wavelength to yield the action spectrum for photoinhibition of MSL as shown in Figure 6 for G. catenella. Action spectra derived for two additional clones of this species and for cultures of G. acatenella and G. tamarensis gave the same single maximum near 562±8 nm with a half-band-width of about 105 nm. Figure 6 also shows the action

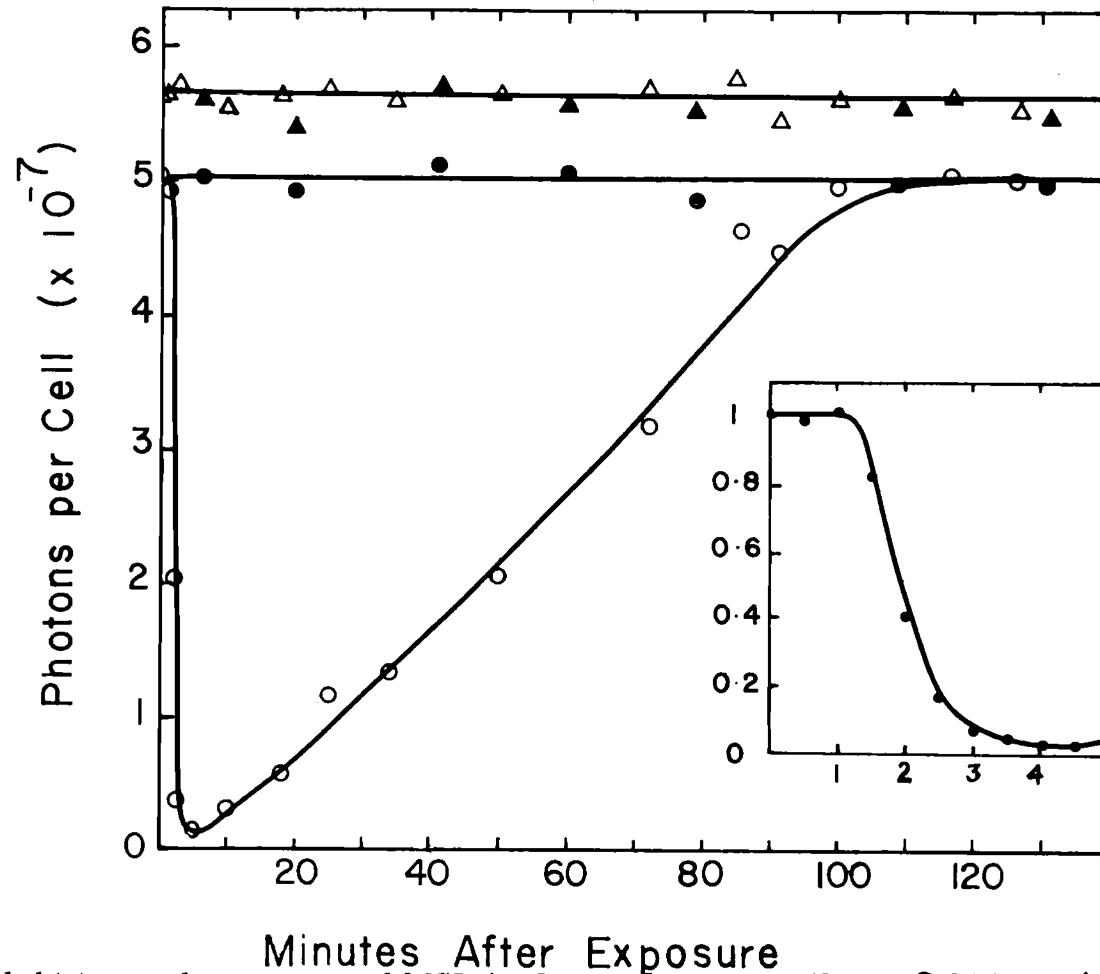

Figure 4. Photoinhibition and recovery of MSL in Gonyaulax catenella. ○ MSL, △ CSL of cells exposed at D_4 to 4 x 10^{12} quanta cm^{-2} sec^{-1} at 550 nm for 15 sec. ● , MSL, ▲ , CSL of controls. Insert-- relative MSL of samples exposed as above with an expanded time scale to indicate the presence of a time lag for photoinhibition.

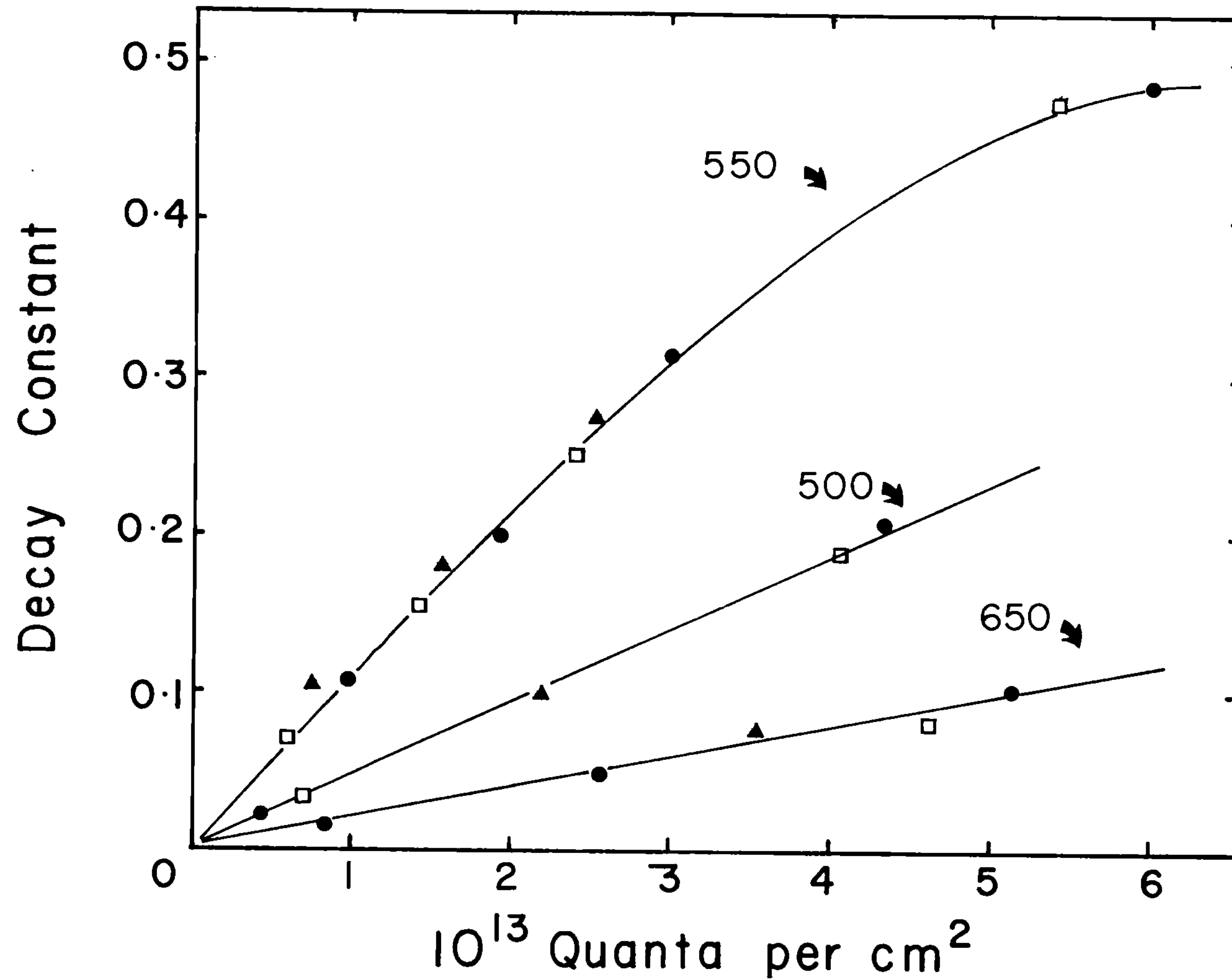

Figure 5. First order decay constants of MSL as a function of exposure in photons per square centimeter. The single point procedure was used. Exposure times - ●, 0.1 sec; □, 0.5 sec; ▲, 0.125 sec.

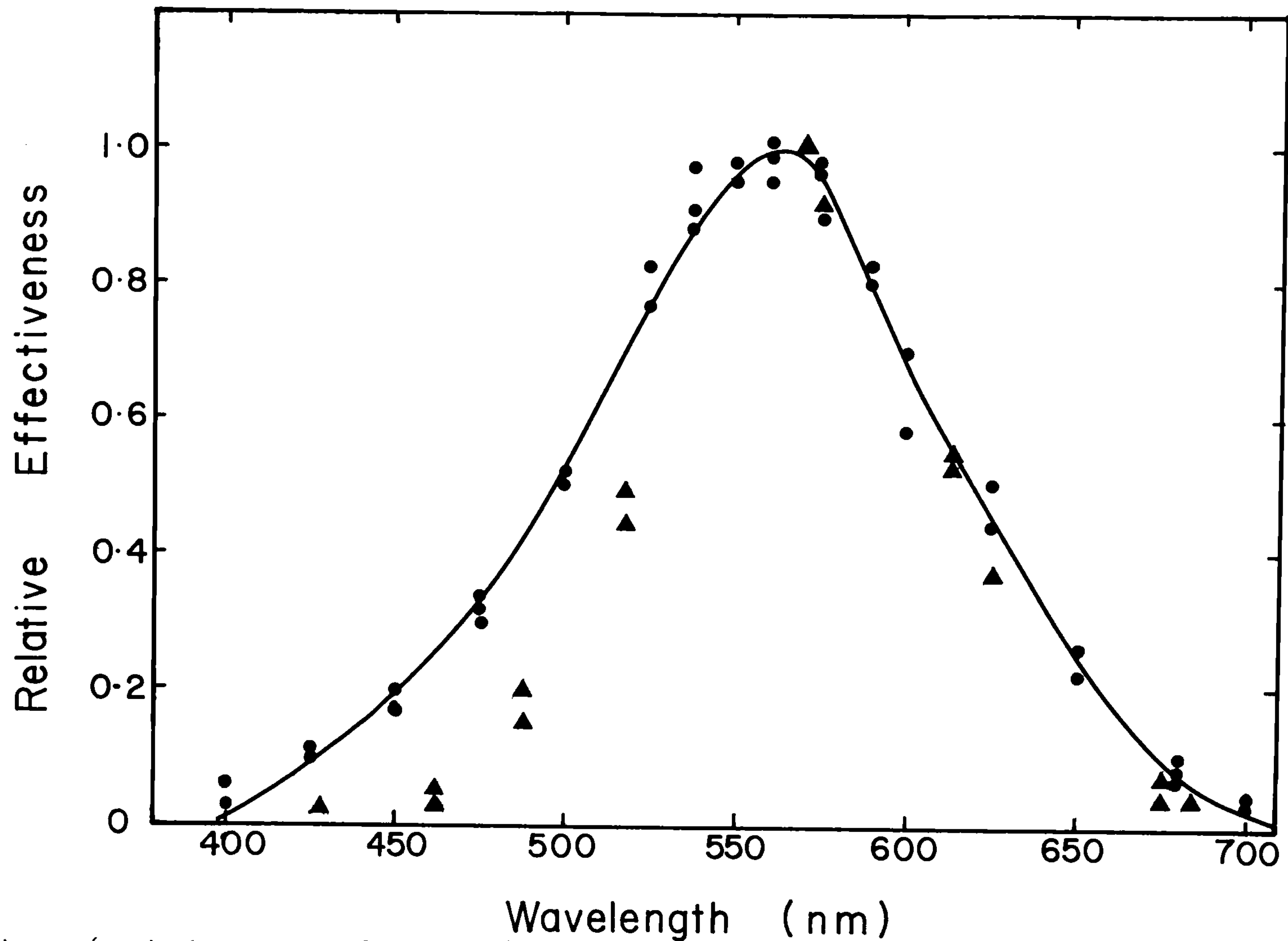

Figure 6. Action spectra for low light intensity, rapid photoinhibition of MSL in <u>Gonyaulax catenella</u>. ● , single point procedure, normalized to 560 nm. ▲ , continuous assay procedure, normalized to 570 nm.

spectrum for *G. catenella* photoinhibition developed by the continuous assay procedure which yielded a maximum at a slightly longer wavelength (570 nm) with a slightly narrower half-band-width.

The magnitude of the maximum inhibition of gently MSL was also dependent upon the spectral quality and total exposure (Figure 7).

Exposures made in the UV to 325 nm and in the red to 775 nm indicated no further pigment involvement. Attempts to reverse the inhibition by immediate re-exposure to red or far-red radiation were unsuccessful.

This rapid-response, low-intensity photoinhibition effect was not observed for *C. fusus*, *G. polyedra*, *P. bahamense* and *Peridinium pentagonum*. *G. monilata* showed decreases in both MSL and CSL following exposure to 10^{18} quanta/cm^2 at 550 nm as compared with 10^{13} quanta/cm^2 for *G. catenella* but with a much slower decay half-time (5 min). Unfortunately, this culture was lost before the initial results could be repeated.

The action spectrum for photoinhibition of mechanically stimulated bioluminescence in *G. polyedra* (Sweeney *et al.*, 1959) showed maxima at 440 and 678 nm, and virtually no activity at 560 nm. Cellular absorbance at 560 nm is relatively much greater in *G. catenella* than in *G. polyedra* as seen in Figure 8. The difference spectrum also shown in Figure 8 indicates that *G. catenella* contains a major pigment in the same region as the action spectrum for rapid

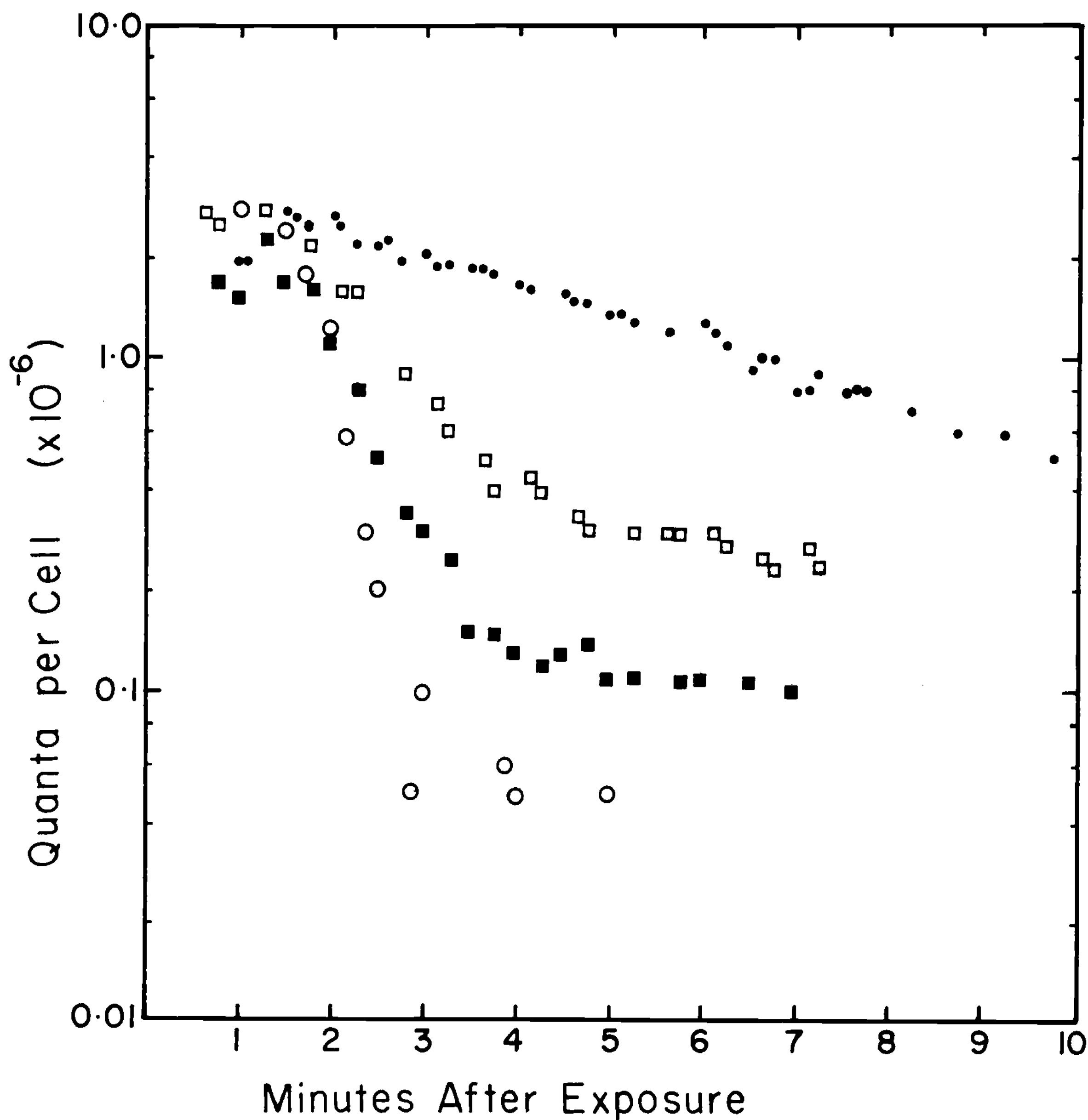

Figure 7. Photoinhibition of gently mechanically stimulable luminescence determined with the continuous assay procedure. The ordinate gives the total quanta emitted during each 9 sec of gentle stimulation. The abscissa gives the time in minutes following the beginning of the 10 sec exposure. ● unexposed controls; ○ 37 x 10^{12}, ■ 18.5 x 10^{12}, □ 9.25 x 10^{12} quanta per square centimeter respectively at 571 nm.

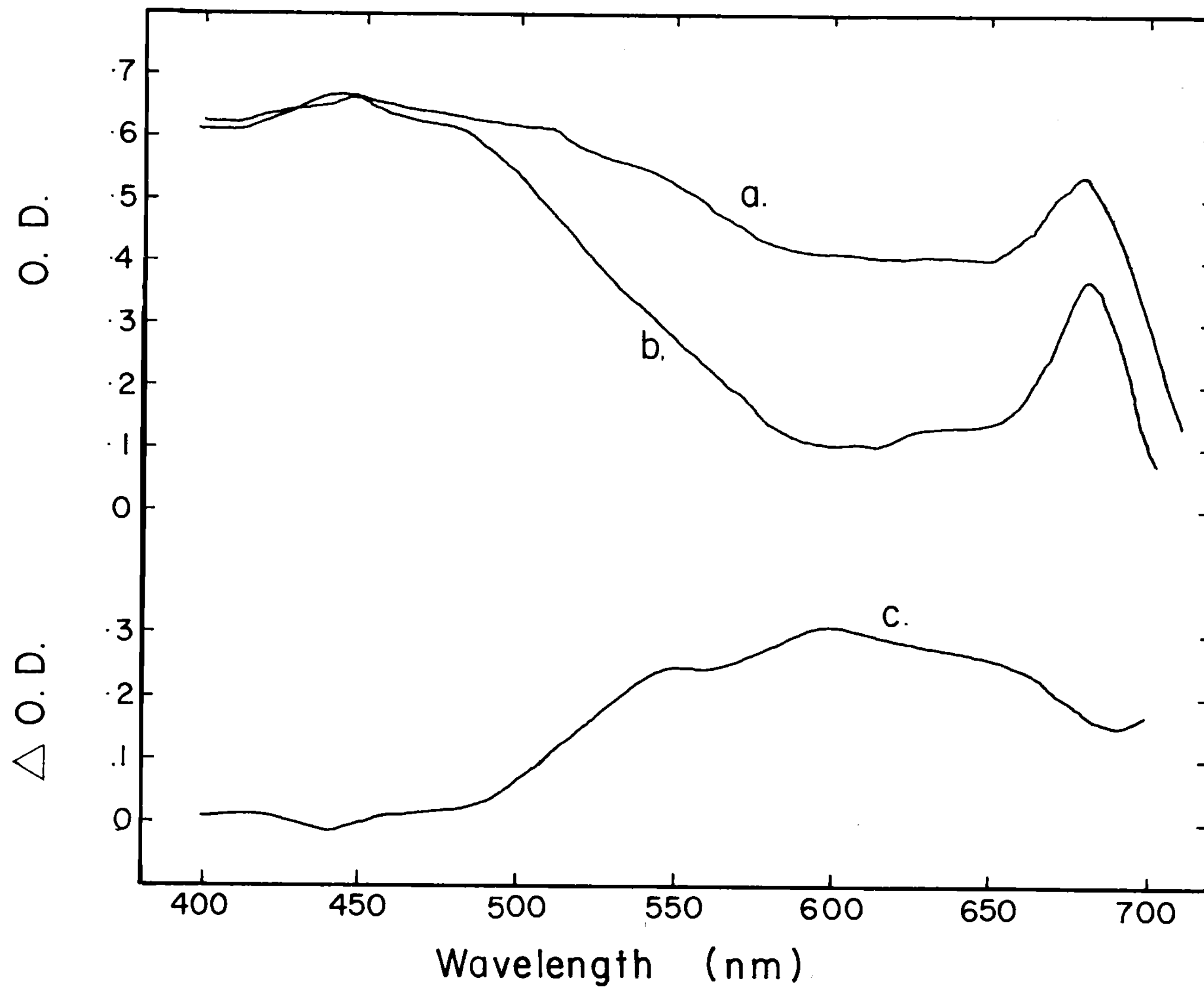

Figure 8. a) Relative absorbance spectra of cultures of Gonyaulax polyedra and G. catenella, normalized at 450 nm, and b) the difference spectrum.

photoinhibition. This may well be the sensitizing pigment for the effect we have shown here.

CONCLUSIONS

All of the photosynthetic dinoflagellates studied show a constant MSL during scotophase and orders of magnitude variations in the ratios of MSL between scotophase and photophase.

The low-intensity rapid photoinhibition of scotophase MSL found in the *Gonyaulax catenella* group is distinctly different from the photophase photoinhibition of *G. polyedra*, *G. monilata*, and other species in terms of kinetics, absolute spectral intensity requirements and shear sensitivity. This supports the taxonomic distinction of the *catenella* group from *Gonyaulax sensu stricto* (Steidinger and Williams, 1970).

The action spectrum implicates a sensitizing pigment other than chlorophyll. Peridinin has been found to be the predominant carotenoid in the Pyrrhophyta (Strain *et al.*, 1944; Loeblich and Smith, 1968) and a peridinin-protein complex has been isolated from *G. polyedra* (Haidak *et al.*, 1966). However this complex had an absorbance maximum at 480 nm. A preliminary analysis of the carotenoids of *G. catenella* revealed that peridinin was the major component, with dinoxanthin, diadinoxanthin and β carotene in lesser amounts.

The low-intensity rapid photoinhibition action spectrum is quite

different from the action spectrum of phototaxis in G. catenella, which showed a single band with maximum activity at 475 nm (Halldal, 1958), but agrees qualitatively with that found for Prorocentrum micans.

From the time lag between exposure and the onset of photoinhibition it follows that at least one photochemical intermediate is present. The rapid flash and CSL obtained by Ca^{++} stimulation of photoinhibited cells makes it unlikely that the rapid photoinhibition involves the scintillon directly (Fogel and Hastings, 1972; Hamman and Seliger, 1972).

The similarity between age-induced rugosity of cells and photoinhibition, where both require higher shear stresses for mechanical stimulation, suggests that both processes involve the cell membrane. The mechanism whereby mechanical stimulation triggers luminescence is not known.

Comparatively, non-photosynthetic dinoflagellates such as Peridinium pentagonum (Harvey, 1926; Hamman and Seliger, 1972), and Polykrikos (Hamman and Seliger, 1972), ctenophores (Harvey, 1925), and sea pansies (Kreiss and Cormier, 1967) do not show photoinhibition of the mechanical receptor apparatus.

The spectral intensity dependence of photoinhibition is of importance ecologically for several reasons. If all of the 562 nm light falling on G. cantenella were absorbed, the data of this paper imply that only 10^8 quanta are required for complete photoinhibition,

coincidently the same as the number of quanta emitted upon complete mechanical stimulation during scotophase. Since mechanically stimulable bioluminescence has been shown to have value in reducing copepod predation on dinoflagellates (Esaias and Curl, 1972), the photoinhibition process may be viewed as a means of conserving energy when ambient light levels are sufficiently high to render the bioluminéscent flash ineffective as a definsive display.

Acknowledgements

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IV. EFFECTS OF DINOFLAGELLATE BIOLUMINESCENCE ON COPEPOD INGESTION RATES[1]

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ABSTRACT

Grazing experiments were conducted with three calanoid copepods and three species of bioluminescent dinoflagellates, using procedures which yielded samples of cultures with high and low capacities for stimulable bioluminescence. In all cases, the ingestion rates were lower for the high bioluminescence capacity samples than for the samples having a reduced bioluminescence capacity. These results indicate that dionflagellate bioluminescence has survival value as a defense against copepod grazing. Of several possible mechanisms, we propose that the flash is a visual, protean display which startles or confuses the copepod sufficiently to allow the dinoflagellate to escape. The net evolutionary value is that predation would be reduced on a dinoflagellate population as a whole.

INTRODUCTION

Dinoflagellates are responsible for most of the bioluminescence observed in the surface layers of the oceans (Kelly, 1968; Tett, 1971), yet the adaptive significance of their flashes is not obvious. Since many of the bioluminescent dinoflagellates are primary producers, probably all serve as food for the smaller zooplankton, and because many of these organisms are extremely toxic to vertebrates it is desirable that we know the value of their bioluminescence and its ecological significance.

Under natural conditions all bioluminescent species emit brief flashes of blue light at night in response to mechanical stimulation, which need only be as slight as the turbulence produced by a small air bubble popping. The capacity for bioluminescence remains constant throughout the night period, and is completely restored in one hour after exhaustive stimulation (Biggley _et al._, 1969). Values of total stimulable luminescence (TSL) range from 10^7 to 10^{10} photons per cell depending on the species (Seliger _et al._, 1969).

Kelly (1968) and Kelly and Katona (1966) have indicated that the characteristics of dinoflagellate bioluminescence strongly suggest that it has a selective advantage. Burkenroad (1943) has proposed that the flash functions as a "burglar alarm" in which the flash, emitted as a result of herbivore feeding activity, would enable a

carnivore to perceive and capture the herbivore at night, thereby benefiting the dinoflagellates on a species level. We chose to examine the direct dinoflagellate - herbivore interaction in copepod grazing experiments, testing the effect of bioluminescence on the rate of cell ingestion.

We thank Dr. W. G. Pearcy for critically reading the manuscript, Dr. E. Sutton for a culture of G. acatenella, and Ms. J. Flynn and Mr. H. O'Connors for help in identification and collection of copepods.

METHODS

Unialgal cultures of photosynthetic dinoflagellates were grown in enriched seawater medium under cool-white fluorescent lights (2600 μw cm^{-2}) at 23° C (Gonyaulax polyedra Stein) or 15° C (Gonyaulax catenella Whedon & Kofoid, Gonyaulax acatenella Whedon & Kofoid, Gymnodinium sp.).

Cell concentrations were determined with a coefficient of variation of less than 8% by making lengthwise scans of a Sedgewick-Rafter chamber for up to six replicates. Assays of bioluminescence capacity were performed on 3 ml subsamples of cultures using a motor-driven stirrer and a photomultiplier photometer in the manner of Biggley et al. (1969). Undiluted cultures four to eight days old were used for grazing, since the shock resulting from large dilutions

decreased motility of the cells and gave non-uniform distributions in the grazing vessels.

Copepods (Calanus pacificus Brodsky, Acartia clausi Giesbrecht, and Acartia longiremis Lilljeborg) were obtained at frequent intervals from Yaquina Bay, Oregon and kept in dim light at 15° C. Only mature females of a given species were used. They were sorted in a Bogarov tray and placed in filtered sea water for 24 hours prior to grazing experiments. Calanus pacificus was kept at 23° for three days prior to the experiments with G. polyedra.

The ingestion rates of copepods were determined by measuring the decrease in cell concentration of a culture sample containing copepods relative to a non-grazed sample over a ten hour period. A rate was calculated in terms of cells ingested per copepod per hour according to the formula

$$I.R. = \frac{(Cc - Cg)V}{N\,t}$$

where C_c and C_g are the cell concentrations in the control and grazed samples respectively, N the number of copepods, V the volume of sample and t the time in hours. It has been shown that copepod grazing rates are very dependent upon the age, size, sex and species of copepod, and also upon the age, size, shape, species and previous history of the food organism, ambient illumination, temperature, and the length of the grazing period. Procedures were developed which

kept these factors constant for a given experiment and allowed us to examine solely the effect of dinoflagellate bioluminescence on the calculated ingestion rate.

The first procedure involved manipulation of the endogenous circadian rhythm exhibited by the genus *Gonyaulax* (Sweeney and Hastings, 1957). Cultures grown for several days under continuous bright light become arhythmic, but the rhythm can be expressed at any time by placing them in darkness. This induces a twelve hour phase of high bioluminescence capacity and stimulability (D_0-D_{12}). The bioluminescence capacity decreases rapidly from twelve to fourteen hours (D_{12}-D_{14}), and then remains fairly constant until 24 hours in darkness (D_{14}-D_{24}), with ratios of Total Mechanically Stimulable Luminescence (TMSL, D_{14}-D_{24}:D_2-D_{12}) on the order of 0.15-0.3 for the organisms we used. We divided a large arhythmic culture into two series of samples for grazing, non-grazed controls, and bioluminescence capacity assays. One series (A) was placed in darkness twelve hours before the second (B). Equal numbers of copepods (10-20/500 ml) were added to the grazing samples of each series 14 hours after the A series was placed in darkness. Thus, while grazing occurred simultaneously, the A series samples were in the second phase of the bioluminescence capacity cycle (D_{14}-D_{24}) and had a lower bioluminescence capacity than the B series samples, which were in the first phase of the cycle (D_2-D_{12}).

At the end of the grazing period (about 10 hours) the animals were removed and subsamples taken for cell counts. The bioluminescence capacity of each series was monitored throughout the dark period. On several occasions the bioluminescence resulting from copepod activity in the grazing vessels was monitored visually, or electronically by placing the photomultiplier above the grazing vessel for 10 minute periods in the middle of the grazing period.

The second procedure used the rapid decrease in sensitivity to mechanical stimulation due to brief exposure to dim lights, which we have found in G. catenella and G. acatenella (Esaias et al., 1973, in prep.) in order to provide two samples of a single culture, one having a normal and the other a reduced bioluminescence capacity. We divided cultures of dinoflagellates grown on normal 12 hour light-dark cycles (LD12:12) into two series in the middle of photophase (light cycle). Grazing occurred during the normal scotophase (dark cycle, D_2-D_{12}) with one series in complete darkness, the other in darkness interrupted by 5 second pulses of white light (200 μw cm^{-2}) every 30 minutes. The pulses were sufficient to maintain the stimulable bioluminescence capacity of these samples at 10^{-2} - 10^{-1} that of the normal scotophase cells in complete darkness. Cultures of the non-bioluminescent dinoflagellate Gymnodinium sp. were treated identically with the bioluminescent species to assess the effect of the light pulses on ingestion rates.

RESULTS

Ingestion rates were determined for four copepod - dinoflagellate combinations according to the first procedure (Table 1, Figure 1). In all cases the copepods ingested fewer of the dinoflagellates in the B series, which had a higher bioluminescence capacity than the A series over the range of cell concentrations used. Since grazing is dependent upon the size and shape of the food organism, we measured the average diameters of cells (ca. 29μ) and the percentage of double cells (ca. 11%) resulting from cell division in both series before and after the grazing periods. We could find no significant differences in the means between series using the Student "t" test (P 0.01). In diatom grazing experiments, completely uniform distribution of food organisms are usually insured by stirring or use of rotational devices, but would have stimulated bioluminescence in these studies. However, the motility of the dinoflagellates in darkness prevented large-scale settling, and we could detect no differences in the distributions between the series either visually or by cell counts taken at various positions in the 1000 ml jars as grazing vessels.

Results of an experiment using the second procedure are shown in Table 2. The copepods, Acartia clausi in this case, consumed approximately equal numbers of Gymnodinium both in

TABLE 1. Ingestion rates of copepods and dinoflagellate bioluminescence during a photoperiod controlled experiment.

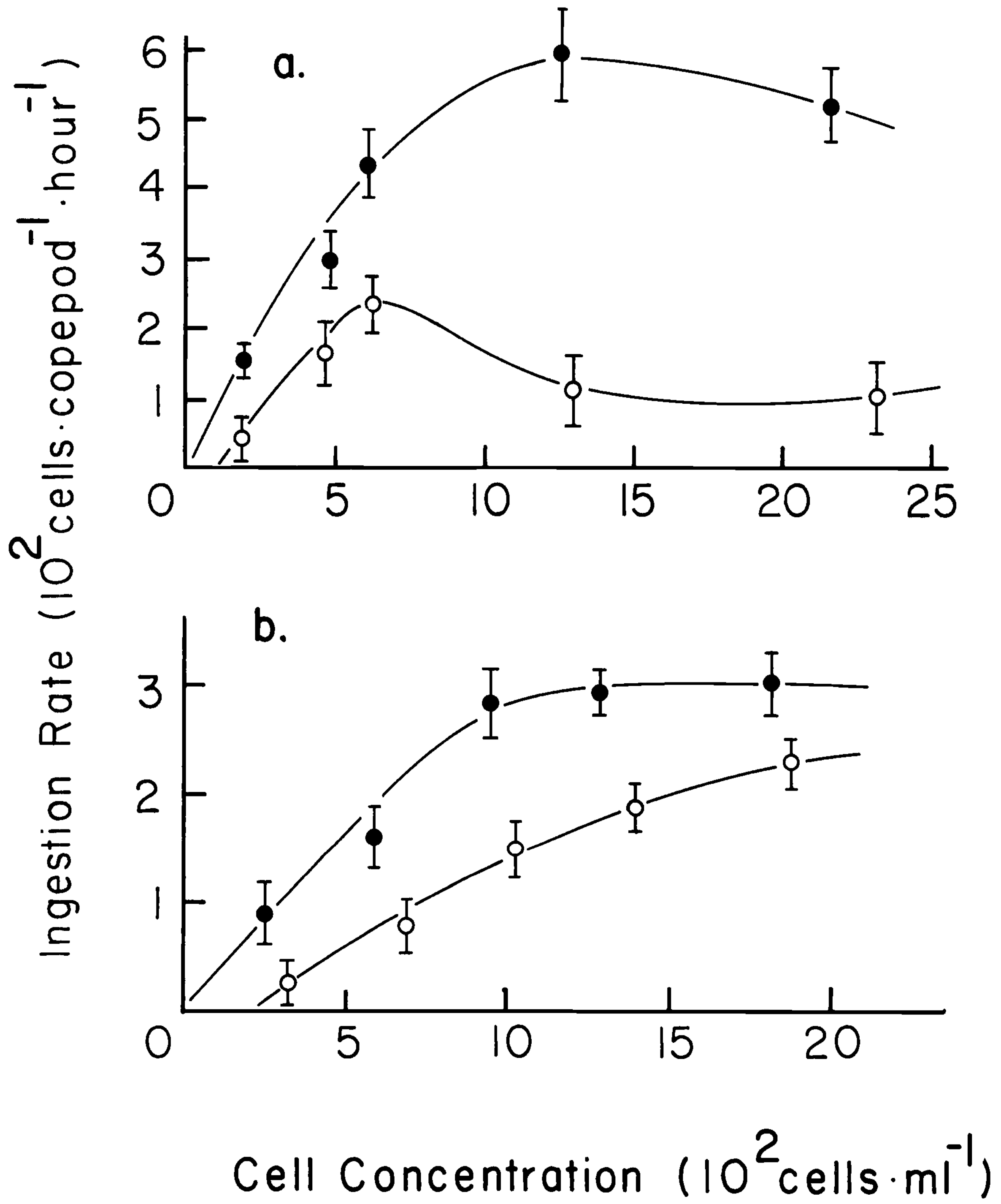
Copepod	Dinoflagellate	A series TMSL*	A series CSL+	A series I. R. #	B series TMSL*	B series CSL+	B series I. R. #
C. pacificus (15/400ml)	*G. polyedra* (600/ml)	4.0 ± 0.6	-	876 ± 85	12 ± 1.5	-	610 ± 45
A. clausi (22/400ml)	*G. acatenella* (1350/ml)	2.3 ± 0.2	0.29	284 ± 17	8.3 ± 0.9	1.0	182 ± 14
A. clausi (20/400ml)	*G. acatenella* (620/ml)	2.8 ± 0.4	-	150 ± 27	8.4 ± 1.2	-	87 ± 19
A. longiremis	*G. acatenella*	2.9 ± 0.3	0.36	811 ± 48	8.5 ± 1.5	1.0	291 ± 78

* Total mechanically stimulable bioluminescence from assays of 20 samples, values in 10^7 quanta$\cdot$ cell^{-1} for 15 sec stimulation, ± SD.

\+ Copepod stimulated bioluminescence - relative light detected photometrically from one grazing vessel over 10 min. periods. B series = 1.0

Averaged Ingestion Rate (cells copepod^{-1} hr^{-1}) for three determinations ± SD.

Grazing periods were 10 ± 0.5 hrs., Temperature 15 ± 0.5° C. except for experiment with *G. polyedra* where T = 23 ± 0.5° C.



a.
b.
Ingestion Rate (10^2 cells · copepod^{-1} · hour^{-1})
0
1
2
3
4
5
6
5
10
15
20
25
Cell Concentration (10^2 cells · ml^{-1})

TABLE 2. Ingestion rates of Acartia clausi (20/400 ml) and bioluminescence of dinoflagellates during a photoinhibition controlled experiment.

Dinoflagellate	cells ml^{-1}	Light Pulses			Complete Darkness		
		TMSL	CSL*	I. R.	TMSL	CSL*	I. R.
G. acatenella	345	0.092	0.005	171 ± 23	8.4 ± 0.9	0.42	45 ± 26
	986	0.085	0.011	372 ± 50	8.1 ± 0.9	1.0	133 ± 34
Gymnodinium	270	0	0	140 ± 28	0	0	149 ± 27
	1040	0	0	397 ± 67	0	0	375 ± 57

Grazing periods were 10 ± 0.5 hrs., Temperature 15 ±0.5° C. Abbreviations same as for Table 1.

*CSL normalized to the higher cell concentration in complete darkness, averaged over two 10 min. periods beginning 5 min. after light pulse, in the fifth hour of grazing.

darkness and with pulsed light, but consumed only one fourth as many of the highly bioluminescent G. acatenella in the dark samples as in the photoinhibited samples. Again we could find no significant differences in cell size, percent double cells, or dinoflagellate distributions between the two series using the Student "t" test (P 0.01).

Observations of grazing samples at high dinoflagellate concentrations revealed two distinct patterns of stimulated bioluminescence. Swimming movements of copepods produced a streak of light of very short duration, while feeding motions occurring when the copepod was nearly stationary produced a bright spot of light lasting several seconds due to continuous stimulation of a constantly renewed supply of dinoflagellates. These patterns were distinctly evident when the samples were monitored with the photometer. With a single copepod at dinoflagellate concentrations lower than 10/ml it became very difficult to locate the copepod by the luminescence it evoked because of "noise" produced by random spontaneous flashing of the dinoflagellates.

DISCUSSION

General relationships between ingestion rates and cell concentration are similar to those found in studies using diatoms (Mullin, 1963; Conover, 1966; Frost, personal communication), in which the rate of cell ingestion increases with cell concentration to a maximum

value. Our data indicate that a capacity for bioluminescence decreases the rate at which cells are ingested throughout the ranges of concentration for _C. pacificus_, and at low to medium concentrations for _Acartia_ species. The difference in ingestion rates between normal and photoinhibited cells was somewhat greater than the differences obtained by the first procedure, which may be due the greater decrease in stimulable bioluminescence capacity in the second method.

The low rates of ingestion found in association with high bioluminescence capacity indicate that stimulated bioluminescence in dinoflagellates has survival value as a defense against copepod grazing. The exact mechanism for this effect is unclear at present. In high concentrations of bioluminescent dinoflagellates, flashing produced by the copepods may produce an ambient light level which could influence their behavior. We would expect this effect to be greatest at high cell concentrations as in Figure 1a. Natural dinoflagellate concentrations are more commonly on the order of 10 - 500/liter, much less than can be used in grazing experiments of this type, and the probability of a copepod encountering a bioluminescent dinoflagellate is much less than for the more abundant non-bioluminescent phytoplankters. We propose that the flash resulting from the filtration action might act as a visual "protean display" (Chance and Russell, 1959; Humphries and Driver, 1967) or an unexpected response by a prey organism which serves to startle or confuse the

predator and allows the prey to escape. At night the sudden flash of the dinoflagellate could have an effect on light sensitive herbivores due to the small distances involved. If we assume that a flash of 0.1 second duration comprised of 10^7 photons occurs when a single dinoflagellate is filtered by the feeding appendages of an Acartia, the energy incident upon the eye 0.2 mm away is on the order of 10^{-3} $\mu w\ cm^{-2}$, well above the apparent threshold of stimulation of other crustacean napliar eyes, which are usually sensitive in the blue-green regions of the visible spectrum (Waterman, 1961). The motility of the dinoflagellate in this interaction may be important, although copepods can reject unwanted particles at will.

Protean displays depend upon a low encounter frequency between predator and prey for effective action, and become ineffective at high encounter frequencies when the response is less unexpected, which may explain the decreasing effect of bioluminescence with increasing cell concentration shown in Figure 1b.

Bioluminescent radiolarians also emit flashes upon mechanical stimulation, which led Brandt (1885) to propose that their display served as a "schreckmittel" to frighten predators away. A protean display function might be a secondary function of bioluminescence in other organisms which respond to mechanical stimulation by flashing and thereby maintain the bioluminescent capability despite the fact that there may be no present selective advantages with respect to a

primary, metabolic function (McElroy and Seliger, 1962).

We have not tested Burkenroad's burglar alarm hypothesis, but at commonly occuring dinoflagellate concentrations it seems unlikely that a predator could determine the position of a herbivore from a single brief dinoflagellate flash sufficiently well to enable successful attack.

It is possible that the effect shown here could be a factor in the formation of red-tides and permanent blooms of bioluminescent dinoflagellates, since zooplankton grazing is recognized to be a significant factor in phytoplankton population dynamics.

Although it is difficult to explain the co-occurrence and often equal abundance of non-bioluminescent dinoflagellates, bioluminescent species generally constitute a majority in those genera which contain bioluminescent members, even though all species have yet to be tested for luminescence.

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APPENDICES

APPENDIX A. A COMPENDIUM OF BIOLUMINESCENT DINOFLAGELLATES

In the course of this investigation it became convenient to compile a listing of dinoflagellates which have been rigorously determined to be luminescent. Previous listings are scattered in the literature, have included very dubious accounts derived from casual associations of luminescence in sea water and dominant species, and errors due to incorrect transcriptions of prior listings (cf. Tett, 1971; and Kelly, personal communication, have reported C. dens and S. sweeneyi luminescent from Sweeney, 1963; although Sweeney, 1963, reports both as non-luminescent).

This listing reports only those which have been found to be luminescent either in culture, or in single cell isolation and subsequent identification. When available, the total photon emission is also reported.

Table A-1. Bioluminescent Dinoflagellates.

Genus and species	References	Total Photon Emission		
		Value	Reference	Source
Ceratium fusus	3,4,6,7,10(13)	$8.7 \pm 2.9 \times 10^8$	-3	(i, c)
Gonyaulax acatenella	3	$9.1 \pm 0.8 \times 10^7$	-3	(c)
G. balechi	9	--		
G. catenata	13	--		
G. catenella	3,10	$6.2 \pm 1.2 \times 10^7$	-3	(i, c)

Table A-1. continued

Genus and species	References	Total Photon Emission Value	Reference	Source
G. digitale	3, 6, 10	$2.1 \pm 1.1 \times 10^{7}$	-3	(i)
G. hyalina	10	--		
G. monilata	3, 10	$1.0 \pm 0.1 \times 10^{8}$	-3	(c)
G. polyedra	1, 10	$1.2 \pm 0.1 \times 10^{8}$	-8	(c)
G. sphaeroida	10	--		
G. spinifera	3, 6	$3.0 \pm 1.7 \times 10^{7}$	-3	(i)
G. tamarensis	3	$3.6 \pm 0.4 \times 10^{7}$	-3	(c)
G. washingtonensis ?	2	--		
Noctiluca scintillans	10	--		
N. Miliaris				
Peridinium brochi	10	--		
P. ceraseus	3	$4.8 \pm 3.0 \times 10^{8}$	-3	(i)
P. claudicans	3, 6 (10)	8.3×10^{8}	-3	(i)
P. conicum	3, 6, 10	$3.4 \pm 0.8 \times 10^{9}$	-3	(i)
P. crassipes	3	$8.7 \pm 3.1 \times 10^{9}$	-3	(i)
P. curtipes	6	--		
P. depressum	3, 6, 10, 13	$1.0 \pm 0.4 \times 10^{10}$	-3	(i)
P. divergens	13	--		
P. globulus	6	--		
P. granii	3, 6, 10	$8.1 \pm 2.0 \times 10^{8}$	-3	(i)

Table A-1. continued

Genus and species	References	Total Photon Emission Value	Reference	Source
P. leonis	3, 6	$3.3 \pm 1.1 \times 10^9$	-3	(i)
P. mite	3 (6)	3.8×10^9	-3	(i)
P. oceanicum	4, 6	--		
P. ovatum	3, 13	$1.0 \pm 0.3 \times 10^9$	-3	(i)
P. pallidum	6, 13	--		
P. pellucidem	3	$2.0 \pm 0.6 \times 10^8$	-3	(i)
P. pentagonum	3, 6, 1	$5.0 \pm 0.9 \times 10^9$	-3	(i, c)
P. punctulatum	6	--		
P. steinii	3, 13	$1.4 \pm 0.3 \times 10^8$	-3	(i)
P. subinerme	3, 6	$1.5 \pm 0.6 \times 10^9$	-3	(i)
Polykrikos schwarzii	5, 13 (3)	--		
Pyrocystis fusiformis	12	1.8×10^{10}	-12	(c)
P. (Dissodinium) lunula	11	$4.0 \pm 0.4 \times 10^9$	-8	(c)
P. pseudonoctiluca	12	$5\text{-}11 \times 10^{10}$	-12	(c)
Pyrodinium bahamense	1, 4	$2.8 \pm 0.5 \times 10^8$	-8	(c)

1. Biggley et al., 1969; 2. Dupuy, 1968; 3. Esaias and Curl, Section II; 4. Gold, 1965; 5. Hamman and Seliger, 1972; 6. Kelly, 1968; 7. Nordli, 1957; 8. Seliger et al., 1969; Steidinger, 1971, personal communication; 10. Sweeney, 1963; 11. Swift, 1967; 12. Swift et al., 1970; 13. Tett, 1971.

(i) = measured as individual cells

(c) = measured as culture samples

APPENDIX B. DESIGN OF THE PHOTOMULTIPLIER PHOTOMETER

Although there are a number of sensitive photometers on the market, none standardly incorporate the necessary features for recording both integrated and running intensity at reasonable prices. We decided to construct our own device using modular commercial units with a minimum of custom wiring. This instrument has proven to be very versatile, and incorporate several unique design features which might prove helpful to others in the field of bioluminescence.

A schematic of the device is shown in Figure 1, as a block diagram, and in Figure 2 as a more complete schematic. The 1P21 photomultiplier was mounted in a welded aluminum housing which was fastened to various black lucite sample tube holders. A standard linear dynode configuration was used. The anode current was fed into a Keithley 301K solid state electrometer amplifier. Feedback elements consisting of 10 and 100 Mohm resistors for intensity readings and 0.01 and 0.15 uf polystyrene capacitors for integrated light readings were mounted in a Keithley 3011 shielded switch. A zero-reset circuit automatically resets the capacitors at a presetable output voltage, and also allows the recorder, a Brush 220 oscillographic dual channel strip chart model, to be zeroed with unity gain. An Acopian dual regulated power supply provided power for the operational amplifiers and reset circuit. Negative regulated high

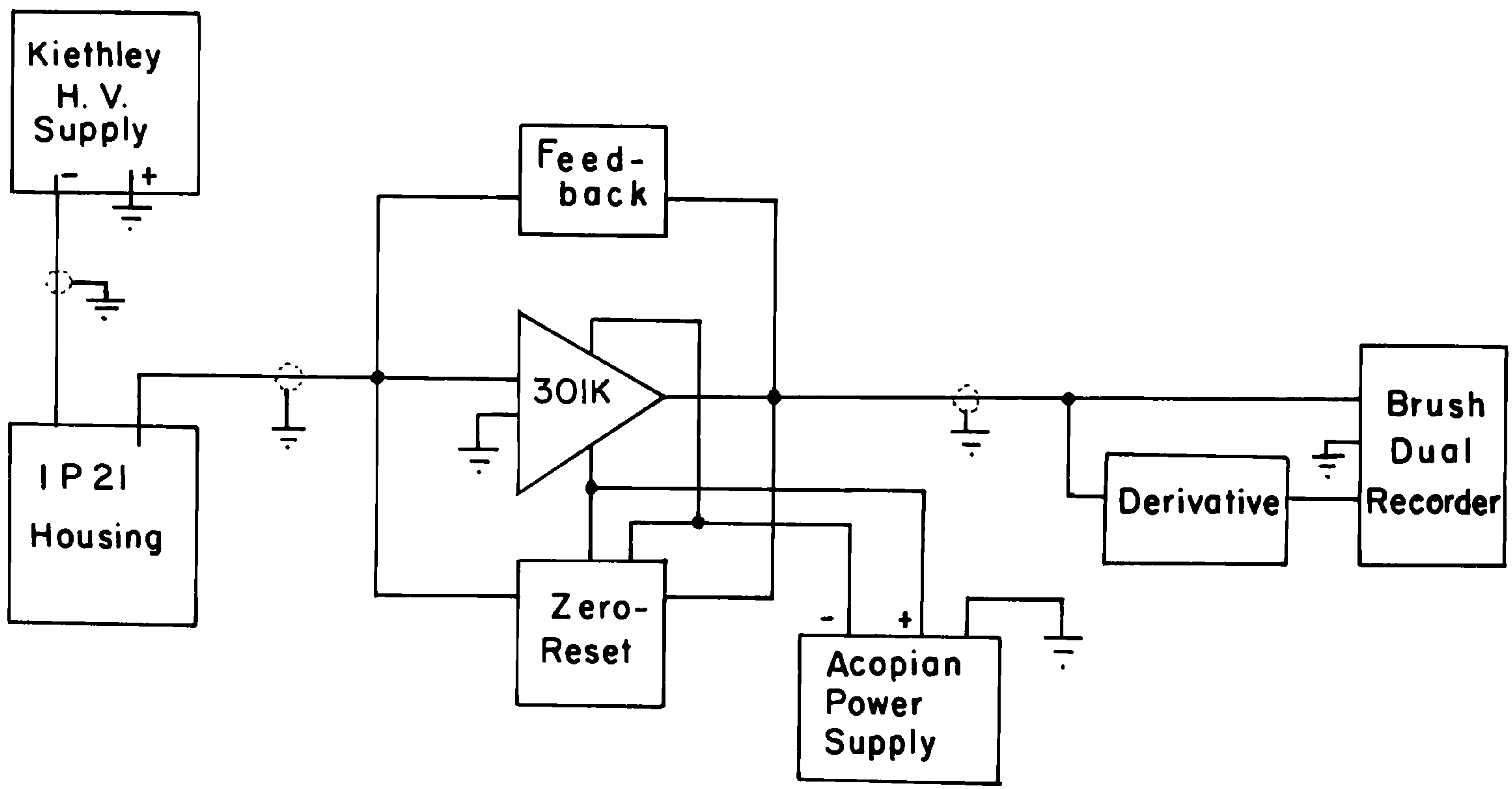

Figure B-1. Block diagram of photomultiplier photometer arrangement.

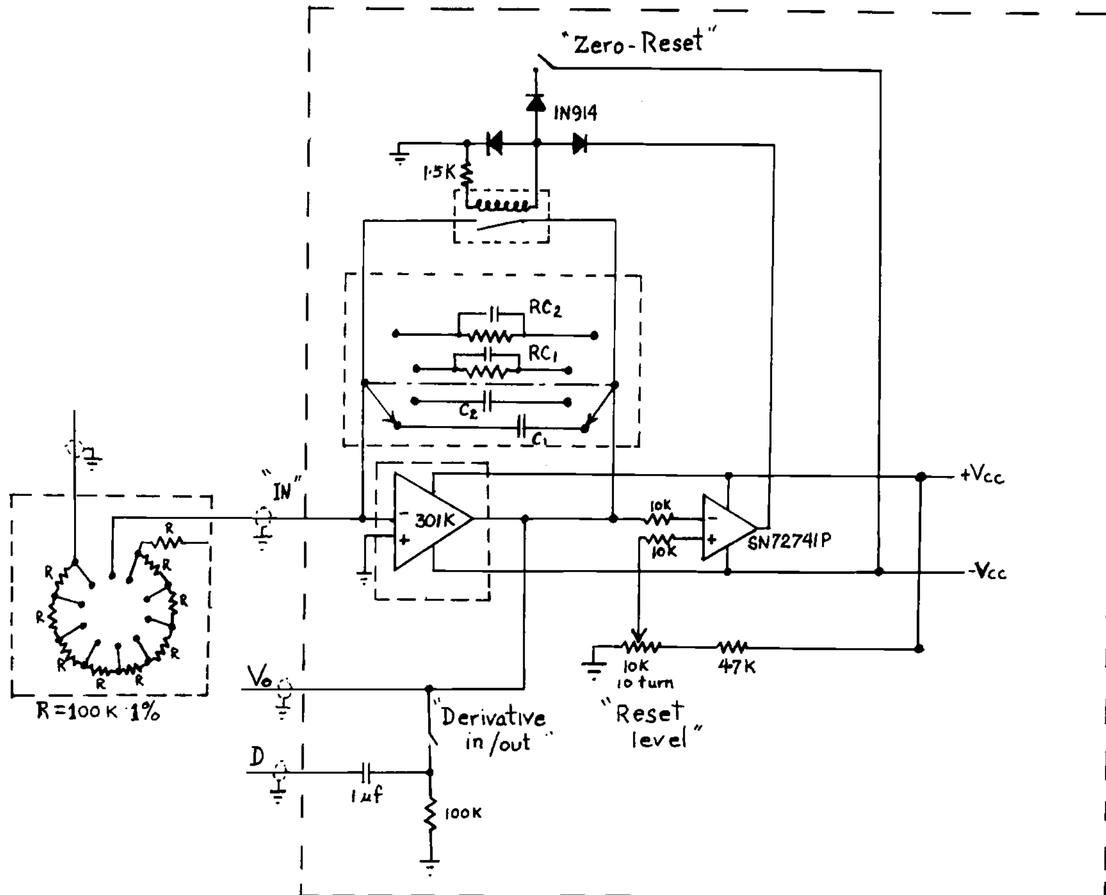

Figure B-2. Schematic diagram of photometer amplifier. RC_1: 100 Megohm, 1%; 27 pf 10%. RC_2: 10 megohm 1%; 270 pf ceramic. C_1: 0.01μf polystyrene, 100 WVDC. C_2: 0.15μf polystyrene 100 WVDC.

voltage for the photomultiplier was provided by a Keithley 240A. This instrument permitted gain changes by virtue of its presetable high voltages.

The reset circuit employs a differential voltage comparator (Texas Instruments - SN72741P), the output of which switches from $+V_{cc}$ to $-V_{cc}$ when the output of the 301K reaches the value of a variable reference voltage derived from the power supply. A diode array allows the reed-coil to activate the reed-switch when $-V_{cc}$ is present, which can also be done manually to zero the recorder. This circuit replaces the troublesome sensitive relay found in other instruments.

The differentiating circuit is that given by Biggley et al. (1969), and allows both the integrated light reading and an intensity analogue to be recorded simultaneously. The RC network was chosen to give a sufficiently small time constant to provide reasonable flash rendition.

The 301K, shielded switch, reset circuit, and power supply were mounted in a single housing, and access provided by "BNC" connectors. Shielded cables were used throughout the set-up. It was necessary to hermetically seal the apparatus when working in the constant temperature room to prevent leakage across the capacitors due to the high relative humidity.

The gains of the instrument were determined at various

voltages and feedback elements with the aid of a self-luminous source consisting of a radium watch dial in a test tube. This was also used to monitor the sensitivity of the instrument, and was calibrated for temperature variations since the instrument was used over a range of 14° C. Absolute calibration of the instrument in terms of photons per cell in the various geometries is described in Section II and III.

Recently Mitchell and Hastings (1971) have redesigned the MacNichol photometer which incorporates presetable high voltages to allow integral gain settings. The present instrument is nearly as versatile, and more simple, but slightly more costly to construct. Also, Santini and Pardue (1970) have designed a dual detector spectrophotometer with solid state components, and a dynode assist circuit which greatly improves the dynamic range of the 1P21 photomultiplier tube.