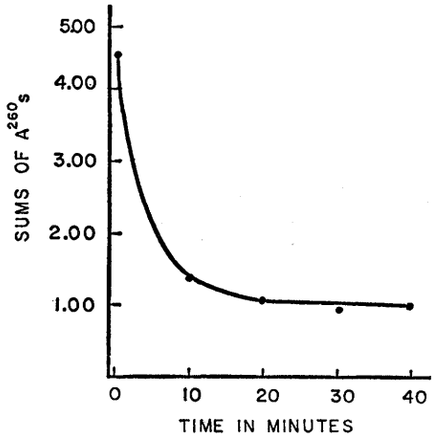

Fig. 4. Decrease in ribosome content of *B. megaterium* during exposure to chloroquine ($1.6 \times 10^{-3}M$). Samples (1000 ml) of experimental mass cultures were taken at intervals, the bacteria were collected and disrupted as described in the legend to Fig. 3, and the samples were clarified by low-speed centrifugation and dialyzed for 24 hours at 4°C against a buffer mixture introduced by Nirenberg and Matthaei into experimentation with ribosomes (*19*). The samples were then subjected to molecular sieve analysis (*20*) by being placed on columns of Sephadex G-100 and eluted with fresh buffer. Relative quantities of ribosomes in eluted fractions were estimated spectrophotometrically (wavelength 260 mμ), and the sums of the absorbancies (A^{260}s) in fractions comprising entire ribosomal peaks were plotted as a function of the time of bacterial exposure to chloroquine.

consumption as a function of chloroquine concentration yielded a straight line with a value of ED_{50} (the 50-percent effective dose) of 760 μg/ml, that is, 2.4×10^{-3} mole/liter. Evidently the inhibitions of macromolecular biosyntheses we report are not results of a general anabolic failure owing to blocking of electron-transfer reactions; oxidative phosphorylation is insensitive to chloroquine in cells whose growth is inhibited by the drug (*13*).

Our finding that DNA synthesis in bacteria is inhibited in vivo by chloroquine is in essential agreement with observations that incorporation of radiophosphate into nucleic acids of plasmodia is inhibited by this drug (*5*); blockage of DNA replication per se explains, in our opinion, the bactericidal effect of chloroquine.

The breakup of ribosomes and the dissimilation of ribosomal RNA were unexpected findings, although another instance is known (*14*) in which blocking of DNA replication was accompanied by similar phenomena. Degradation of ribosomes and their RNA in our experiments with chloroquine explains the observed net loss of RNA from *B. megaterium* as well as the failure of protein synthesis and may have contributed to the bactericidal effect of the drug upon this organism.

We propose that inhibition of DNA replication, based upon a direct action of the drug on DNA, is the general mode of antimicrobial action of chloroquine. This idea has certain implications for observations of natural or acquired resistance to the drug. The molecular architecture of double-stranded DNA is evidently universal, and susceptibility or resistance to chloroquine cannot be explained on the basis of structural or compositional differences between the DNA's of susceptible or resistant cells. It is more likely that susceptibility to chloroquine, like that to actinomycin D (*15*), is based upon the capacity of susceptible cells to permit passage and accumulation of critical concentrations of the drug while natural or acquired resistance may be results of impermeability or of an impaired concentration mechanism. This is borne out by two observations. (*i*) Chloroquine-exposed and packed cells of susceptible *B. megaterium* contained ten times as much of the drug as did identical volumes of packed, chloroquine-resistant *B. cereus* (*11*). (ii) The DNA-polymerase reaction in cell-free experiments in vitro is highly susceptible to chloroquine (*4*) in spite of the fact that the priming DNA as well as the enzyme has been prepared from *Escherichia coli* which is resistant to chloroquine (*6*).

JENNIE CIAK
FRED E. HAHN
Department of Molecular Biology, Walter Reed Army Institute of Research, Washington, D.C. 20012

References and Notes

1. H. Andersag, S. Breitner, H. Jung, German patent 683,692 (1939).
2. F. S. Parker and J. L. Irvin, *J. Biol. Chem.* **199**, 897 (1952); D. Stollar and L. Levine, *Arch. Biochem. Biophys.* **101**, 335 (1963); S. N. Cohen and K. L. Yielding, *J. Biol. Chem.* **240**, 3123 (1965); J. L. Allison, R. L. O'Brien, F. E. Hahn, *Science* **149**, 1111 (1965).
3. N. B. Kurnick and I. E. Radcliffe, *J. Lab. Clin. Med.* **60**, 669 (1962).
4. S. N. Cohen and K. L. Yielding, *Proc. Nat. Acad. Sci. U.S.* **54**, 521 (1965).
5. K. A. Schellenberg and G. R. Coatney, *Biochem. Pharmacol.* **6**, 143 (1961).
6. F. Kradolfer and L. Neipp, *Antibiot. Chemothap.* **8**, 297 (1958); L. B. Robinson, T. M. Brown, R. H. Wichelhausen, *ibid.* **9**, 111 (1959); V. Houba and M. Adam, *Cas. Lek. Ceskych* **103**, 540 (1965).
7. J. Ciak and F. E. Hahn, *Fed. Proc.* **24**, 454 (1965).
8. Z. Dische, "Color reactions of nucleic acid components," in *The Nucleic Acids*, E. Chargaff and J. N. Davidson, Eds. (Academic Press, New York, 1955), vol. 1, p. 285.
9. W. Mejbaum, *Z. Physiol. Chem.* **258**, 117 (1939).
10. C. Levinthal, A. Keynan, A. Higa, *Proc. Nat. Acad. Sci. U.S.* **48**, 1631 (1962).
11. These experiments were carried out by Dr. R. L. O'Brien of our laboratory.
12. H. P. Treffers, *J. Bacteriol.* **72**, 108 (1956).
13. H. Greiling and G. Doerner, *Z. Rheumaforsch.* **21**, 316 (1962); M. W. Whitehouse, *J. Pharm. Pharmacol.* **15**, 556 (1963); M. W. Whitehouse and H. Boström, *Biochem. Pharmacol.* **14**, 1173 (1965).
14. H. H. Kersten and W. Kersten, *Z. Physiol. Chem.* **334**, 141 (1963); H. Suzuki and W. W. Kilgore, *Science* **146**, 1585 (1964).
15. B. Mach and E. L. Tatum, *Science* **139**, 1051 (1963).
16. F. E. Hahn, C. L. Wissman, Jr., H. E. Hopps, *J. Bacteriol.* **67**, 674 (1954).
17. A. Gierer and G. Schramm, *Nature* **177**, 702 (1956).
18. J. D. Mandell and A. D. Hershey, *Anal. Biochem.* **1**, 66 (1960).
19. M. W. Nirenberg and J. H. Matthaei, *Proc. Nat. Acad. Sci. U.S.* **47**, 1588 (1961).
20. M. Takanami and G. Zubay, *ibid.* **51**, 834 (1964).
21. We gratefully acknowledge the technical assistance of H. L. Whitfield and Angelo Zegna and the efforts of the Medical Audio-Visual Department of our Institute in producing the figures.

5 October 1965

Subcellular Sources of Luminescence in Noctiluca

Abstract. *The perivacuolar cytoplasm of* Noctiluca miliaris *contains approximately 10^4 microsources of luminescence, with dimensions of 0.5 to 1.5 microns, which exhibit marked fluorescence with ultraviolet excitation. Local invasion by an action potential elicits light emission (microflashes) from these sources with a coupling latency of about 2 milliseconds. Magnitudes of the microflash vary directly with the dimensions of the source. Time courses of the microflash resemble that of the macroflash emitted by the whole cell but have somewhat shorter time constants. The small discrepancy in duration between micro- and macroflash can be explained by the 5- to 10-millisecond asynchrony of microsource triggering that results from the conduction time of the action potential. Reversible gradations in amplitude of the macroflash, as from potentiation or fatigue, result from parallel summation of graded changes in microflash intensity. Thus the macroflash gives a reasonably true picture of the subcellular kinetics of luminescence.*

The luminescent flash of the dinoflagellate *Noctiluca miliaris* is triggered by an all-or-none action potential that propagates nondecrementally in the complex peripheral layer of cytoplasm at a rate of about 60 μ/msec (*1, 2*).

Initiation of light emission spreads over the cell at a similar velocity. In addition, simultaneous photometric and electrical recordings from restricted areas of cytoplasm showed that light emission follows local action current with an approximate latency of 2 msec at 20° to 25°C. The flash recorded from the whole organism reaches maximum intensity in 10 to 20 msec, decreases in intensity by 50 percent in about the same time, and is largely terminated after 80 msec.

Examination of the flashing specimen with high-magnification light optics reveals apparently synchronous flashes from a myriad of microscopic sources within the peripheral sheet of cytoplasm. This observation, first made over 100 years ago (*3*), indicates that the total luminescent flash of the organism (termed "macroflash") results from summation of a great many "microflashes" arising from separate organelles termed "microsources" (*4*). Since the microsources are scattered in the peripheral cytoplasm and have no direct continuity with one another, each one must be independently triggered to luminesce by the passing action potential or by a concomitant of the potential. These considerations indicate that the events which underlie excitation-flash coupling occur at the microsource. Examination of the microflash is therefore important for an eventual understanding of the coupling mechanism.

Are the macroflash wave shape and time course due to a temporal distribution of short, intense microflashes, or do they result from a simple parallel summation of microflashes having time courses similar to that of the macroflash? Meaningful interpretation of macroflash data in excitation-flash studies requires clarification of this question.

Microscopic examination of the cytoplasm of *Noctiluca* revealed many (about 2.5×10^5 per cell) strongly phase-retarding inclusions ranging in diameter from less than 0.5 to about 1.5 μ. Photographic studies with image intensification (*5*) showed that light emission during the flash is associated with about 5 percent of these structures, giving a figure of 10^4 microsources per cell (*6*, *7*). The same proportion of phase-retarding inclusions were stimulated by ultraviolet light (360 to 404 mμ) to fluoresce blue-green. No fluorescence was detected

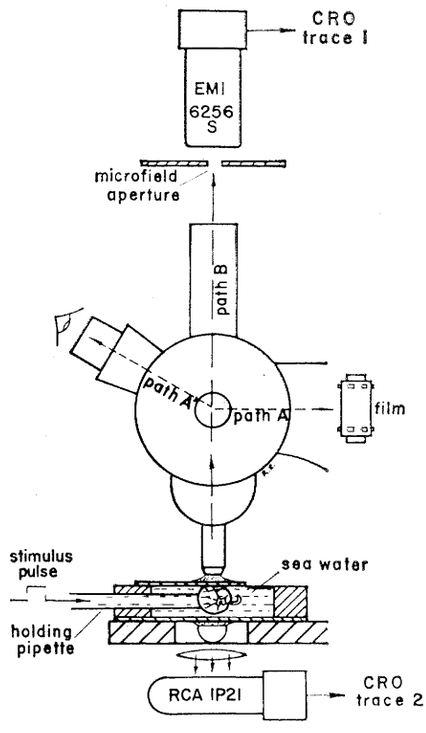

Fig. 1. Apparatus for simultaneous photometry of micro- and macroflash of *Noctiluca*. Specimen on stage of Zeiss Photomicroscope was immobilized in sea water at end of suction pipette. The 100× Neofluar objective was focused, through immersion oil and cover glass, on peripheral cytoplasm. Inclusions were selected for microphotometry, on the basis of fluorescence and phase-retarding properties, and were brought into recording field by visual alignment with an ocular cross hair (via light path A'). A photomicrograph was then made to record position of cytoplasmic structures with respect to photometer field (light path A). Finally, the side-window photomultiplier of the macrophotometer was swung into position below condenser, and the selector prism was repositioned to let the image project onto the plane of the photocathode of the multiplier tube of the microphotometer (light path B). Stimulation was by a 1.0-msec electropositive current pulse delivered via the holding pipette. Micro- and macrophotometer outputs were registered on two traces of an oscilloscope and recorded on film. In some experiments the microphotometer signal was electronically integrated and displayed on a third trace. The time constant of the integrating feedback network was 0.01 seconds. The microphotometer recording field was restricted to 3 μ at specimen level by means of an aligned, calibrated aperture in front of the photocathode of the EMI 6256S multiplier. Dark current in the latter was reduced by cooling to −40°C. The phototube was operated at 1700 volts, with anode current monitored by a Philbrick SP2A operational amplifier in a current-to-voltage transimpedance circuit (*11*). Both micro- and macrophotometers had time constants below 1 msec.

from the remaining inclusions or from organelles of the nonluminescent form of *Noctiluca* (*8*). Fluorescent inclusions have a somewhat larger size range than others and exhibit slightly less phase retardation. Moreover, fluorescent inclusions brighten perceptibly in response to stimulation of the cell when viewed under ultraviolet illumination, whereas nonfluorescent structures show no luminescence. The correlation between fluorescent and luminescent properties of cytoplasmic organelles was subsequently confirmed by microphotometric measurements. In this study fluorescence served as a means of identification of microsources.

The average number of photons, 5×10^5, per microflash was calculated by dividing the average number of photons (*1*) in a macroflash, 5×10^9, by the approximate number of sources per cell, 10^4. Photometric recordings of sufficiently high amplification and low dark noise were obtained from selected areas of cytoplasm 3 μ in diameter by means of a cooled EMI 6256S photomultiplier (Fig. 1). Areas selected for photometry were positioned within the recording field by alignment with an ocular cross hair, and the position of the photometer field was registered photographically for later reference several seconds prior to each recording. The preparation was then darkened, and a flash was elicited by stimulation of the cell with a 1.0-msec positive-current pulse delivered through the suction pipette holding the cell. When the organism flashed, a background signal was always recorded from the microfield, regardless of its position with respect to cytoplasmic structures. This light probably represents emission from numerous sources within the microfield but several hundred microns below the plane of focus on the far side of the cell. The microphotometer signal was displayed on one trace of an oscilloscope. In some experiments the integral of this signal was obtained and also displayed. The macroflash was monitored with an RCA 1P21 side-window multiplier, positioned under the substage condenser. It served as a temporal reference and as a monitor of amplitude changes due to fatigue or potentiation of the flash.

Luminescence exceeding background levels was recorded only from inclusions showing fluorescence. Moreover, a clear correlation was established be-

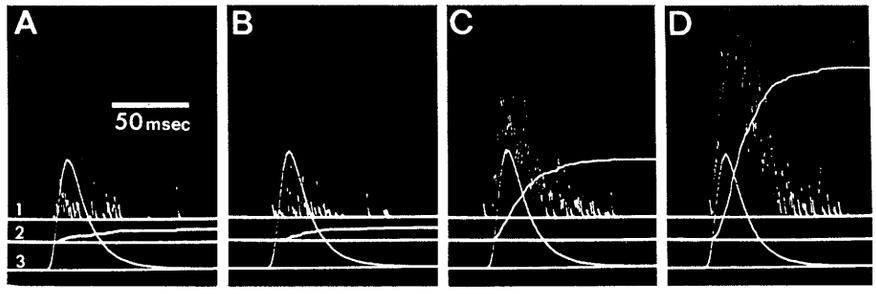

Fig. 2. Correlation between fluorescence and bioluminescence in cytoplasmic structures of *Noctiluca*. Trace 1, microphotometer output (see Fig. 1); trace 2, electronically integrated output of microphotometer; trace 3, macrophotometer output displaying light emitted from whole cell (macroflash). (*A*) no fluorescent object in field of microphotometer; (*B*) small (<0.5 μ diameter) fluorescent source within field; (*C*) medium (about 1 μ) fluorescent source; (*D*) large (about 1.5 μ) fluorescent source. Note correlation between size of fluorescent source and its luminous output as determined by integration. Each frame contains two sweeps, one providing signal base lines.

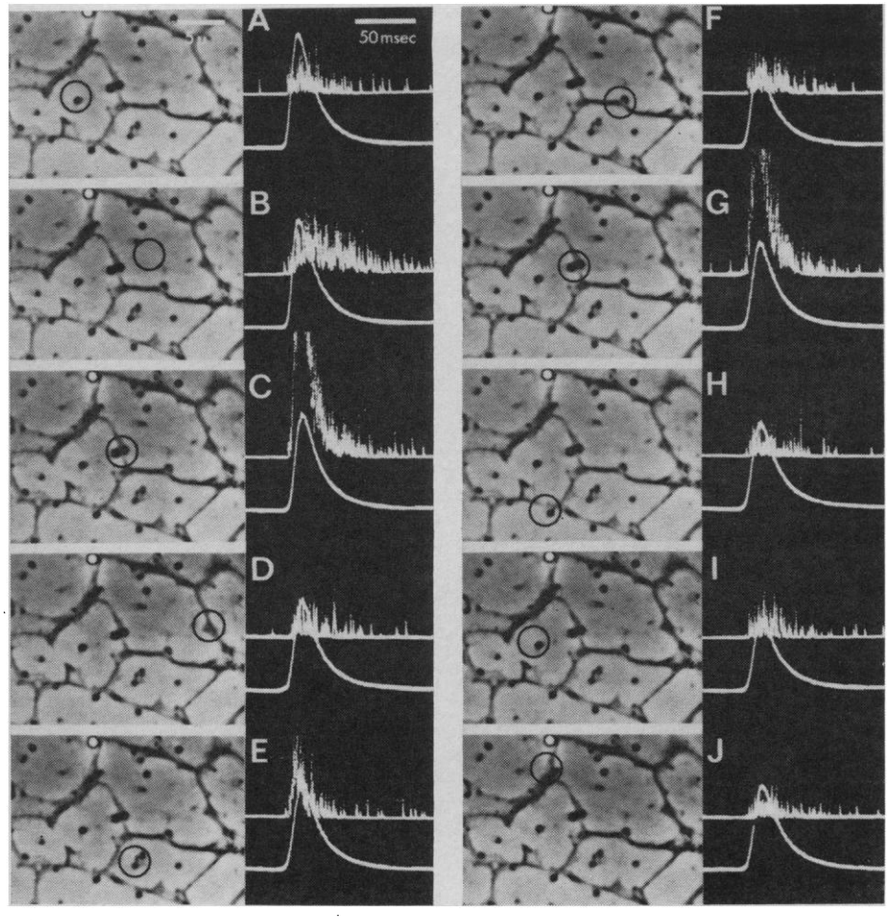

Fig. 3. Microphotometric records from selected areas in the peripheral sheet of cytoplasm in *Noctiluca*. To the left of each recording is a photomicrograph with a black circle indicating the position of the 3-μ photometer field with an accuracy of $\pm$ 1 μ. Upper trace of each cathode-ray–oscilloscope sweep is the microphotometer recording from the field outlined by the black circle. Brief deflections of this trace indicate single photons or groups of closely spaced photons. Lower trace of each pair shows macroflash recorded from entire cell through substage condenser. *A* to *J* are shown in the chronological order of their recording. The location of photometer field was documented by a photograph made several seconds prior to recording, and microfields were subsequently indicated in ink on ten copies (*A* to *J*) of the clearest photomicrograph of the series.

tween size of the source and the photon content of its microflash (Fig. 2). Prior to each recording the approximate size of the fluorescent source positioned within the microfield was noted and recorded. The examples shown constitute a representative consecutive series of frames (reproduced in rearranged sequence). Electronic integration (trace 2) of the microphotometer signal (trace 1) provided a relative value of total flux within the field. Failure of "small" sources to add significantly to background signal was probably due to the large (about 40 : 1) ratio of microfield area to source area. This ratio was reduced to approximately 4 : 1 in the case of "large" sources. Except for differences in amplitude, signals from microsources of different sizes appeared similar.

Figure 3 shows a series of recordings from selected loci within a restricted field (*9*). Strong fluorescence was exhibited by the double structure near the center of the field. Although it is assumed that this structure represents two closely adjacent sources, the microflash characteristics it displayed (recordings *C* and *G*) were kinetically indistinguishable from those of single sources. Recording *E* was apparently from a weak source, whereas the origin of the photometer signal in *B* is not entirely clear (*10*). The remaining recordings were from nonluminescent areas of cytoplasm, showing only background levels of luminescence.

The signal noise inherent in low-intensity photometry renders quantitative analysis of wave shape difficult. Visual inspection, however, of numerous recordings indicates general similarity in the wave shape and time course of micro- and macroflashes. Minor variations in time course are noted from one microsource to another, but individual sources flash with quite reproducible wave shapes (for example, Fig. 3, *C* and *G*). Inspection of electronically integrated signals of both indicates that the microflashes generally rise and fall somewhat more rapidly than the macroflashes. This was not surprising in view of the finite conduction rate of the action potential that triggers microflashes as it propagates from its origin over the remainder of the cell (*2*). Time course differences appear consistent with the observed conduction latency of 5 to 10 msec. No latency differences were noted among microsource responses other than those ex-

plicable by the conduction rate of the triggering potential.

The macroflash exhibits rigid coupling to the action potential (*1*). This apparently is also true of the microflash, which, in photometric recordings from individual microsources, occurred in a strictly one-to-one response to each invading action potential. Asynchronous spontaneous flashing of microsources was visually noted in some deteriorating specimens, but never in fresh material. Occasionally, however, large regions of the cell irreversibly failed to luminesce (*7*), and attenuation of the macroflash resulted. This was due, presumably, to failure of invasion of those areas by the action potential, an occurrence occasionally seen in electrical recordings.

Reversible graded alterations in amplitude of the macroflash (that is, summation, potentiation, fatigue) commonly occur as a function of temporal stimulus sequence (*1*). This could occur in at least two ways. (i) Gradations in macroflash intensity could arise from changes in the proportion of sources responding to the triggering potential, and could have their basis in statistical changes in the triggering threshold of the microsource population. This does not, however, appear to be the case, for as noted above, individual luminescent sources flash in a rigid one-to-one response to invading action potentials. Microsources possess little or no triggering lability. (ii) Macroflash gradations could result from summation of gradations in the intensity of the microflashes. This is supported by results of simultaneous recordings of micro- and macroflashes: temporal summation of two closely spaced responses occurs similarly in each. Likewise, macroflash fatigue with repeated stimulation was paralleled by a similar decline in microflash intensity. Thus, the macroflash parallels events at the level of the microsource and can serve as a reliable indicator of microflash kinetics.

Since the triggering potential is all-or-none (*1, 2*), gradations in microflash intensity must result either from changes occurring in the luminescent system itself (for example, as a result of substrate depletion) or at the "control" or "coupling" steps linking the luminescent chemistry to changes in membrane potential. That is to say, summation, potentiation, and fatigue of luminescence must have their physical basis within or adjacent to the microsources.

These data indicate the following. (i) Fluorescent luminescent organelles, 1.5 μ and less in diameter, emit flashes with intensities proportional to their physical size and appear to be the sole origin of light contained in the flash emitted by the organism. (ii) Time courses of microflashes resemble those of macroflashes, but typically show somewhat shorter time constants. (iii) Flashes from different microsources show only minor differences in time course. (iv) Measurable differences in latency between micro- and macroflashes can be explained by the transcellular conduction time (5 to 10 msec) of the action potential. (v) Each source flashes in unison with its immediate neighbors in response to each invading action potential. (vi) Reversible graded changes in intensity of macroflash result from graded changes in amplitudes of microflash. (vii) The kinetics of both flashes are parallel and can be used to investigate fundamental subcellular events in studies on excitation-flash coupling.

ROGER ECKERT

Department of Zoology, Syracuse University, Syracuse, New York, and *Marine Biological Laboratory, Woods Hole, Massachusetts*

References and Notes

1. R. Eckert, *Science* **147**, 1140 (1965).
2. ———, *ibid.*, p. 1142.
3. A. de Quatrefages (1850), cited by E. N. Harvey in *Bioluminescence* (Academic Press, New York, 1952); E. B. Harvey, *Carnegie Inst. Wash. Publ.* **251**, 235 (1917).
4. A more specific term for these organelles must await electron micrographic clarification of their fine structure. Although there is evidence (R. DeSa and J. W. Hastings, unpublished) that the extracted particulate luminescent systems of *Noctiluca* and *Gonyaulax* exhibit certain physical and chemical similarities, the precise relation between the microsources of *Noctiluca* and the "scintillons" of *Gonyaulax* [R. DeSa, J. W. Hastings, A. E. Vatter, *Science* **141**, 1269 (1963)] cannot be defined at this time.
5. G. T. Reynolds, R. D. Allen, R. Eckert, J. W. Hastings, S. Inoué, *Biol. Bull.* **125**, 389 (1963); G. T. Reynolds, *IEEE Inst. Electrical Electronic Eng. Trans. Nucl. Sci. NS* **11**, 147 (1964).
6. R. Eckert, G. T. Reynolds, R. Chaffee, *Biol. Bull.* **129**, 394 (1965).
7. ———, unpublished.
8. R. Eckert and M. Findlay, *Biol. Bull.* **123**, 494 (1962).
9. The photomicrograph is focused in the plane of the thin, complex layer of cytoplasm bounded on the upper surface by the plasma membrane and pellicle, and below by the vacuolar membrane. Dark grey strands are seen in electron micrographs (by Dr. H. S. DiStefano) to consist of cytoplasmic thickenings which include mitochondria, golgi bodies, trichocysts, vacuoles of several kinds, and granules. In the light grey areas between strands, extraordinarily little cytoplasm is found between the plasmalemma and vacuolar membrane, the membranes frequently coming into apparent mutual contact. The dark, round, phase-retarding inclusions have not yet been unequivocally identified in electron micrographs. This photograph fails to convey the slight difference in phase retardation exhibited by microsources (such as paired structures in center of photo) and inactive inclusions.
10. Owing to small movements of the specimen in the plane of the field, the position of the photometer field, as determined photographically prior to each recording, had an estimated uncertainty of 1 μ. Hence, the photometer field in Fig. 3*B* may have included one of the structures closely adjacent to the circle.
11. D. H. Sheingold, *Lightning Empiricist* (Philbrick Researches, Boston) **12**, No. 1, p. 1 (1964).
12. I thank P. Specht for building the microphotometer circuit and Miss Francine Brady for culturing the organism. Supported by grants NSF GB-1908 and GB-3566, and PHS B-3664 and NB-05301, and in part by an ONR grant to the Marine Biological Laboratory.

5 November 1965

Enhanced Distress Vocalization through Selective Reinforcement

Abstract. *Eighteen Peking ducklings were imprinted and tested for their tendency to emit distress calls during the presentation and withdrawal of the imprinted stimulus. A subsequent arrangement in which each distress vocalization led to a 5-second presentation of the imprinted stimulus resulted in an enhanced tendency to emit distress calls.*

When young ducklings (like the infants of many species) are separated from their mother, they typically emit a stream of loud distress-like vocalizations. Several researchers have noted that these calls influence the mother in that they increase her tendency to return to the young (*1, 2*). In this study we sought to examine the complement to this process by determining how the duckling's tendency to emit distress calls is influenced by the mother's return. More specifically, we wished to assess the effects of an arrangement whereby the mother appears each time a distress call is emitted.

The problem is complicated by several factors. If the distress call is solely a reflection of emotionality produced by the mother's absence, then the constant availability of the mother by means of a distress call should minimize emotionality and thereby reduce distress calls. If, however, the mother's return serves to strengthen the behavior which precedes it, these arrangements should lead to the reinforcement