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MARINE MODELS IN MODERN MEDICINE

The horseshoe crab, Limulus polyphemus, *is an important resource in medically related basic research. (Russ Kinne, Photo Researchers)*

BY LEWIS THOMAS

The assorted life of the sea contains workable models for the close study of any number of fundamental issues in biomedical science. Some of these have relevance for the underlying mechanisms of defense against pathogens, reactions to injury, self-preservation, and the physiological processes involved in homeostasis. A few deal directly with disease itself, and the way appears open for a new broad approach to experimental pathology.

With so rich a source of available chemicals, flowing within and around so great a variety of living forms, you'd think the sea would have served, long since, as a sort of immense pharmacy for medicine. By this time we should have extracted stores of things: antibiotics, cancer cures, tranquilizers, and the rest; but it is not so.

The truth is, medicine cannot work this way as a science, never has (although it has a long record of trying), and never will. The small handful of therapeutically effective agents surviving the millennia of empirical trials of terrestrial extracts—from herbs, lizards, snakes, the barks of trees, powdered insects, precious stones, and the like—are evidence enough that this is an unproductive way to go. The fact that there have indeed been a few magically effective drugs obtained in this way—digitalis, morphine, ephedrine and quinine, and a variety of cathartics—informs us only that such agents do exist in nature and should be looked for, but warns that they are unlikely to be found by blind chance.

The trouble is that we are still lacking the essential kinds of information about the inner mechanisms of most diseases, and because of this lack we really do not know what we should be looking for. The great exception is the field of infectious disease, in which major advances in therapy began explosively four decades ago with the discovery of sulfonamides, penicillin, and streptomycin. Although it is true that these agents were first found by more or less accidental, trial-and-error methods, there was nothing blind or empirical about the whole process. The investigators possessed pieces of information of profound significance, long before the search for chemotherapy and antibiotics could have begun. The basic research, or the most essential part of it, had already been done, beginning around 1875 and reaching fruition in the 1930s. Because of this work, it was known that specific, indentifiable diseases were caused by

specific, identifiable bacteria. To be sure, the actual mechanisms by which disease was produced were not understood in detail for any microorganism (and still are not, for most), but it was at least known that the entry and presence of the bacteria were necessary for the disease to occur, and this, as it turned out, was enough. Given the information that streptococci caused scarlet fever, erysipelas, and rheumatic fever, or that the pneumococci caused lobar pneumonia, it made good scientific sense to search afield for substances capable of killing these organisms without damaging the host tissues.

Regrettably, we do not yet possess facts at this level of meaning about cancer, heart disease, stroke, schizophrenia, arthritis, nephritis, or most of the others, and therefore we cannot guess intelligently at where and how to intervene in the mechanism. For the time being we are stuck, with a few empirical, less than conclusive drugs, or with palliative measures, or with nothing at all.

What we need, of course, is a better understanding of disease processes, and for this we need, to begin with, a clearer comprehension of how cells and tissues work, under both normal and pathologic conditions. It is here that we are most likely to reap benefits from the new kinds of research in marine biology.

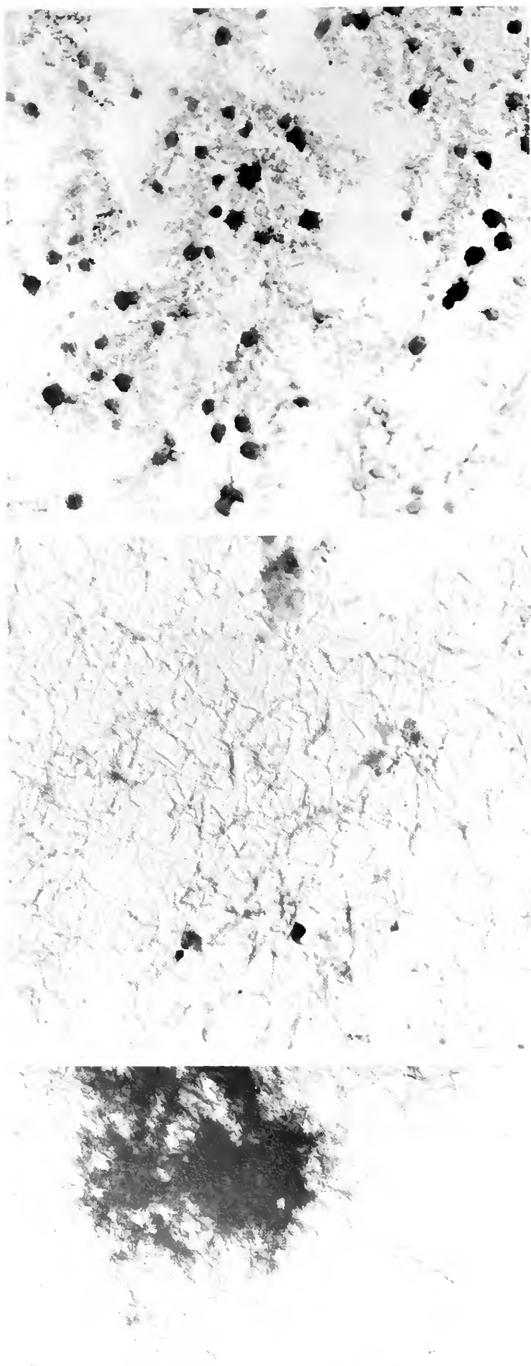
The advantages of marine models for work of this kind are spectacular. Many of the invertebrate animals represent early stages of planetary life and have evolved with somewhat less complexity and elaboration of internal structure than is the case for land creatures. Symbiosis is a more conspicuous way of life in the sea, and the mechanisms involved in specific recognition and accommodation between different but interdependent organisms, and between the separate parts of organisms, are more directly approachable. Developmental physiology, aging, dying, and recycling are displayed in an abundance of models. Tissues, cells, nerves and neural networks, sense organs, and reproductive systems are easier to get at, more feasible to study. The phenomenon of inflammation, perhaps the most fundamental of all disease processes, can be examined with and without the participation of various immunologic reactants, since immune mechanisms evolved, piecemeal, long after the perfection of recognition and defense systems in invertebrate forms. Toxins of great subtlety and variety, designed for predation or protection, or for no discernible reason, apparently by accident, are present in abundance, awaiting application as probes into disease processes.

What is needed is a new biological discipline of experimental marine pathology. It would really not be new at all, of course, since a long line of investigators, extending from Metchnikoff and his contemporaries (see page 56) to Frederick Bang and his colleagues today, has already opened up the field; but it is now in need of more people and more resources.

One example will be provided here of the kind of model I have in mind, which may illustrate the applicability of marine biological problems to human medicine.

Several years ago Bang (*1*) was interested in making direct observations of the microcirculation in the horseshoe crab, *Limulus polyphemus,* and he examined the behavior of the blood cells in vivo (in transilluminated gill appendages) and also in vitro (in wet preparations on glass slides). In the course of this study, he encountered a series of crabs in which the hemocytes were aggregated in dense clumps, with obvious interference with the normal circulation. These animals subsequently died, and Bang found that they were infected by a gram-negative microorganism that had produced septicemia. He then found that extracts of the killed bacteria caused similar intravascular clumping and that the toxic material was the lipopolysaccharide endotoxin, common to all gram-negative bacteria.

Endotoxin has been an unsolved problem of special interest in medicine for many years (*2*). This ubiquitous toxin is responsible for lethal shock in human beings and is the basis for some of the most puzzling phenomena in experimental pathology. Endotoxin is the well-known "bacterial pyrogen" responsible for febrile reactions after injections of contaminated blood or fluids. It produces the local and generalized Shwartzman reactions in rabbits, in which destructive hemorrhagic lesions affect many organs, most notably the kidneys. In pregnant animals, it regularly causes abortion. Some of the physiological disturbances mimic the effects of epinephrine, and the action of epinephrine on peripheral blood vessels is greatly exaggerated by endotoxin. In addition, endotoxin enhances the clotting of blood, reacts with a component of complement, causes platelets to clump together, stimulates phagocytosis, increases the level of immunologic response to various antigens, and brings about the selective destruction of certain rapidly growing tumors in animals (it is probably



the basis for the effects of "Coley's Toxin," used at one time for the treatment of cancer in human beings). With all of these extraordinary properties, its mode of action has remained a mystery for more than fifty years. A central difficulty has been the lack of any experimental system in which endotoxin can be seen to produce a *direct*, toxic action on any cell or cell product. All of its damaging effects in rabbits, for example, appear to be the result of indirect, secondary or tertiary effects. The hemorrhagic necrosis of the Shwartzman reaction is due to the plugging of small blood vessels either by masses of platelets and leucocytes or by deposits of fibrin within the lumen. The reaction of lethal shock, which closely resembles the syndromes of traumatic and hemorrhagic shock, remains unexplained. The actions on the immunologic system and on experimental tumors are unaccountable.

Thus Bang's discovery of a new model for the lethal action of endotoxin, involving an invertebrate lacking the major constituents of the vertebrate immunologic, coagulation, and leucocyte systems, is of major importance. Moreover, subsequent work from the laboratories of Bang and Levin (*3*) revealed, for the first time, an in vitro reaction involving endotoxin that should prove of great value in further elucidating the biological properties of this material. They found that aqueous extracts of *Limulus* hemocytes become solidly coagulated when endotoxin is added. The amounts of endotoxin capable of clotting the extracts are extremely small; as little as 0.1 nanogram per milliliter of lysopolysaccharide will cause clotting, thus providing the most sensitive method for the bioassay of endotoxin now available. The latter finding has already become practical and useful, even commercial; there is a considerable market in the pharmaceutical industry for a technique capable of detecting traces of endotoxin. Horseshoe crabs have suddenly become popular and increasingly expensive laboratory animals. One hopes they are not about to become another endangered species. Explorations are underway to discover whether other marine invertebrates possess similar receptors and coagulable proteins.

Electron micrographs of serum of normal Limulus *before addition of toxin (top) and at 7 minutes (center) and 15 minutes (bottom) after toxin was added. Effect of toxin was the formation of fibers and, eventually, a gel. Magnifications, from top to bottom: 6600X; 10,700X; 6400X. (From F. B. Bang, "A bacterial disease of* Limulus polyphemus," Bull. Johns Hopkins Hosp., *vol. 98, 1956, pp. 325-51. Reprinted by permission from* The Johns Hopkins Medical Journal.)

Meanwhile, a new approach to the mechanism of disease seems to be at hand. The lethal response of *Limulus* to lipopolysaccharide, taken as a sort of disease model, does not appear to be due to any primarily toxic property of endotoxin. On the contrary, it seems more like a programmed, orderly response to a biological signal, but one in which the response, originally designed for defense, is so wildly exaggerated that the outcome is a disorganization of the entire animal, and death. In nature the coagulable protein is probably extruded from hemocytes when they sense a gram-negative pathogen in nearby tissues; this may provide a means of entrapping the microorganism prior to phagocytosis. But in the experimental model, when lipopolysaccharide is injected into the blood compartment, all of the hemocytes receive a signal that bacteria are there, all around, and they all extrude the protein, which coagulates the blood and stops the circulation, and the animal dies.

If there are, as is likely, human diseases in which the tissue damage and lethal outcome are due more directly to the mistaken and inappropriate use of host defense mechanisms than to any frontal injury by invaders from outside, this *Limulus* model should prove ideal for further study.

There may be other models, awaiting investigation and manipulation, where the first benefit for medicine will turn out to be an understanding of normal and pathological mechanisms. Starfish cells, for example, contain a substance that mimics certain intermediary reactants in the delayed hypersensitivity response of vertebrates (*4*). The polyps of neighboring *Gorgonaceae* live at close quarters, sometimes touching, but they do not fuse; they prevent this by a mechanism that permits the prompt elimination of whichever happens to be the smaller of a too-close pair. Theodor (*5*) has shown that a signal of some sort is exchanged, after which metabolic activity ceases in the smaller one, and it then undergoes dissolution due to its own lytic enzymes. Both the signalling system and the detailed mechanism enabling one tissue to cause another to destroy itself have yet to be explained. The problem contains obvious points of interest to pathologists, particularly to those interested in cancer.

Bang and Bang (*6*) have recently been engaged in studies of the urn cells of the marine invertebrate *Sipunculus*. These cells originate in the epithelial lining of the coelom and detach to swim freely in the coelomic space, where they elaborate long strands of sticky mucus. They are evidently scavenger cells, and the mucus strands serve to remove bacteria and debris from the fluid. The

Gorgonians display an "induced suicide" mechanism whereby the smaller of a too-close pair ceases metabolic activity and is destroyed by its own lytic enzymes. (Benthos Inc.)

Bangs have found that the rate and character of mucus production can be readily manipulated in vitro; they can detect macromolecular substances in human serum, urine, and tears that cause extraordinary hypersecretion of mucus, and they suggest the use of this technique for studying the regulation of normal and abnormal secretory systems on human disease.

The potential richness of other models for the detailed scrutiny of disease mechanisms, from other disciplines in marine science, will be made evident in the articles that follow. If you believe, as I do, that there is a necessary, indispensable basic science underpinning modern medicine, and that we must greatly expand this foundation before medicine can achieve its ultimate objective of disease control, then the relevance of contemporary marine biology to medicine is self-evident. In the essential features of our day-to-day transactions with our environment, we are not as different from the range of creatures in the sea as we used to think.

Lewis Thomas is president of Memorial Sloan-Kettering Cancer Center, New York, and author of The Lives of a Cell.

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The Squid Giant Axon

by William J. Adelman, Jr., and Robert J. French

To a large extent, our modern knowledge of the workings of the nerve cell, or neuron, has come from studies of the giant nerve fiber or axon of the squid. The giant fiber system was first noted by L. W. Williams in 1909, but it was not until its rediscovery by J. Z. Young in 1933 that its potential for biophysical and physiological study was recognized.

Nerve cells are generally referred to as being *excitable* in that they respond to a variety of environmental changes or stimuli by generating a characteristic electrical response. Among effective stimuli are forms of energy such as electrical, mechanical, heat, and light energy. Some nerve cells are adapted to respond particularly well to a given form of energy—for example, visual cells to light, and mechanoreceptors to force displacements. However, irrespective of the type of effective stimulus, the response produced involves the generation of an electrical event that is conveyed from its point of initiation over the nerve cell to some distant point.

In order to carry information over long distances, nerve cells generally have an elongated form. Long filamentous fibers project from the nerve cell body and connect to distant parts of the animal organism. Axons are those fibers that primarily are the communication lines for conducting messages rapidly over relatively long distances in the body. For example, leg muscles are innervated with axons whose cell bodies lie in the spinal cord. Impulses arriving from the brain can activate these nerve cells, which then send impulses along the axons to the leg muscles to initiate muscular contraction.

Axons are therefore parts of cells, which are the units of biological tissue structure. A cell maintains an internal aqueous phase that is separated from the external fluid environment by a dense surface layer (or membrane) about 100 Å thick, composed of lipid and protein molecules, thought to be arranged as depicted in Figure 1. (An Angstrom unit, Å, is one ten-billionth of a meter, or about one two-hundred and fifty millionth of an inch.)

Nervous Systems

In order to understand how nervous systems work, let us consider one way in which we are made aware of the external world. Distributed over the surface of our body are many sensory receptors responding to those mechanical displacements we call touch. In the cerebral cortex there is a specific array of nerve cells, each of which corresponds to a small area of the skin. The body surface is thus spatially represented in the brain. Each of these brain cells is constantly receiving impulses from its specific receptive field. The intensity of touch is signaled to the brain by the frequency, or number per unit time, of impulses sent to the brain. Therefore, intensity of stimulation is conveyed by a frequency-modulated code.

Let us consider another example of nervous function: In the retina of the eye are special cells called visual receptors whose chemical structure enables them to absorb light energy, or photons (see page 28). In the light-absorption process a set of chemical events is put in motion and results in the generation of electrical events in the cell. These electrical events are conveyed along a number of interconnected nerve cells until they eventually reach the visual cortex of the cerebrum. Since there are thousands of visual cells, each of which is part of the grain of the visual image on which the eye is focused, the cerebral cortex receives an image of the world coded in terms of thousands of nerve fibers continuously sending billions of impulses. Analysis

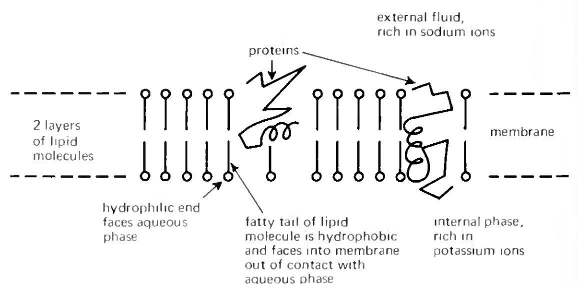

Figure 1. Diagram showing the current view of the cell membrane, a bilayer of lipid molecules with proteins attached and inserted here and there in the lipid. The "channels" discussed later are probably protein molecules such as these, reaching through the lipid part of the membrane.

of these phenomena is extremely complex and will not be discussed here. Rather, we will focus on how just one nerve fiber produces a single impulse and how that is transmitted or conducted.

Bioelectricity

Animal electricity was known by its effects to the ancient Egyptians who made observations of the electric catfish, *Malapterurus,* about 2600 B.C. The first systematic study of bioelectricity was made in 1790 by Galvani. Controversy between Galvani and Volta over the phenomenon of excitation led to the development of the storage battery (the stimulus source) by Volta and the founding of the science of electrophysiology (the systematic study of excitable responses) by Galvani's successors, Matteucci, du Bois-Reymond, Helmholtz, and Hermann, over the half-century from 1830 to 1880. Their studies made clear that excitable tissues, such as nerve and muscle cells, had a relatively constant voltage between cell interiors and the extracellular body fluids, and that nerve and muscle messages were signaled by momentary or transient changes in this voltage ("negative variations"). These brief pulses of electrical activity associated with the nerve impulse are now called *action potentials.*

Membranes and Permeabilities

Since Helmholtz's time, physicists and biologists have attempted to describe the mechanisms whereby the nerve fiber produces a nerve impulse. The most successful theory of the early twentieth century was the membrane hypothesis of Julius Bernstein, developed from 1868 to 1902. Bernstein conceived the idea that cells were coated by a thin surface layer or membrane that separated the cell interior from the extracellular fluid medium. Noting that the concentration of potassium ions inside the cell was much higher than that outside, he proposed that the membrane was selectively permeable to potassium. Bernstein suggested that an excess of positively charged potassium ions would tend to escape from the cell, down the concentration gradient from the high potassium concentration inside to the low concentration outside. This tendency leads to a voltage gradient across the membrane—positive on the outside, negative on the inside. In the resting cell, the force of the concentration gradient pushing potassium ions outward is balanced by the electrical force propelling them toward the negative interior of the cell.

Bernstein further proposed that during a nerve impulse the membrane momentarily lost its potassium selectivity and became permeable to all ions. Under such a condition all the various ionic electrical forces would be balanced and the membrane potential would approach zero. Electrically, the nerve impulse would then be a transient pulse, or action potential, going from the inside negative resting potential to zero, and then back to the resting potential. Adequate testing of the Bernstein hypothesis awaited the rediscovery of the squid giant axon.

Nerves

In humans most peripheral nerves contain thousands of nerve fibers (axons and dendrites) whose cell bodies lie in or near the central nervous system. A nerve cell is composed of a nucleus-containing cell body from which the long projecting processes, the axons and dendrites, radiate. In the human body a sciatic nerve in the leg contains thousands of axons, each of which has its own cell body in the spinal cord. If one were to record the electrical activity from the surface of a sciatic nerve, the recording would represent a composite of signals of many nerve fibers, greatly reduced in amplitude because the external electrodes record only a small fraction of each fiber's activity.

With the introduction of the vacuum-tube amplifier to neurophysiology in 1921 by Gasser and Newcomer, and the cathode ray oscilloscope by Erlanger and Gasser in 1924, these small composite signals could be fractionated into a number of wavelike components, each of which was attributed to a specific axon type. On the basis of these American studies, it was found that anatomically different fiber types conduct impulses at different velocities and that nerve impulses are electrical transients whose existence at any one point lasts only a few thousandths of a second. In England Lord Adrian had proposed that single axons

produce unit responses whose amplitudes are constant but whose frequency of repetition varies. It was found that any one axon transmits information by means of the frequency of the impulses propagated. For these advances, Adrian won the Nobel Prize in 1932, and Erlanger and Gasser, in 1944.

An Escape System for the Squid

During the 1930s the squid giant axon was introduced as a preparation for nerve studies and became the object of a series of intensive investigations designed to reveal the molecular machinery of the nerve impulse.

The giant axon system was evolved to enable squid to escape their enemies. The squid (Figure 2A) has two modes of movement through the water: A gentle swimming is accomplished by tail fin propulsion, and a rapid motion is achieved by true jet propulsion. The muscles activating the latter form of movement are innervated by the giant axon system. The giant fibers radiating from the two stellate ganglia receive neural messages from the brain and conduct nerve impulses to the mantle muscles initiating contraction. Figure 2B illustrates diagramatically the left stellate ganglion and the giant axon system of the squid.

To produce jetting, the muscles throughout the mantle must simultaneously receive nerve impulses triggering muscular contraction. Those axons that go to remote muscle fibers are larger in diameter than those that go to nearby muscle fibers. The relation between axon diameter and distance the nerve impulse must travel to the muscles is so precise that all muscle fibers are signaled to contract simultaneously regardless of distance from the stellate ganglia, because large fibers conduct messages faster than smaller fibers. The water volume in the mantle cavity is thus subject to a uniform increase in pressure and escapes through the siphon in a smooth jet. While serving the squid to escape its predators, these giant axons have been a boon to neurophysiologists.

The Giant Axon

Soon after Young's discovery, it was found that the squid giant axon could be isolated from the animal and that upon proper bathing in sea water it would remain active for many hours in vitro. The first important development using the isolated squid giant axon came at the Marine Biological Laboratory in Woods Hole, Massachusetts. In 1936 K. S. Cole and H. J. Curtis measured the transverse impedance of the isolated squid giant axon with a Wheatstone bridge. They determined the membrane capacitance as 1 microfarad per square centimeter of surface. This extraordinarily high value was consistent with Fricke's concept of a 25 to 75 Å thick lipid membrane with a dielectric constant of 3 to 9, separating two conducting solutions and forming an almost perfect capacitor. These measurements gave validity to the membrane hypothesis. When the electron microscope became available, it was found that the nerve membrane in common with all cell membranes was indeed about 60 Å thick.

In 1938 Curtis and Cole were able to demonstrate that the membrane resistance transiently decreased by about a hundredfold during the passage of the nerve impulse. There was little or no change in membrane capacity during this event. If the membrane resistance were related to ion permeability, then perhaps Bernstein's postulated loss in ion selectivity being responsible for the impulse might also be true.

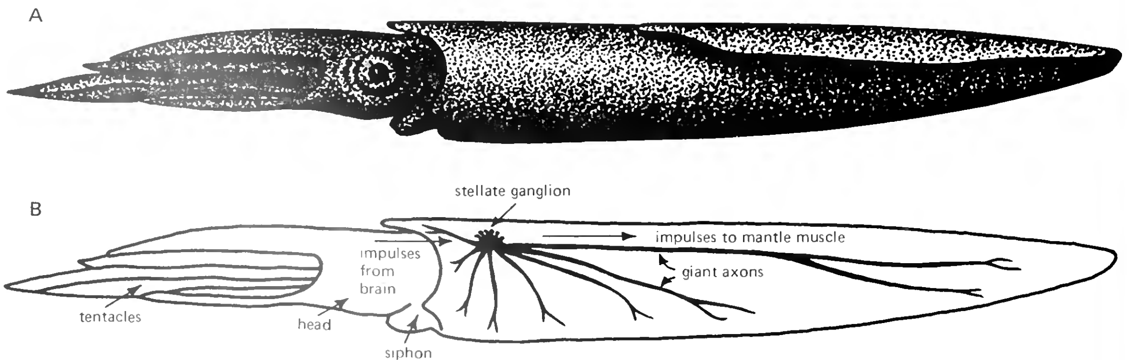

Figure 2. (A) The North Atlantic squid, Loligo pealei. *(B) Diagrammatic representation of the squid giant axon system in the left side of the mantle. Impulses from the brain activate the nerve cells in the stellate ganglion. The giant axons radiating from the stellate ganglion carry impulses to synchronously excite the whole mantle muscle, causing it to contract and eject a jet of water through the siphon, propelling the squid rapidly backwards.*

One of the great advantages of the squid giant axon is its large diameter (from 0.4 to 1 millimeter). In 1939 Curtis and Cole in Woods Hole and A. L. Hodgkin and A. F. Huxley in Plymouth, England, were able to insert an electrode inside the giant axon and measure the potential difference across the membrane between this internal electrode and an external electrode.

Prior to this all measurements of nerve potentials had been made from the outside. The internal electrode was a tiny glass tube, about 0.1 millimeter in diameter, filled with a conducting salt solution. It was pushed 2 to 3 centimeters down the axis of the nerve fiber from a cut in one end. With this electrode it was possible to test Bernstein's hypothesis that the membrane potential approached zero at the peak of the impulse. Both the Woods Hole and the Plymouth workers found that during the impulse, the membrane potential did not merely approach zero, but was reversed so that the interior of the axon became about 40 millivolts positive with respect to the external electrode. Thus one aspect of Bernstein's hypothesis was disproven. Intracellular recordings have been made in many nerve and muscle cells, in a wide variety of species, from invertebrates to mammals. The overshoot of the action potential has been found to be a general property of most neurons.

An explanation for the overshoot of the action potential was set forth by Hodgkin and B. Katz in 1949. They suggested that during the rising phase of the action potential, the axon membrane becomes highly and selectively permeable to sodium ions. This hypothesis was based on external ion substitution experiments done on squid giant axons in Plymouth during the summer of 1947, which clearly showed that the value of the overshoot is a function of the external sodium concentration. They suggested that the action potential returned from its peak by a simultaneous decline in sodium permeability and an increase in potassium permeability. Following the response, both the sodium and the potassium permeabilities would return to their resting values.

The Regenerative Process

Such time-variant permeability changes could adequately account for the appearance of an action potential at a given point on the membrane. But how does the action potential propagate down a nerve axon? The development of electrophysiology paralleled the development of the early physics of electricity. Following the appearance of Ohm's Law in 1827 and the mathematical analysis of the properties of submarine cables by Lord Kelvin in 1855, many comparisons were made between propagation of messages in nerve fibers and transmission of messages in metallic core conductors. The transatlantic cable, composed of a copper core coated with an effective but somewhat leaky insulator, was a good conductor of electricity: A pulse of electrical energy put into one end of the cable would travel down the cable with a velocity approaching the speed of light.

Nerve fibers or axons have a similar appearance to the Atlantic cable—a core conductor, the axoplasm, containing dissolved ionized salts, surrounded by an insulating lipid bilayer membrane separating the core from the extracellular fluid electrolyte. A key quantitative difference between the cable and the axon lies in the nature of the core conductors. The conduction by flow of electrons along the copper wire of the cable is much more effective than the conduction by movement of ions in solution in the axoplasm. The cable is therefore capable of much more rapid and efficient transmission of a signal.

In the 1930s and 1940s many experiments were done on both sides of the Atlantic to test the adequacy of the cable equations in describing the propagation of the nerve impulse. On the basis of these experiments, the traveling of the nerve impulse down the axon appeared to depend not only on the passive (cable) properties of the axon membrane and axoplasm but also on a regenerative (excitable) process. For without the regenerative process, a depolarizing (positive-going) voltage stimulus equal in magnitude to a normal nerve impulse, applied at a given point across the membrane, would produce virtually zero change in the transmembrane potential only a few millimeters away. Under normal circumstances, such a depolarization is sufficient to stimulate the regenerative process in an adjacent patch of membrane and set off a propagating impulse. The regenerative events require times on the order of a millisecond or two to activate, leading to propagation speeds on the order of meters per second.

In contrast, for a telegraph cable with its metallic core of very low resistance and its outer insulation of extremely high resistance, signals dependent upon only the passive cable properties may be transmitted over thousands of miles with only a small decrement in amplitude. Thus it is the "active" or regenerative process in the nerve that distinguishes it from the man-made, passive telegraph cable. Although the cable properties of nerves to produce faster-propagating impulses are improved by decreased internal resistance (through increased axon diameter) and increased resistance of the outer, insulating sheath, even the fastest-conducting

giant axons of invertebrates and the yet speedier myelinated nerves of vertebrates are dependent upon the regenerative process to produce an action potential that propagates over distances greater than a few millimeters.

The Voltage Clamp

After World War II, in Woods Hole, Cole and G. Marmont suggested two innovations using the squid axon that became routine and essential techniques in subsequent work that unraveled the events responsible for the nerve impulse. First, they inserted a fine platinum wire through the axoplasmic core. This reduced the internal resistance to such an extent that the regenerative events occurred essentially simultaneously at all points along a few centimeters of axon, enabling these events to be studied separately from the normal propagation in distance along the axon. An axon set up in this manner is said to be *space clamped.* Second, they used electronic feedback circuits to control either the voltage or the current across the membrane. In a *voltage-clamped* axon the potential difference across the membrane of a space-clamped axon is jumped to a new value chosen by the investigator and held constant while membrane currents are recorded. Conversely, under *current clamp* the transmembrane current is fixed and the voltage recorded. After a brief discharging of the membrane capacity, any changes in current during voltage clamp could thus be attributed to changes in the resistance (or its reciprocal, the conductance) of the axon membrane, the driving force being held constant. We can illustrate this by writing Ohm's Law in the following form:

current = driving force x conductance.

The equation is expressed in terms of conductance rather than resistance, since the conductance is directly proportional to the ease with which ions cross the membrane. Now, if we observe changes in current over a period in which the driving force is constant, that is, the membrane is voltage clamped at a constant voltage, the changes in current must be reflecting changes in membrane conductance. The current does change in time during voltage clamp, as may be seen from a quick preview of Figure 3. Similarly, during current clamp a voltage change implies a membrane resistance change.

In 1947 Marmont showed that it was possible to record an action potential under zero net current conditions, implying that changes of membrane resistance were a function of voltage rather than current. From that point Cole abandoned the current clamp and devoted his full attention to the voltage clamp.

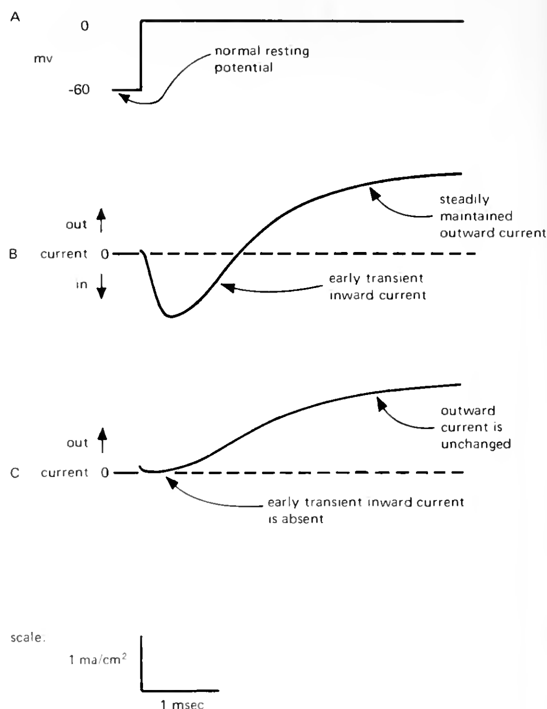

Figure 3. Horizontal axis for all parts of the figure is time. (A) Membrane voltage showing a depolarizing voltage-clamp pulse of 60 millivolts. (B) Current seen when the voltage pulse is applied with axon bathed in sea water. Notice that there are two distinct phases: an early inward flow that gives way to a maintained outward current flow for the remainder of the period that the voltage pulse is continued. (C) Current record for the same voltage pulse, this time with the axon bathed in modified sea water of greatly reduced sodium concentration. The early transient current has disappeared, but the later, maintained outward current appears to be unaffected. These data suggest that the early transient current is carried by sodium ions.

In 1949 Cole published the results of his voltage-clamp experiments. By controlling the membrane potential in discrete steps, he was able to show that positive (depolarizing) step-changes of membrane voltage rapidly discharged the membrane capacity, then produced an inward current flow that turned to a steady outward current (Figure 3B). No abrupt or threshold behavior was seen. Cole concluded that the slower outward steady-state currents were related to potassium ion flow across the membrane, but this did not explain the earlier noncapacitative transient current component. The peaks of these early transient currents were shown to be a smooth function of membrane potential with a negative slope in the voltage region between the resting potential and zero membrane voltage.

After a visit to Cole's Woods Hole laboratory,

Hodgkin took back to England Cole's technique, which he, Huxley, and Katz modified and set to work. Hodgkin and Huxley were able to demonstrate with this technique that the early transient current was carried by sodium ions through a specific membrane conductance. The delayed current was found to be carried through another conductance by potassium ions. They showed that no early transient inward current flowed when axons were voltage clamped while bathed with sodium-free solutions (Figure 3). Previous experiments using radioactive tracers, largely done in the laboratory of R. D. Keynes, had shown that sodium flowed inward and potassium outward in equal quantities during a series of action potentials. The evidence thus seemed conclusive that the specific conductances for sodium and potassium were responsible, respectively, for the rising and falling phases of the action potential as shown in Figure 4. Hodgkin and Huxley derived mathematical relations to describe the membrane currents seen in voltage-clamp experiments. Solutions of these equations predicted most classical neurophysiological phenomena.

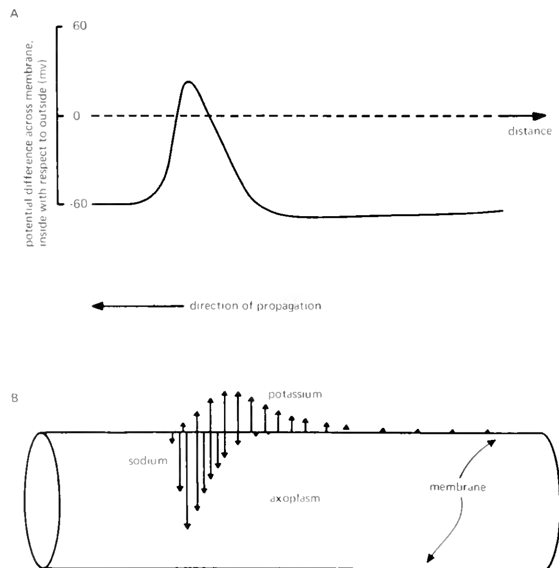

Figure 4. (A) Voltage wave-form of a propagated action potential in squid giant axon. Note that in contrast to the voltage-clamp records of Figures 3 and 5, the horizontal axis here represents distance along the axon. The direction of propagation of the impulse is from right to left. It is possible to see what the sequence of events in time *at a fixed point would be by reading the graph from left to right. (B) Arrows indicate the direction of flow of sodium and potassium corresponding to the point in the action potential aligned directly above in A. Lengths of the arrows are proportional to the conductances to sodium and potassium.*

Within a few years the voltage clamp, with minor modification, was applied to many other excitable tissues. The Hodgkin-Huxley equations, with minor variations, were used to describe the behavior of these tissues. Of particular interest was the analysis by J. C. Eccles of the behavior of nerve cells in the central nervous system of vertebrates in terms of the ionic hypothesis. For their brilliant achievements Hodgkin, Huxley, and Eccles shared the Nobel Prize in Physiology and Medicine in 1963, and Cole was awarded the National Medal of Science by the U.S. Government in 1967.

The Hodgkin-Huxley Theory

In essence, the Hodgkin-Huxley theory states that two specific voltage-dependent components of membrane conductance are responsible for the action potential. For an axon in the absence of voltage clamp, any stimulus producing a membrane depolarization beyond a low, but definite, threshold value leads to a self-enhancing increase in the conductance of the membrane to sodium. When the sodium conductance pathway or channel opens, sodium ions rush inward, down the steep concentration gradient from the high external sodium concentration to the low internal concentration. This influx causes the membrane to depolarize faster and faster. Within a millisecond or so, the potential inside the axon takes on a positive value. Hodgkin and Huxley further proposed that the rising sweep of the voltage triggered two other independent events. A turning-off process shuts down the sodium conductance until the membrane is repolarized and, somewhat more slowly, a conductance to potassium ions opens up. The potassium ions flowing down their outwardly directed concentration gradient swing the membrane potential back toward its normal inside negative value. The concentration differences for sodium and potassium ions maintained across the membrane of the resting nerve thus provide the immediate source of energy to drive the currents across the membrane during the action potential.

The Sodium Pump

Almost all animal cells are externally bathed in body fluids having a composition similar to that of sea water. These fluids contain an abundance of sodium chloride, the common table salt, as well as lesser concentrations of other salts. How then does the nerve fiber maintain a high internal potassium concentration and a low internal sodium concentration? It is now believed that the answer lies in the action of a molecular mechanism located in the cell membrane.

During the past twenty-five years, intensive studies on squid giant axons have revealed the essential details of this mechanism, which is called the sodium-potassium pump or, more commonly, the sodium pump. R. B. Dean was the first to propose this concept in 1941. In 1955 Hodgkin and Keynes found that in squid giant axons poisoned with the metabolic inhibitor dinitrophenol, the normal extrusion of sodium ions and uptake of potassium ions were greatly reduced. As the inhibitor was washed away, the sodium-potassium exchange returned to normal values. It was clear that the sodium pump was dependent upon metabolic energy, and the source was thought to be the energy-rich substance adenosine triphosphate (ATP), produced during oxidative metabolism in the cell. P. C. Caldwell analyzed squid axons for ATP and found that metabolic inhibitors reduced both ATP levels and sodium extrusion in parallel. By means of microinjection, ATP was introduced intracellularly into metabolically poisoned axons, dramatically restoring sodium pumping.

More recently, L. J. Mullins, F. J. Brinley, and P. DeWeer, working at the Marine Biological Laboratory in Woods Hole, have used an internal dialysis technique to determine the precise relations between ATP and sodium pumping in the squid giant axon. Comparison of the squid axon results with findings obtained in red cells and muscle fiber membranes has suggested that the key part of the sodium pump is an enzyme called adenosine triphosphatase, ATPase. This enzyme has been identified as being present in or associated with axon membranes. ATPase breaks down ATP, releasing energy for biological work. The internal dialysis experiments have clearly identified ATP as the immediate fuel for the sodium pump and have provided the stoichiometry for the action of membrane ATPase in promoting sodium extrusion and potassium uptake in the axon.

All animal cells studied so far have been found to have membrane-bound ion pumps. In some organs, such as the kidney, ion pumping has been refined and specialized so as to represent the major function of the organ. The squid axon studies have not only provided important information about the workings of nerve cells, but also have suggested much of value in understanding the functions of other cells and tissues.

Artificial Axoplasm

In 1960 workers in two independent laboratories made use of the squid giant axon to move a step closer to an isolated living membrane preparation, separated from the influences of cytoplasmic constituents. By reaming out a core of axoplasm, I. Tasaki and co-workers in Woods Hole internally perfused the squid axon with inorganic salt solutions flowing down the length of the axon from an inlet canula, or glass capillary tube, in one end to an outlet canula inserted in the other end. T. I. Shaw and co-workers in Plymouth, England, squeezed out the axoplasm through an opening in one end of a single squid axon by means of a roller, much like squeezing toothpaste from a tube. A canula was inserted into one end of the flattened and almost axoplasm-free axon. Upon introducing a flow of appropriate solutions into the canula, they were able to reinflate and continually internally perfuse this axon "ghost."

A number of other laboratories soon adopted the internally perfused axon to study the relationship between both internal and external ion concentrations and the membrane potential. It was soon demonstrated that internally perfused axons were excitable, conducted nerve impulses, and generally behaved similarly to intact axons. On the basis of these findings, the majority of workers agreed that bioelectrical phenomena in the squid axon were limited to the membrane. The internal perfusion studies provided the strongest support to date for the membrane hypothesis of bioelectricity. In general, membrane potentials were recorded that could be related to the experimentally controlled ionic gradients by means of an equation derived from thermodynamic considerations by D. E. Goldman in 1943.

Working in Woods Hole in 1961, W. J. Adelman, Jr., and D. L. Gilbert developed a method for voltage clamping the internally perfused squid axon. They were able to show that currents flowing across the voltage-clamped membrane were essentially the same in perfused axons as those recorded in intact axons, when the perfused axons had ionic concentrations similar to those found in vivo. These experiments therefore suggested that the ionic conductances are membrane conductances and that the ionic concentration gradients provide the immediate source of energy for the movement of ions through these conductances. W. K. Chandler and H. E. Meves, working in Plymouth a few years later, voltage clamped the internally perfused axon and found that the sodium conductance admitted ions other than sodium. The relative permeabilities of ions through this conductance were found to follow a definite sequence. A similar finding was made with respect to the potassium conductance, except that the permeability sequence was different. However, among the ions normally present in vivo, the sodium channel preferred sodium and the

potassium channel preferred potassium. Therefore, most workers still refer to the two ionic conductances as the sodium and potassium conductances. Implications of the ionic selectivity sequences for estimating the size of the ionic channels will be discussed later.

Drugs and Toxins

The Hodgkin-Huxley idea of two separable ionic currents flowing through independent channels received strong support from the discovery of chemical agents that selectively block one of the currents but not the other. The chemically related substances tetrodotoxin (TTX) and saxitoxin (STX) both block the sodium conductance, eliminating the action potential of intact nerves and the early transient currents (Figure 5C) of voltage-clamped preparations. Both TTX and STX are extremely potent nerve poisons active at minute concentrations. Both are also of vital interest to those with a taste for seafood. Tetrodotoxin, which fortunately loses its potency in the cooking process, is isolated from the puffer fish, a great delicacy in Japan. Saxitoxin is responsible for "paralytic shellfish poisoning" and is the bane of would-be clam diggers in some seasons.

A different drug, diphenylhydantoin, which has a much milder and more easily reversible blocking effect on the sodium channel, has found a valuable medical application in the treatment of epilepsy. During an epileptic attack some of the nerve cells of the brain become much more highly excitable than usual, producing wild, uncontrolled bursts of impulses that cause the convulsions typical of a seizure. Administration of a carefully calculated dose of diphenylhydantoin, which blocks a fraction of the sodium channels, helps to control the seizure and alleviate the symptoms.

The organic ion tetraethylammonium (TEA) greatly prolongs the action potential when injected inside a squid giant axon. It does so by slowing the falling phase without affecting the rate of the rising sweep of the membrane voltage. Hodgkin and Huxley argued that the falling back of the membrane potential to its resting value is brought about by an inward current carried by potassium ions. In voltage-clamp experiments, one sees that the more slowly appearing inward component of the current is blocked in the presence of TEA (Figure 5D). The remaining component of current—the rapid inward transient—closely matches that which Hodgkin and Huxley estimated to be carried by sodium ions. Conversely, in the presence of tetrodotoxin, the observed current matches that which Hodgkin and Huxley calculated for the potassium current. This turns on more slowly and, in contrast to the sodium current, is maintained more or less constant as long as the depolarizing voltage pulse is maintained.

Experiments with drugs such as TTX and TEA thus provide strong corroborating evidence for the hypothesis of independent sodium and potassium channels. In addition, these substances have been identified as valuable tools enabling researchers to study properties of one conducting channel without the observations being complicated and obscured by currents flowing through the other.

Analyses of the relation between toxin dose (for STX and TTX) and the blockage of the sodium current by B. Hille and by L. Cuervo and Adelman indicate that one toxin molecule binds to a single sodium channel to cause the block. Knowing

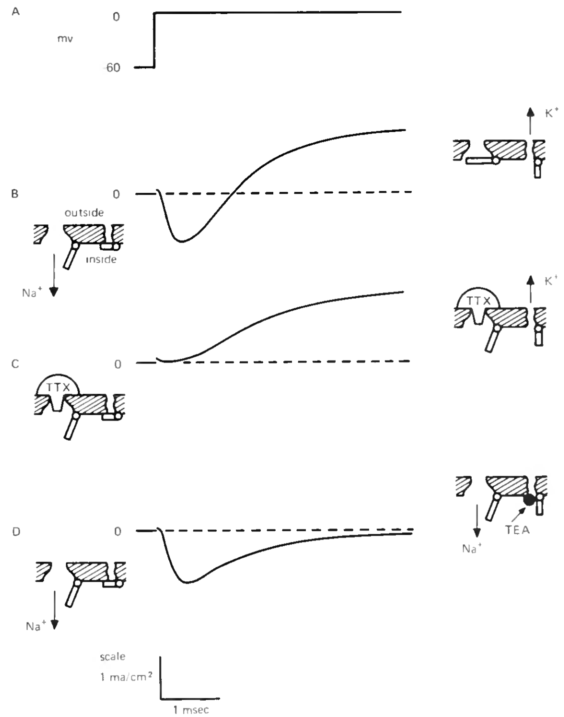

Figure 5. Voltage-clamp records from a squid axon showing separation of the current into sodium and potassium components by the use of channel blocking agents. (A) Record of membrane voltage plotted against time. (B) Normal current record as in Figure 3B. (C) Current record taken with tetrodotoxin (TTX) in the extracellular fluid. The early transient sodium current is absent. Only the potassium component remains. Compare with record in Figure 3C, obtained after removal of most of the sodium ions. (D) Current record taken after injection of tetraethylammonium ions (TEA) into the axoplasm. The sodium current is seen without the later, maintained component. The insets accompanying each current record show when the gating structures of the channel are open, and when the added chemicals are in position to block the channels.

this, investigators have used the binding of TTX to estimate the number of channels in the membrane. The first channel-count experiments were carried out on lobster axons by J. W. Moore, T. Narahashi, and T. I. Shaw. Since then values of approximately 300 channels per square micron of membrane surface have been obtained using squid giant axon. (A micron is a millionth of a meter, or about a twenty-five-thousandth of an inch.)

Knowing the total conductance of the membrane to sodium, and the density of conducting channels on the membrane surface, one simply has to divide the total conductance by the number of sodium channels to obtain the conductance of a single sodium channel. Analogous calculations have been done for the potassium channels based on their blockage by tetraethylammonium ions.

In much of the foregoing discussion the sodium and potassium "channels" have been mentioned as if they were indeed minute holes through the membrane. An alternate hypothesis is that the functional conducting unit is a molecule, free to move across the membrane lipid, and able to bind ions on one side, carry them across the membrane, and release them on the other side. Such a carrier hypothesis is made unlikely by the values for the unit "channel" conductances that have been estimated. The values calculated for the single-channel conductances are higher than one would expect for a single carrier moving back and forth across the membrane. As the best current hypothesis, then, we are left with the idea that the sodium and potassium channels are actually holes through the membrane possessing "doors" or "gates" that are opened and closed by changes in the membrane voltage.

How Many Channels Make a Light Bulb?

The channels about which we write are tiny conductors that carry electric currents, as flows of sodium or potassium ions, across an insulating membrane two fat molecules thick. Common inanimate conductors are made of wire, so in order to obtain an intuitive feel for the magnitude of a single-channel conductance, let us ask how many channels it would take to conduct current as readily as a single 40-watt bulb from an ordinary desk lamp, powered from a 120-volt supply. Knowing the following relation

electrical power (watts) = voltage x current (amperes),

we can calculate that the current carried by the light bulb filament is 1/3 ampere. Using Ohm's Law, which states that

voltage = current (amperes) x resistance (ohms),

we found the resistance of the filament to be 360 ohms. To convert this to an equivalent conductance, one simply turns the number upside down and spells the name of the units backwards. Thus, the conductance of the light bulb is

$$1/360 = 2.8 \times 10^{-3} \text{ mhos.}$$

The conductance of a single sodium channel, estimated as described in the previous section, is approximately 10^{-11} mhos. It would therefore require 280 million channels in parallel to conduct current as readily as a 40-watt light bulb.

Going a step further, to compare the size of the current carried by the light bulb to that conducted by the axon channel, one must also compare the voltages driving the currents. The voltage pushing the ions through a sodium channel might reach up to about 120 *milli*volts, or thousandths of a volt, as compared to the 120 *volts* for the desk lamp. This means that the current through a 40-watt desk lamp filament is about 280 billion times greater than the maximum current flow through a single nerve membrane channel during an action potential.

This calculation shows emphatically that the currents flowing in single channels during a nerve impulse are miniscule compared to the currents passing through everyday electrical appliances. It is well that this is so, for with the astronomical number of impulses continually being conducted, we would not otherwise be able to meet by food intake the metabolic energy requirements of our nervous systems.

Future Directions

The following sections provide a glimpse of recent work and something of our own sense of anticipation and excitement for what is to come. On first contact with the work of Hodgkin and Huxley, one is apt to be struck by its remarkable completeness. What more could be said about the axon's workings? Digging deeper into their papers, one can hardly help but marvel at the breadth of the horizons opened up by their incisive questions, questions born of their amazing foresight and grasp of the problem, yet unanswerable with the experimental resources of their day.

Membrane Noise

At present it is not possible to observe single-channel openings and closings in any biological membrane. Some information, however, may be obtained from the analysis of the myriad fluctuations in current and

voltage records from the squid giant axon and other preparations. One of the several sources of these fluctuations, or noise, is presumably the flip-flop opening and closing of channels. The object of the noise analysis is, first, to identify these components, then to interpret them in terms of the molecular events. Begun in the mid-1960s by A. A. Verveen and H. Derksen at Leiden, Netherlands, this task will doubtless occupy investigators for a number of years to come.

Channel Selectivity and Size. How Many Channels Can Be Packed in a Pinhole?

Recently experimenters have begun to ask how the open channels are able to distinguish between different ions, selectively allowing either sodium or potassium to rush across the membrane.

By measuring currents under voltage clamp with axons bathed in solutions in which the sodium or potassium ions normally present have been replaced by other ions, one can show that the sodium and potassium channels are not absolutely selective. A number of small organic and inorganic ions are able to replace sodium and carry measurable currents through the sodium channel. From an elegant study using a series of organic ions of different sizes and shapes, B. Hille was able to suggest that the sodium channel must be about 3 Å x 5 Å at the narrowest portion, which limits the movement of ions through the channel. This conclusion follows from the observation that all of the ions able to pass through have planar structure. Drawings of scale models of two different ions illustrate this point (Figure 6). The larger ion, guanidinium, is readily able to carry current through the sodium channel, whereas the smaller, more symmetric ion, methylammonium, carries no current. This is presumably because the planar arrangement of atoms in the larger ion enables it to pass through a narrow channel of about 3 Å width, where the methyl ($-CH_3$) group of methylammonium is too big and hence is excluded.

Just how big is a channel of 3 Å x 5 Å? An ordinary household sewing pin is about half a millimeter in diameter. If we could cut out a series of templates, 3 Å x 5 Å, to just fit inside our hypothetical sodium channel, and then place a series of these templates edge to edge across a pinhole, we would need about one and a half million to go from one side to the other. To cover the entire opening of the hole would require about one billion of the channel-sized templates.

Studies like those described above suggest that the potassium channel is probably more symmetric and about 3 Å in diameter at its narrowest point. It is important to note, however, that the

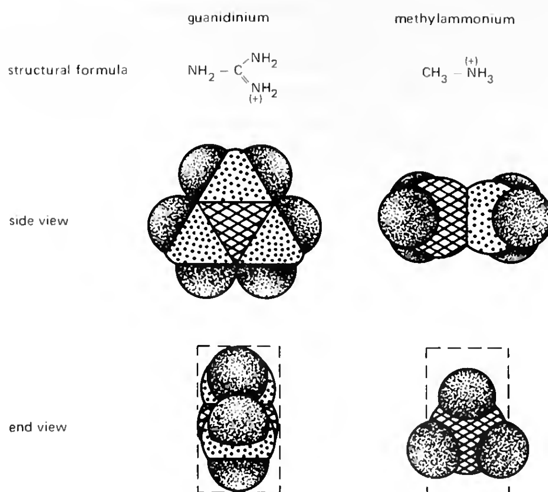

Figure 6. Molecular models give a clue about the structure of the sodium channel. The larger ion on the left, guanidinium, passes through the sodium channel fairly readily, whereas the smaller ion on the right, methylammonium, is impermeant, that is, does not pass through. On such evidence it is suggested that the sodium channel has a roughly rectangular cross-section, measuring about 3 Å x 5 Å. In the diagrams of the ionic models, cross-hatched atoms are carbon (C); stippled atoms, nitrogen (N); and shaded atoms, hydrogen (H).

relative size and shape of ion and channel are not sufficient to determine, in all cases, which ions pass through, though it seems certain that channel size does set an upper limit to the size of permeating ions. At first consideration, it might seem paradoxical that neither lithium, an ion substantially smaller than potassium, nor cesium, which is considerably larger than potassium, can pass through the potassium channel. Indeed, experiments suggest that both of these ions can enter the potassium channel but that they get stuck before passing completely through, thus blocking the passage of potassium, too, if it is also present. This occurs even though both these ions are closely related by their chemical properties to potassium.

Research on the way the channels select among similar ions such as sodium, lithium, potassium, and cesium—all are singly charged metal ions belonging to the same chemical group—is complicated by the fact that there is, associated with the ions in aqueous solution, a cloud of loosely bound water molecules. At least some of these molecules of "water of hydration" must be stripped off a sodium or potassium ion before it passes through a channel. In all probability, the ion passing through the channel binds loosely and momentarily to the channel wall in transit, before being reenveloped in a watery cloud on the other side of the membrane. The experimental task of

gaining insight into the details of these processes is extremely difficult, but it is of fundamental importance to a complete understanding of the workings of the excitable membrane.

The reader might well inquire about the possible benefits of studying the effects of ions to which a normal nerve is never exposed. Such studies could have significant medical implications. Lithium, in recent years, has become an important stabilizing drug for the treatment of certain forms of manic depression. It is far from clear at present just what significant changes are wrought by the administration of chronic small doses of lithium. Certainly the drug would be expected to modify neuronal activity, but to what extent and in how many ways remain open questions. It is not even known whether the significant medical effect of lithium is due to its direct influence on the cells of the nervous system. But perhaps basic studies of its effects at the level of the single neuron, using such an ideal experimental preparation as the squid giant axon, may give information allowing its use to be safer and even more effective in the future.

Gating Currents

Hodgkin and Huxley proposed that there were charged molecular entities responsible for the opening and closing of the ionic conductance pathways. These structures had to be charged to be able to move in response to changing electrical forces when the membrane voltage changed. Any movement of the gating structures would be a movement of charge and, therefore, should have been detectable as a component of the current flow across the membrane. Since the investigators could not distinguish any gating current in the presence of the relatively large currents that they observed, they simply concluded that the gating current was too small to be measurable.

Some twenty-one years and many technical advances later, this conclusion was vindicated by C. M. Armstrong and F. Bezanilla at Woods Hole, where they were able to record a tiny transient current associated with the opening of the sodium channel (Figure 7). The observations were confirmed recently in Plymouth. As this lively investigation continues on both sides of the Atlantic, we can expect work on the squid giant axon to shed further light on the secrets of the function of the nerve cell membrane.

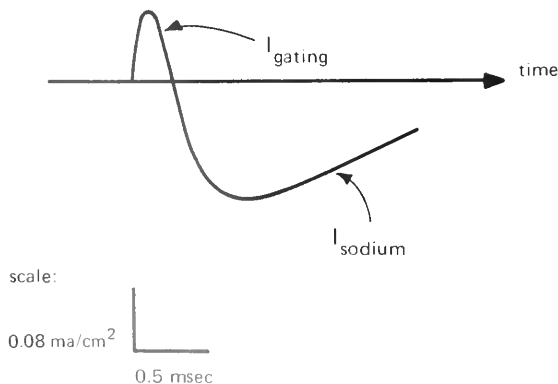

Figure 7. Gating current of the sodium channel. To gain a feel for the size of the gating current, notice that the sodium current values are only about 1/10 of those in Figures 3 and 5. External sodium concentration was reduced to lower the sodium current and facilitate the detection of the gating current. This type of record is obtained by the averaging of many signals to remove noise, a technical feat not possible at the time of Hodgkin and Huxley's work. (After C. M. Armstrong and F. Bezanilla, "Currents related to the movement of gating particles of the sodium channels," Nature *(London), vol. 242, 1973, pp. 459-61.)*

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Bioluminescence

Awak'd before the rushing prow,
The mimic fires of ocean glow,
  Those lightnings of the wave;
Wild sparkles crest the broken tides,
And flashing round, the vessel's sides
  With elfish lustre lave;
While far behind, their livid light
To the dark billows of the night
  A blooming splendour gave.

Sir Walter Scott
The Lord of the Isles

by J. Woodland Hastings

Anyone who has gone swimming or boating at night in the ocean has seen the beautiful sparkling luminescence, the so-called phosphorescence that occurs after the water is disturbed. The true image of its sometimes brilliant and even spectacular appearance is difficult to capture, but it has been well represented in poetry, prose, and painting. Collecting from red tides off Southern California, one sees the undulating luminescent pattern left behind by fish fleeing as the boat approaches. World War II aviators based on carriers in the South Pacific tell of the ease with which they relocated their ship after a mission: a luminescent wake extends many miles behind a ship as a consequence of the persistent turbulence that stimulates the luminescence of the cells. A submarine or a diver under water may be located just as easily by the luminescent track. More poetic prose is found in C. W. Thomson's *Voyage of the Challenger* (volume 2, page 85, 1877) as she passed southeast of the Cape Verde Islands:

From the time we entered the current, immediately after leaving the Cape Verde Islands, the sea had been every night a perfect blaze of phosphorescence. The weather was very fine, with a light breeze from the south-westward. There was no moon, and although the night was perfectly clear and the stars shone brightly, the lustre of the heavens was fairly eclipsed by that of the sea. The unbroken part of the surface appeared pitch black, but wherever there was the least ripple the whole line broke into a brilliant crest of clear white light. Near the ship the black interspaces predominated, but as the distance increased the glittering ridges looked closer, until towards the horizon, as far as the eye could reach, they seemed to run together and to melt into one continuous sea of light. The wake of the ship was an avenue of intense brightness. It was easy to read the smallest print sitting at the after-port in my cabin; and the bows shed on either side rapidly widening wedges of radiance, so vivid as to throw the sails and rigging into distinct lights and shadows.

For many years the origin and nature of this "phosphorescence" were debated. (True phosphorescence, unlike bioluminescence, depends on prior absorption of light.) Descartes thought that when a wave hit an obstacle, the agitation imparted to the salt particles caused them to separate from the water particles and to "generate sparks" similar to those emitted by flint. Over the years, many other ideas and variations thereon were put forward, including theories involving electricity, phosphorus, and putrefaction. Although many serious students were concerned with the question, including Robert Boyle, J. J. D. de Mairan, and Benjamin Franklin, it is significant that none of the great microscopists of the period (Malpighi, Leeuwenhoek, and Hooke, for example) recorded

any interest or observations concerning the phenomenon. It was not until the end of the eighteenth century that luminescence of the sea was definitely attributed to living organisms, and even then dispute continued, both as to whether or not all cases could be so explained, and how living organisms are capable of emitting light.

Marine bioluminescence is mostly due to the dinoflagellates–microscopic organisms, ubiquitous in the oceans of the earth, that are sensitive to the movement of the water and respond with bright and rapid (1/10 second) flashes (Figure 1). (The same kind of organisms are responsible for "red tides"–massive blooms of a single species.)

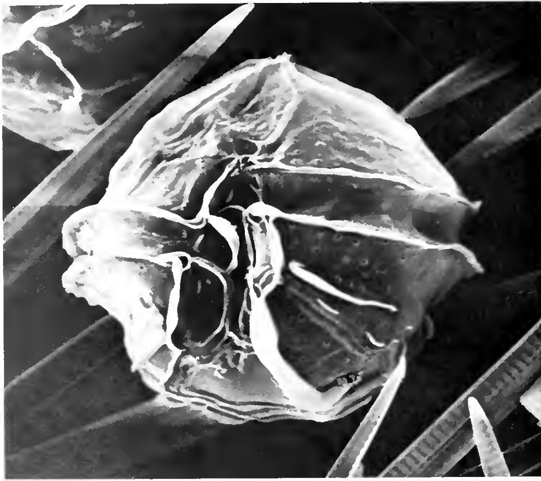
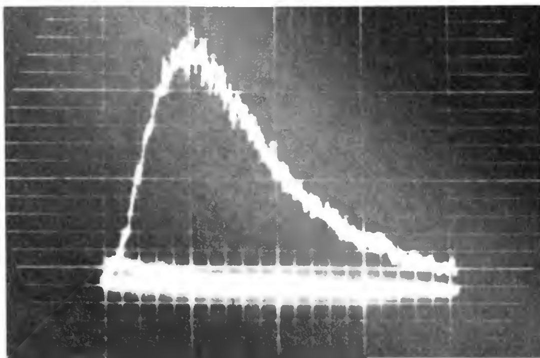


Figure 1.(Above) Scanning electron micrograph of Gonyaulax acatenella, *ventral view. Magnification is 1460X Material collected during a red tide in Malaspina Inlet, B. C., Canada, on June 10, 1965. Bioluminescence occurs as a rapid bright flash due to mechanical or electrical stimulation. (Below) Luminescent flash of the dinoflagellate* Noctilaca. *A single cell is isolated in a test tube and stimulated. Ordinate is light intensity; abscissa, time. One division equals 2 milliseconds. (Micrograph courtesy of A. R. Loeblich III and L. A. Loeblich. From* Proceedings of the First International Conference on Toxic Dinoflagellate Blooms, *edited by V. R. LoCicero, Massachusetts Science and Technology Foundation, 1975.)*

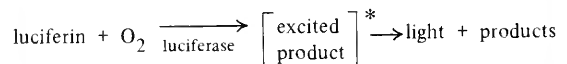


But dinoflagellates are not the only luminous organisms. Table 1 gives a condensed listing of the many different types, and although there are some terrestrial representatives, such as mushrooms, fireflies, and earthworms, the greatest number of luminous species occur in the oceans, often in places or at depths not easily reached. Actually this abundance of luminous species in the marine environment, as compared to fresh water (where only one species is known) or land, is one of the major unexplained facts, one that may hold the key to some of the unanswered questions concerning the origin, evolution, and function of bioluminescence.

The impressive number of luminous species, apparently independently evolved, is indeed a testimonial to the evolutionary process. Representatives are widely distributed among most invertebrate phyla (Table 1); among the vertebrates, only fish have light-emitting capability, and there it is widely and variously developed (Figure 2). No amphibia, reptiles, birds, or mammals possess the property; and except for the dinoflagellates, some of which are photosynthetic, there are no luminous green plants.

'Cold' Light

In the physical sense, luminescence is the emission of light without heat. Chemically it involves a special type of chemical reaction in which the energy, instead of being released as heat (as occurs in most chemical reactions), is used for the specific excitation of a product molecule. This excited molecule then releases the energy, in one big step as a photon–much greater than the energy steps in most cellular reactions. Bioluminescence is a special type of chemiluminescence because the chemicals involved in the reaction are synthesized by living cells and, more especially, because each of the reactions involved is catalyzed by an enzyme (a biological catalyst). Thus, as shown in the scheme, a generalized bioluminescent reaction involves the oxidation of a substrate, referred to as luciferin, catalyzed by an enzyme, called luciferase. The



$$\text{luciferin} + O_2 \xrightarrow[\text{luciferase}]{} \begin{bmatrix}\text{excited}\\ \text{product}\end{bmatrix}^* \longrightarrow \text{light} + \text{products}$$

Bioluminescence involves a chemical reaction in which the energy released is emitted as light without heat. The enzyme luciferase catalyzes an oxidation of luciferin, giving an "excited" product, designated by an asterisk, that emits a photon as it loses this energy.

Type of Organism and Common Names	Genus	Color	Type, Biochemistry, and Function of Display
Bacteria	*Photobacterium* *Beneckea (Vibrio)*	Blue-green	Steady bright glow. Oxidation of reduced riboflavin phosphate. Function as symbionts. Possibly also some non-symbionts.
Fungi, mushrooms Jack-my-lantern Moonlight mushroom Coffee leaf fungus	 *Clitocybe* *Pleurotus* *Armillaria*	Yellow-green	Steady dim glow. Chemical basis unknown. Function unknown.
Dinoflagellates	*Gonyaulax* *Noctiluca* *Peridinium*	Blue	Rapid (0.1 sec) flash after stimulation. Luciferin oxidation. Function uncertain; "alarm" hypothesis.
Coelenterates Jellyfish Hydroid Sea pansy	 *Aequorea* *Obelia* *Renilla*	Green and blue	Train of rapid flashes, sometimes exuded material. Photoproteins stimulated by calcium. Functions to frighten; perhaps other uses.
Annelids Marine polychaete Syllid fireworm Earthworm	 *Chaetopterus* *Odontosyllis* *Diplocardia*	 Blue Blue Green	Exuded as a slime, some effect of stimulation. Some knowledge of chemistry. Function uncertain; may frighten predators.
Molluscs Limpet Clam Squid	 *Latia* *Pholas* *Heteroteuthis*	 Green Blue Green and red	Several different types: exuded luminescence, photophores. Some chemical knowledge. Functions differ.
Arthropods Crustacea Ostracod Shrimp	 *Cypridina* *Meganyctiphanes*	Blue	Squirt enzyme and substrate into sea water. Some flashing. Chemical basis well understood in some forms. Diverts or frightens predators.
Insects Coleoptera Firefly Railroad worm	 *Photinus* *Phengodes*	 Yellow Green and red	Rapid flashes, elaborate signal systems. Luciferin oxidation requires ATP. Communication in courtship, and other functions.
Diptera New Zealand glowworm	 *Arachnocampa*	Blue	Exuded slime, but control can be exerted. Chemistry poorly understood. Attracts prey.
Chordates: fish Cartilaginous Sharks	 *Squalus* *Spinax*	Green	Emission from surface. Chemistry and function unknown.
Bony Pony fish Flashlight fish Knight fish Angler fish	 *Leiognathus* *Photoblepharon* *Monocentris* *Ceratioid*	Blue-green	Emission from specialized organs that culture luminous bacteria. Chemistry the same as bacteria. Control of light by mechanical shutters. Functions differ.
Midshipman Myctophid Hatchet fish	*Porichthys* *Myctophum* (deep sea) *Argyropelecus* (deep sea)	Blue	Emission involves rapid flashing (nervous control) or glow (possibly hormones). Luminous system synthesized by fish. Chemistry largely unknown. Functions differ.

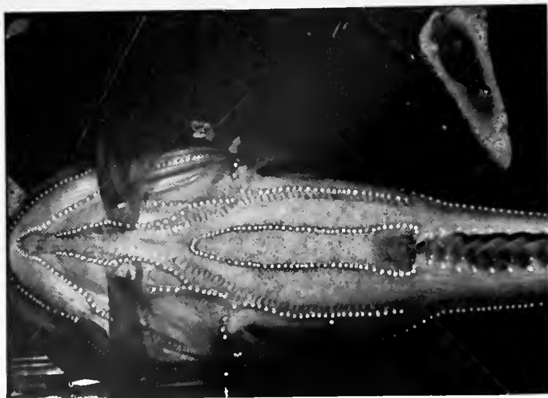


Figure 2. Ventral view of the midshipman fish, Porichthys, *showing the numerous (about 800) pearl-like photophores, whose arrangement like the buttons on a military uniform is responsible for its common name. Normal control of the luminescence is believed to be hormonal; light emission occurs not as a flash but as a longer-lasting glow. The substrate (luciferin) for this luminescence has recently been shown to be obtained in the diet, by the eating of luminous crustacea. In regions where these crustacea do not occur, the fish is nonluminous, even though it possesses both the photophores and the enzyme luciferase. (J. G. Morin)*

chemical structures of luciferins and luciferases differ among groups of organisms, as do the details of the reactions and their specific requirements. Also, the color of the light may vary, ranging from the deep blue of shrimp and dinoflagellates to yellow and even red in fireflies and the railroad worm. This color difference is believed to be significant with respect to the specific functions of different organisms.

Functions of Bioluminescence

The ability to emit light is evidently a valuable and versatile property. For the organisms, light emission seems to serve three functions: offense, defense, and intraspecies communication. In the first case bioluminescence seems to aid in attracting and capturing prey. An elegant example is the angler fish, which possesses a luminescent organ that, as the name implies, acts like a fishing lure: it dangles in front of the mouth at the end of a long filament projecting from the top of the head, and when an interested predator investigates, he promptly becomes prey (Figure 3). Especially interesting is the fact that the actual light comes from luminous bacteria that are cultured by the fish in a special compartment. Similar symbiotic arrangements occur in other (but not all) fish (Table 1) and in some squid. There is an unusual group of dipteran insects in which the carnivorous larvae trap prey, the most celebrated being the New Zealand glowworm, a famous tourist attraction at the Waitomo Caves (Figure 4). The animals attach to the ceiling of the cave and exude long, sticky, luminescent threads that apparently function to attract and catch insects.

Luminescence also appears to be used in many cases and in several different ways to help avoid predation. In the waters off the coast of Japan, there is a small ostracod crustacean, *Cypridina hilgendorfii,* that synthesizes its enzyme and substrate in two separate glands. Upon excitation, these glands squirt their contents into the sea, where the chemical reaction occurs, apart from the animal. This is probably used as a diversion in a type of "squirt and run" behavior. They squirt the luminescence into the water and dart elsewhere, thereby deceiving the predator. Certain deep-sea squid use a similar technique. In the darkness of the depths a black "ink" secretion could be of no value, whereas a luminescence, manufactured in the ink sac, can readily divert or deceive would-be predators.

Communication is a third and quite distinct function of bioluminescence. In the Bermuda fireworm (*Odontosyllis*), a truly extraordinary display occurs as the males and females engage in mating, shedding eggs and sperm in a circle of brilliant luminescence. In fireflies it is well known that flashing is used for signaling during courtship and mating. In European species the partners apparently simply locate one another by the light. In the Western Hemisphere, however, there is a well-defined and species-specific signal system in which the individual female is recognized by the male by her precisely timed response to his own

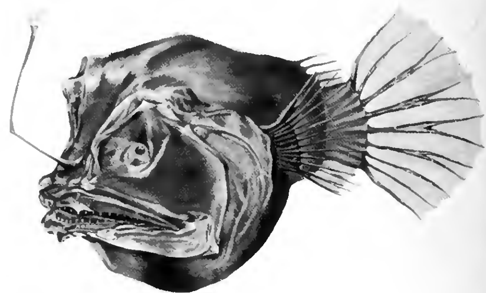
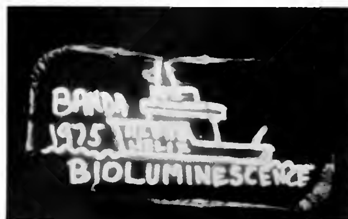


Figure 3. The deep-sea angler fish Oneirodes eschrichtii *Lütken, 1871. Specimen on which drawing is based was about 10 centimeters long, from tip of snout to base of tail. The luminescent organ, or esca, at the end of the modified first dorsal fin spine, inserts on the tip of the snout and serves to attract prey. (Drawing by J. Nakanishi. From T. W. Pietsch, 1974,* Natural History Museum of Los Angeles County, Science Bulletin *18:1-113.)*

Bioluminescence Aboard R/V Alpha Helix



Luminous bacteria were used to make this memento of Alpha Helix*'s Banda Islands cruise. Photographed by its own light, the message was "painted" with a few cells that grew into this rich culture overnight. (J. G. Morin)*

Over the past six years there have been two major scientific expeditions based on the R/V *Alpha Helix,* a ship built expressly for biological and biochemical research. Supported in large part by the National Science Foundation and managed by Scripps Institution of Oceanography, these cruises have allowed scientists to carry out sophisticated experimental research in more remote areas of the world where some of the unusual bioluminescent species are found.

In 1969, under the leadership of John Buck (National Institutes of Health), an expedition was made to New Guinea. Twenty scientists from several countries (the U.S., Australia, France, Japan) joined together in research focused on many different aspects of bioluminescence. Ten of us usually worked in a small but highly efficient "field" laboratory set up in a small concrete-block building in the mission at Maiwara, about 24 kilometers from Madang. Since the laboratory was only about 30 meters from the dock, there was easy access to the ship and expeditions out to sea; small work boats were used to make collections in lagoons and on the coral reef.

Several of us studied the pony fish and other marine luminous species, including dinoflagellates. In research on the pony fish, we were able to demonstrate for the first time that the luminous bacteria known to be maintained within the light organ were all luminous and all viable, packed in at a density of about 100 billion per cubic centimeter. Buck and his colleagues studied the synchronously flashing fireflies (page 24, Figure 5)—a phenomenon that has provided a major new experimental system for biologists interested in rhythmical events. Using fireflies brought into the laboratory, and an artificial tree with small electronically controlled lights to simulate firefly flashes, the scientists were able to demonstrate the endogenous rhythm of flashing and to show that phase shifting and mutual entrainment result in the remarkable synchrony.

In 1975 James Case (University of California, Santa Barbara) organized a second *Alpha Helix* cruise to study bioluminescence. This time we went farther west, to Indonesia, specifically to the Banda Islands where the famous flashlight fish, *Photoblepharon* (page 25, Figure 6), and its close relative *Anomalops* occur together and in great abundance. Again, a shore-based laboratory was established only a short distance from the dock, allowing for expanded experimental programs based on material collected both from midwater trawls and from more shallow water.

But much of the research was carried out in the ship's laboratories, which themselves are extensive and well equipped. For example, in our experiments with the pony fish, we set up fish tanks aboard ship (while underway) in a dark room with a variable-intensity blue light overhead. A photometer was used to measure the intensity both of this light and of the fish bioluminescence (the two could be measured separately). It was found that the brighter the overhead light, the brighter the bioluminescence. This bioluminescent emission thus can serve to match the background light, thereby obscuring the animal's silhouette and protecting it from predation.—J.W.H.

flash, the pattern of which she, in turn, recognizes. In Southeast Asia there is yet another behavior: a population of males of a given species takes up residence in a tree. They then flash in precise synchrony once every second or so (depending on the species) throughout the night, apparently attracting females to that particular tree (Figure 5).

A most extraordinary case, which I have had the occasion to study in recent years, is the flashlight fish, *Photoblepharon.* These fish occur over a wide range in the Indo-Pacific region, from the coral reefs of the Sinai Peninsula, to the Comoro Islands off Madagascar, to the Banda Islands in Indonesia (see box). In these fish the organs of bioluminescence are situated, almost like headlights, just beneath the eyes, with lids that close from bottom to top to extinguish the light, and contain a rich culture of truly brilliant luminous bacteria (Figure 6). Like a flashlight, this light organ appears to be highly versatile, being used under different circumstances for all three major functions discussed above. To capture prey the fish aggregate on dark nights with their lights on, attracting and feeding on small crustacea that are positively phototactic. To escape predators the fish engage in a highly effective "wink and run" technique, somewhat analogous to the "squirt and run" behavior mentioned above. Swimming in a zigzag manner, the fish have their lights on during the "zig"; then in in a wink, with lids closed and lights off, they change direction ("zag") and swim away. For communication the light is used both by male-female pairs and by the large aggregations (usually from 50 to 200 individuals).

All of the above functions involve the emission of light during the night. Another and somewhat different function has been suggested for certain fish and other organisms that have the ability to emit light from their ventral side. During the day the silhouette of an animal seen from below (as in the ocean) could be obscured if it emitted light to match exactly the intensity and color of that coming from the sky. The pony fish (*Leiognathus*) of the Indo-Pacific appears to be an example. It cultivates symbiotic luminous bacteria (Figure 7) in an organ surrounding the esophagus, deep inside the body. Through an elaborate optical system, which involves a slit to regulate intensity, a

Figure 4. The famous glowworm of New Zealand's Waitomo Caves, which is actually the larva of the dipteran fly, Arachnocampa luminosa. *From the ceiling, where they cling, the animals exude a sticky threadlike substance, attracting positively phototactic flying insects, which are then trapped, reeled in, and devoured. One worm may dangle as many as 70 threads. (Photo by Paul Zahl, © National Geographic Society)*

Figure 5. Mangrove tree in Malacca where fireflies aggregate and engage in synchronous flashing throughout the night. It is speculated that [illegible] are attracted to the tree. Photographed by a double exposure (to get the tree by day and the fireflies by night). Long exposure of luminescence shows the dotted tracks of flying fireflies, flashing regularly. (Photo by Ivan Polunin © National Geographic Society)

swim bladder modified to serve as an integrating sphere, and muscle fibers that function as fiber optics, the light is emitted as a glow distributed over the entire ventral surface. I have also had the opportunity to study this fish, both in New Guinea and in Indonesia, and our recent experiments during the *Alpha Helix* expedition (see box) have shown that the ventral bioluminescence is indeed stimulated by and proportional to light coming from above.

Bioluminescent Systems as Research Tools

For the medical research scientist the study of bioluminescent systems has many facets and important implications above and beyond the basic biology and biochemistry of the phenomenon itself. At the level of molecular biology, bioluminescence involves a fundamental process by which chemical energy is converted to light energy. This is of basic importance, both as a physiochemical process and as it relates to the more general phenomenon of energy conversion and utilization by living organisms. All life depends on chemical energy for its maintenance, and all life processes involve the conversion of this energy from chemical energy to other forms, be they muscular, electrical (nervous function), or transport (kidney function) in nature. In none of these cases is the basic nature of the energy conversion process yet understood; luminescence provides a model system for this basic problem that is accessible to highly sophisticated experimental analysis.

Even more extensive are the possibilities that bioluminescent systems offer as probes to investigate other problems in medicine, ranging from pharmacology to anaesthesiology. Basically, in bioluminescence research the methods of measuring light are simple and rapid, as well as extremely sensitive, and provide instantaneous, continuous, and faithful information concerning the status of the system being studied. One feature

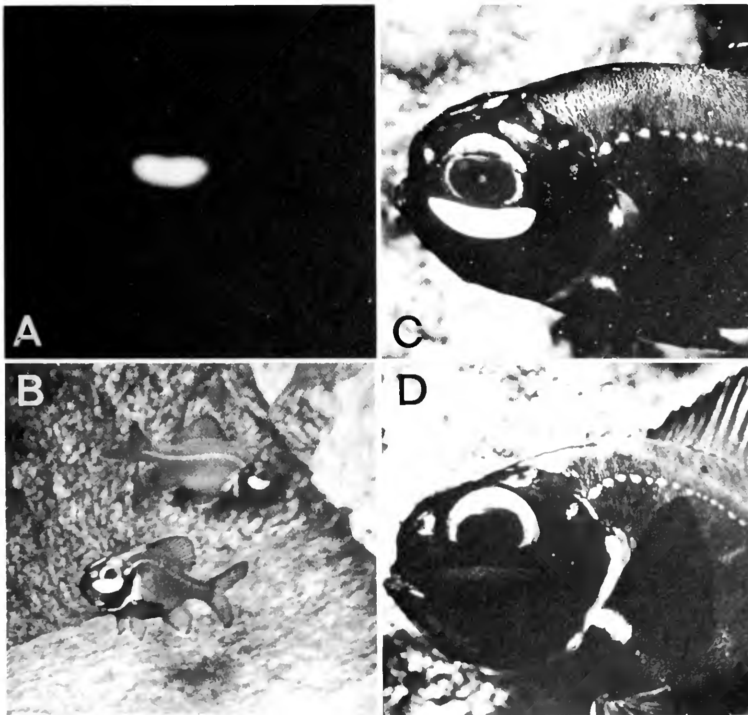


Figure 6. The flashlight fish, Photoblepharon palpebratus, *photographed at night along the reefs in the Gulf of Elat, Israel, by the light emitted from its own luminescent organ (A) and with an underwater strobe light (B, C, and D). The reflective areas of the lateral line, the edges of the fin rays, and the operculum are not luminescent. (B) A pair of* Photoblepharon *in their intertidal territory. (C) Close-up of* Photoblepharon *with the lid of the luminescent organ open. (D) Close-up with the lid closed. Fish are about 6.5 centimeters long. (Photos by J. G. Morin. From J. G. Morin et al., "Light for all reasons: versatility in the behavioral repertoire of the flashlight fish,"* Science, *vol. 190, 3 October 1975, p. 74. Copyright 1975 by the American Association for the Advancement of Science.)*

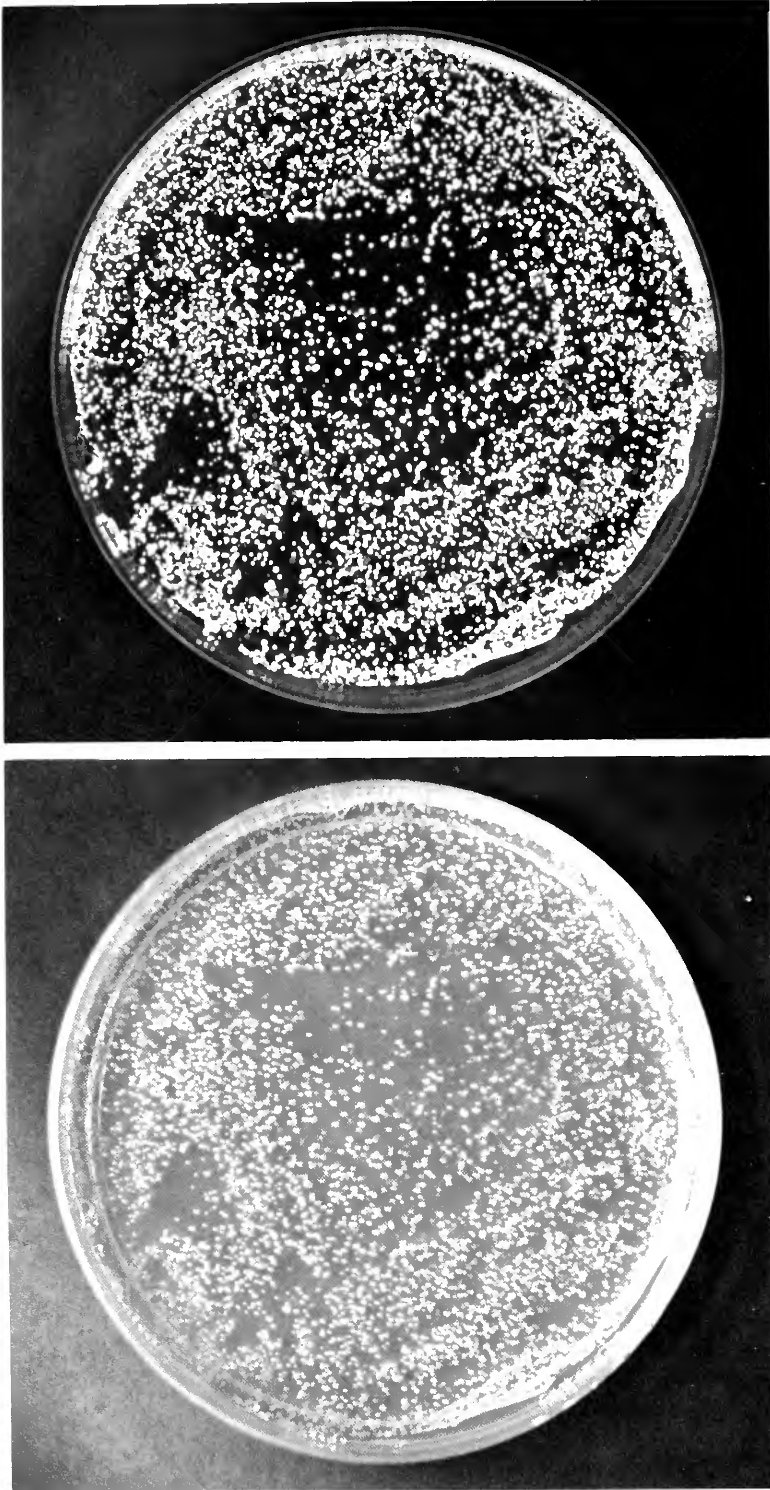


Figure 7. Colonies of luminous bacteria photographed by their own bioluminescence (top) and by room light (bottom). Such bacteria can be isolated directly from special light organs of certain fish, such as the pony fish of the Indo-Pacific, and can also be grown in the laboratory for biochemical and genetic studies. Colonies shown here are from laboratory cultures. (J. W. Hastings)

in particular is important: scientists can actually observe the activity level of the luminescent system *in a living cell,* without disturbing the organism. With any other enzyme one must perturb the cell in some way or break it open in order to determine enzyme content, and even then it is enzyme *content* that one measures, not the physiological activity of the enzyme in the cell at the time in question. Bioluminescence allows one to see into the cell. An excellent example is the recent demonstration that luminous bacteria are dimmed by anaesthetics almost precisely in proporation to their anaesthetic potency in man. Here the living cell can be used to evaluate dosage, but more than that, extracts may be used to pinpoint the site and mode of anaesthetic action, neither of which is yet known—in 1975, some 150 years after the discovery of ether. Luminous bacteria that live as symbionts have also been shown to produce antibiotics, apparently to prevent the growth of nonluminous contaminants in the organ. These antibiotics are presently being screened and evaluated for their potential use in treatment of disease.

At the level of more practical application, there has already been extensive use of bioluminescent systems. Different systems require different biochemical factors and cofactors for activity, and these are usually highly specific. Many bioluminescent systems can thus be used as a sensitive analytical method for determining one or more of these specific factors. The best known and most often used system is the firefly luciferin and luciferase, which specifically requires adenosine triphosphate (ATP)—the central nucleotide in energy metabolism in all organisms. The materials for estimating the amount of ATP by this system are now available commercially in kit form and are used in many laboratories. The method is routinely employed in testing for heart disease and in several other clinical situations. Levels of ATP can be detected one million times lower than by conventional methods, and very few, if any, other substances interfere.

Equally interesting is a protein, aequorin, extracted from the jellyfish (coelenterate) *Aequorea,* which requires calcium for activity. With the use of microinjection techniques, aequorin has been placed inside a muscle cell, where it flashes as the muscle contracts, concomitant with and faithfully reporting intracellular movements of calcium related to the contractile process. This method has also been used in measuring calcium in many other medical and clinical applications.

An inspection of the biochemical basis for some of the other bioluminescent systems will show that there are indeed many other substances for which analytical determinations are possible using light emission as the output—and indeed quite a number of these are already in use.

Bioluminescence, although sometimes considered of trivial scientific and social relevance, is in fact providing important contributions in fields ranging from medicine to molecular biology and evolution. Moreover, luciferases provide examples of an entirely novel function of enzymes. Luminescent reactions often involve precise controls, for example, in cases where the kinetic pattern of the flashing serves as a distinguishing signal for the species and for the sex. In these cases there is no turnover of the luciferase in the classic sense. Instead, it involves a molecular control on the rapid initiation and termination of a reaction, a physiological activity not yet elucidated in other areas.

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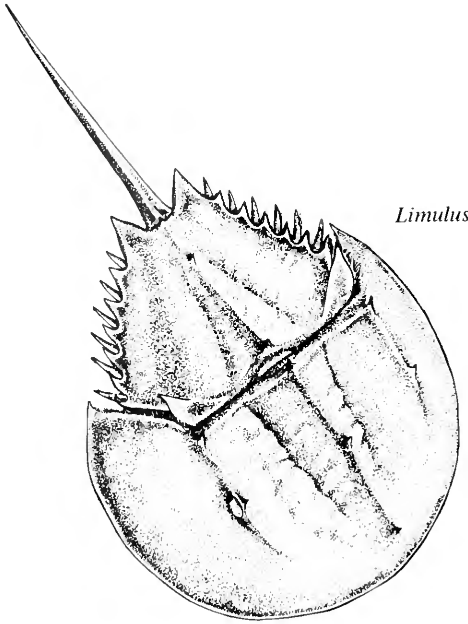
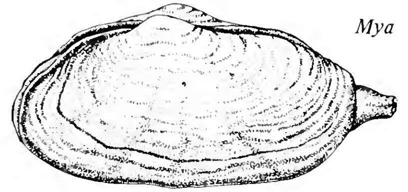
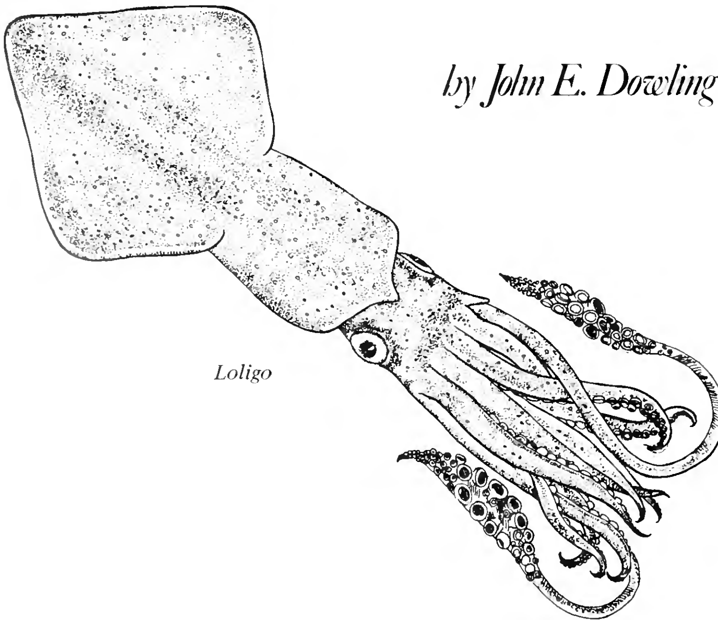




From Sea to Sight

by John E. Dowling and Harris Ripps



Some of the marine invertebrates used in vision research. (Drawings by Tor Hansen)

The use of marine organisms for studies on the visual system has a long and distinguished history. An early example is the quantitative analysis by Selig Hecht of the light-induced siphon-withdrawal reflex in the clam *Mya arenaria.* This research led to the formulation of a photochemical scheme that attempted to explain much of the visual behavior of these organisms, including the loss of visual sensitivity in the light (light adaptation) and its recovery in the dark (dark adaptation). It is through these crucial adaptation processes that the human eye adjusts to the 10-billionfold range of light intensities that it encounters between high noon and twilight. Hecht's studies were of paramount importance in the development of modern concepts of visual mechanisms because they suggested for the first time that such physiological phenomena could be explained in terms of physics and chemistry. As Hecht put it, "There is no need either for the postulation of a learning process or for the presence of a 'higher behavior' in order to rationalize the results obtained."

Later research has shown that the mechanisms underlying the phenomena of light- and dark-adaptation are considerably more complex than Hecht thought, but his basic notion that these processes are controlled by peripheral events (those occurring in the eye itself) has received impressive support. In the normal vertebrate eye, for example, the slow return of visual sensitivity during dark adaptation depends on, and runs parallel to, the regeneration of the light-sensitive pigments of the rods and cones, the receptors that capture the incoming light and initiate the visual process.

The sea has also provided the classic organism for electrophysiological studies of the processing of visual information. In the early 1930s H. K. Hartline, working at the Marine Biological Laboratory in Woods Hole, Massachusetts, found that it was possible to dissect out and record electrical activity from single nerve fibers from the eye of the horseshoe crab, *Limulus polyphemus.* The long, accessible optic nerve of this animal and the relatively small number of fibers in the nerve as compared with those of vertebrates made this an ideal preparation for such experiments. The recordings, the first to be obtained from individual units in the visual system, contributed greatly to our understanding of how the visual message is encoded and transmitted by single nerve fibers comparable to those which comprise the human optic nerve.

The range of information obtained from the *Limulus* preparation is truly impressive, and the short discussion presented here can hardly do it justice. However, the discovery by Hartline, F. Ratliff, and their co-workers of the reciprocal, inhibitory interactions that occur between cells in the *Limulus* eye deserves special mention. The way in which these cells interact with one another has led to the formulation of an elegant model describing how visual systems—including our own—can detect contours and enhance contrast at the borders of objects. While it had long been supposed that some intrinsic mechanism was at work to increase our ability to discern forms and objects, Hartline and Ratliff's quantitative account was of particular significance in that it placed this complex psychological phenomenon on a sound physiological basis. In 1967 Hartline, together with G. Wald and R. Granit, was awarded the Nobel Prize in Medicine and Physiology for his contributions to visual physiology.

Another marine species, the squid *Loligo pealei* has provided fundamental information for both the visual biochemist and the electrophysiologist. The visual pigment molecules found in squid photoreceptors have somewhat different properties from those of vertebrates, and the analysis of these differences led R. Hubbard, Wald, and their colleagues to conclude that the primary action of light in vision is to alter the shape of (that is, photoisomerize) a part of the visual pigment molecule.

We still do not fully understand how the activation of visual pigment molecules by light leads to excitation of the visual cells, but much of what we do know stems from studies of the photoreceptors of the squid and horseshoe crab. All known pigments contain a derivative of vitamin A (vitamin-A aldehyde, or retinal) linked to a protein (opsin). The absorption of photic energy affects the shape of retinal, thereby changing its relationship to opsin and initiating a series of events that lead to an electrical perturbation in the visual cell (Figure 1). In the vertebrate retina the photoisomerization ultimately results in the splitting of the retinal away from the protein to form a colorless product—a change known as *bleaching.* In the squid, however, the retinal remains attached to the protein after light exposure, thus showing that visual excitation does not depend on the dissociation of the chromophore from the protein, that excitation occurs before bleaching. But how are the photochemical events converted into electrical signals?

Employing slices of the squid retina, W. Hagins and his colleagues demonstrated that one of the early light-induced effects is an inward flow of sodium ions across the photoreceptor cell membrane in the region of the activated visual

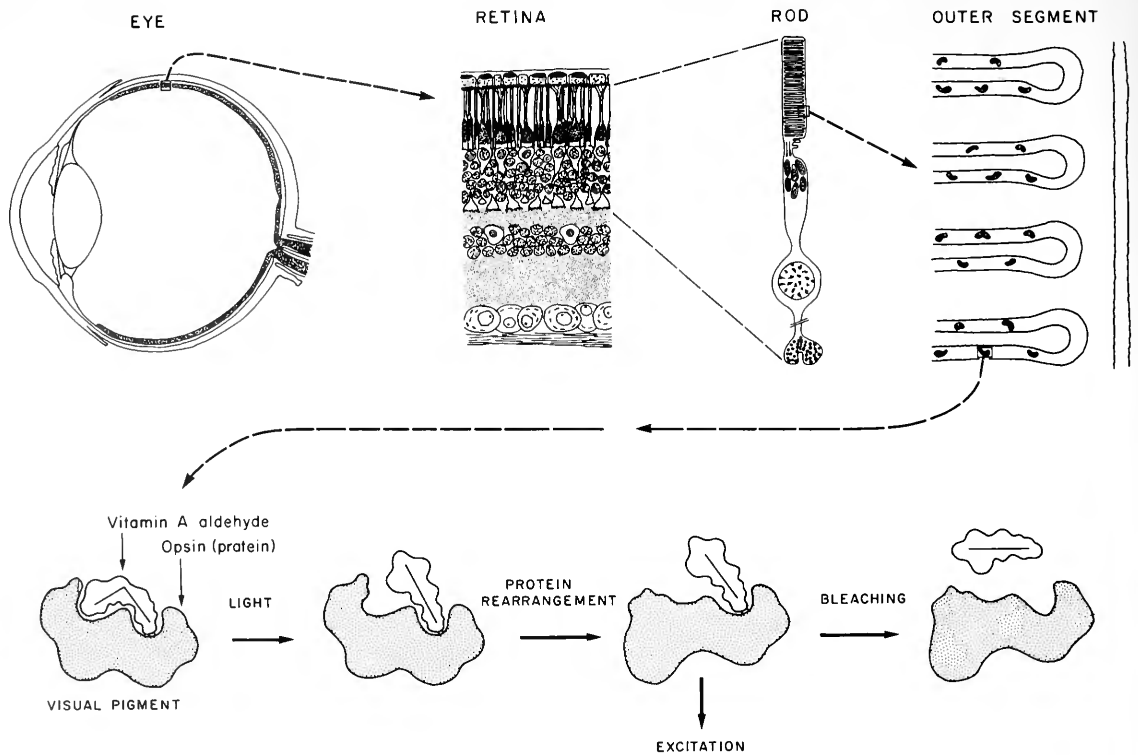


Figure 1. Drawing of a vertebrate eye, the retina, a rod photoreceptor cell, and a portion of its outer segment. Visual pigment molecules are located within membranes in the outer segment of the photoreceptor cell. Light changes the shape of the vitamin-A aldehyde part of the visual pigment molecule, initiating an alteration in the protein (opsin), which leads to visual excitation. In vertebrates the vitamin-A aldehyde eventually splits away from the protein, a process called bleaching.

pigment molecules. The influx of sodium decreases the electrical potential across the cell membrane and leads to the generation of nerve impulses for the transmission of information to the brain (see page 6). Quite recently, J. Brown and J. Lisman have demonstrated that calcium ions accompany the light-induced sodium influx into *Limulus* photoreceptors. Since an increase in the level of calcium within the cell decreases its sensitivity to light, intracellular calcium has been implicated as an important component in the processes of light- and dark-adaptation.

The invertebrate kingdom has not been the only source of marine life to have served visual science; equally noteworthy information has been culled from aquatic vertebrates. For example, striking results have been obtained from the study of single cones in a fresh water relative, the common goldfish, *Carassius auratus.* More than a century and a half ago, T. Young suggested that at least three independent mechanisms within the retina account for the remarkable properties of human color vision. In 1962 W. Marks and E. F. MacNichol, Jr., by passing microbeams through the pigment-bearing outer segments of individual goldfish cones, provided unequivocal evidence that color vision in these fish depends on the presence of three different visual pigments, each confined to its own class of cone receptor. These findings and those obtained by P. Liebman in a variety of vertebrate species, and by P. Brown and Wald in the primate retina, have confirmed Young's hypothesis and have shown that the division into three color channels occurs in the receptors, at an initial stage of the visual process.

The Skate Retina and Dark Adaptation

Turning again to marine organisms brings us to a consideration of the skate, *Raja erinacea.* This elasmobranch appears to be unique in that its retina contains only rod receptors, the percipient elements that give the eye its exquisite sensitivity and permit us to function in dim surroundings (Figure 2). The absence of cone photoreceptors leaves the skate color blind, unable to resolve fine details, and lacking other attributes of the photopic (day) system. However, this feature enables us to examine the

scotopic (night) system without the complications introduced when, as in man, both rods and cones are present.

For the past several years we have been studying the functional organization of the skate retina in an attempt to identify the mechanisms subserving dark adaptation, and to better understand some of the visual defects that arise when these mechanisms are upset in the human retina. The inability of the eye to dark-adapt results in night blindness, a disorder that affects millions of the world's population. A few examples of how these disturbances are manifest can be seen in Figure 3, which compares the measurements of dark adaptation in two night-blind patients with those in a normal observer. In each instance a region of the peripheral retina was exposed for a few minutes to a bright light, the light was extinguished, and the recovery of light sensitivity (that is, the fall in visual threshold) was determined as the subject remained in darkness. For the normal observer the results describe a bipartite curve: the early photopic (cone-mediated) branch adapts quickly to reach a plateau in 10-15 minutes, and is followed by the scotopic branch, which takes over as the rod system becomes more sensitive to light. As dark adaptation proceeds, scotopic thresholds fall (less light is needed to elicit a visual sensation), and within 30 minutes they reach the lowest level attainable, the absolute threshold, indicated here as zero on the scale of ordinates.

In night blindness (nyctalopia) the results depart significantly from the normal function, and one set of data differs markedly from the other. Note, for example, that in the case of Oguchi's disease, a rare form of night blindness, the final threshold lies at the normal dark-adapted level, but that it requires *four hours* for this degree of sensitivity to be attained. Moreover, the rod portion of the dark-adaptation curve does not appear until almost two hours have elapsed. Thus, while this patient is not truly night blind, a severe abnormality of the rod system places him at a disadvantage in almost all situations requiring night vision.

The other patient represents the more common form of night blindness, which is inherited as a recessive trait. This individual is indeed night blind: the scotopic branch of his dark-adaptation curve is absent, and there is no sign of a cone-to-rod transition no matter how long the period of dark adaptation.

Two questions immediately arise with regard to the night-blinding conditions: Where in the visual pathway are the disturbances that give rise to these anomalies, and which aspects of the adaptive machinery are upset by the disturbances? Initially, the data on human dark adaptation suggest rather simple solutions to these problems. We mentioned earlier that the changes in visual sensitivity associated with the scotopic branch of the normal dark-adaptation curve are dependent upon the rate at which visual pigment re-forms after its degradation by light. In the rod photoreceptors the visual

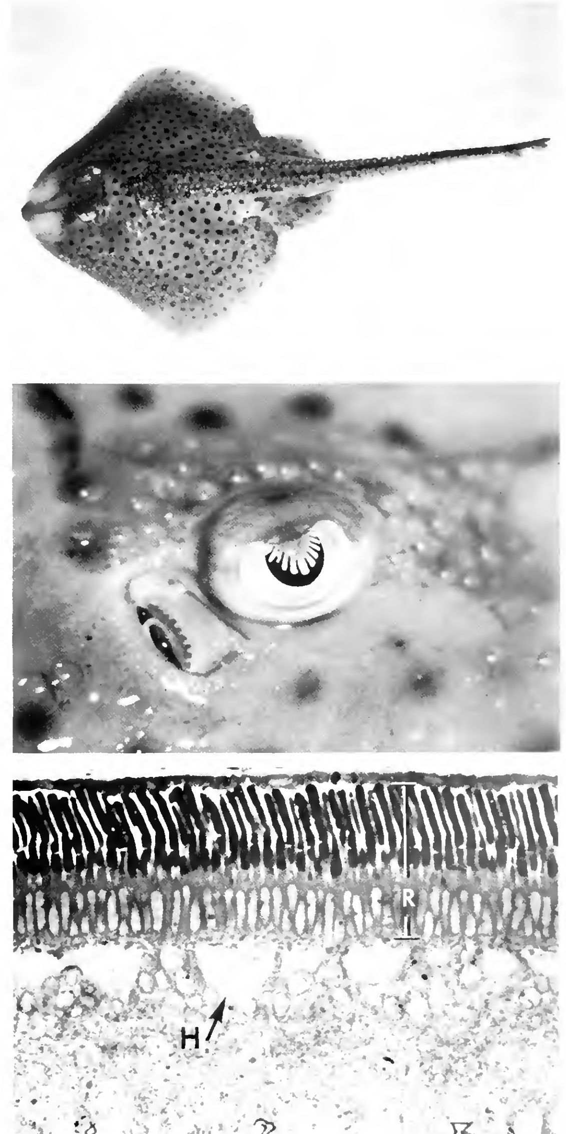


Figure. 2. Photographs of a skate, Raja erinacea *(top), and its eye (center). The skate eye has an elaborate structure, called the operculum, that regulates the amount of light entering the eye. In the dark the opercular processes contract, allowing maximum access of light into the eye. In the light the opercular processes extend across the pupil opening, restricting light access to the retina. (Bottom) Light micrograph of the skate retina showing its homogenous receptor (rod) layer (R) and large horizontal cells (arrow, H).*

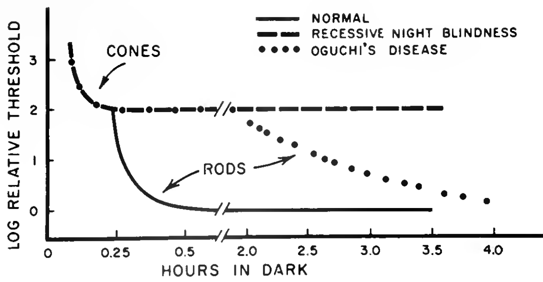


Figure 3. Dark-adaptation curves for a normal human subject and two patients suffering from night-blinding conditions. The initial fall of threshold reflects the dark adaptation of the cones. The later fall of threshold in the normal subject and in the patient with Oguchi's disease reflects the dark-adaptation process of the rods. The patient with recessive night blindness shows no rod function at all: his dark-adaptation curve shows only cone recovery.

pigment is rhodopsin, and it might be supposed that in Oguchi's disease rhodopsin regenerates at an extremely slow rate, whereas in the case of essential night blindness the rhodopsin concentration of the rods is severely depleted.

But the situation is not resolved so readily. It is possible to measure the quantity and kinetic properties of rhodopsin in the retinas of these night-blind patients, and some surprising results are obtained. *The retinas of both contain normal amounts of rhodopsin; it bleaches on exposure to light and is resynthesized completely in less than 40 minutes.* How then can dark adaptation continue for four hours in Oguchi's disease when the bleached rhodopsin of the rods regenerates fully within the first hour? And why does the patient with recessively inherited night blindness fail to dark-adapt at all?

Because of its extreme susceptibility to injury, the human retina can be explored only with noninvasive probes. One that has proven of great diagnostic value is the electroretinogram (ERG), a light-induced electrical response from the retina that is recorded with the aid of a contact-lens electrode (Figure 4). In the normal eye the ERG consists of two main components of opposite polarity: a negative *a*-wave followed by a larger, positive deflection, the *b*-wave. Recent studies have shown that the *a*-wave provides an indicator of receptoral function, whereas the *b*-wave derives from the activity of cells in the inner retinal layers.

A record of the ERG in essential night blindness offers an important clue as to the locus of the defect in this disease. The *a*-wave exhibits a perfectly normal amplitude, but the *b*-wave is practically nonexistent. The gross depression of the *b*-wave when the receptors seem to be functioning normally suggests either that there is some impediment to the transfer of information from the receptors to the second-order cells, or that the latter are incapable of responding to these signals.

The way in which signals are transferred at the receptor terminal has not been fully resolved, but the retina of the skate is admirably suited to the study of this (synaptic) transmission. In the skate, as in most fish, there is a layer of large, closely packed horizontal cells nestled just beneath the receptors (Figure 2, bottom). Fine processes from the horizontal cells extend to the terminal end of the receptor, and it is across this junction that signals are transmitted by a chemical agent. Because of their size, the horizontal cells can be penetrated with microelectrodes and their responses (so-called *S*-potentials) monitored while various solutions are applied to the retina. Of particular interest are the effects on the *S*-potentials and the ERG produced by increasing the concentration of

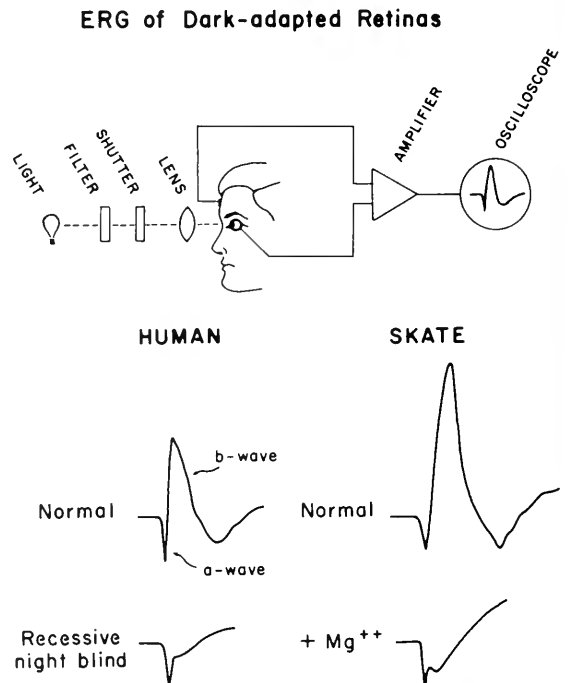


Figure 4. Electroretinograms (ERGs) recorded from a normal and a night-blind human subject and from a normal skate retina and a skate retina treated with a high concentration of magnesium (Mg^{2+}). The ERG is evoked with a flash of light projected onto the retina of the eye; it is recorded with an active electrode placed on the cornea of the eye and an indifferent electrode on the forehead. The response is amplified and observed on an oscilloscope. The ERG of the night-blind subject shows a loss of the b-wave component of the response. Application of excess Mg^{2+} to the skate retina causes a similar selective loss of the b-wave component.

magnesium ions in the bathing solution. Magnesium tends to inhibit the release of transmitter agents at nerve terminals, and we have found that it exerts a similar effect at the photoreceptor terminal of the skate. Since this in no way interferes with the functional integrity of the receptor, its electrical response, the *a*-wave, retains its normal appearance. But shutting off the transmission eliminates the *S*-potential since post-receptor cells are no longer being signaled. Accordingly, the *b*-wave is also suppressed, leaving a response that looks surprisingly like the ERG of essential night blindness (Figure 4). The similarity seems hardly fortuitous, and we are currently exploring the possibility that defects in neurotransmission are responsible for some forms of night-blinding diseases.

Another piece of information culled from our studies on the skate is that although the receptors constitute an integral part of the adaptive machinery, a neural "network" deeper within the retina controls an important phase of the dark-adaptation process. The effect of this post-receptoral mechanism can be seen after exposing the skate retina to a dim light that bleaches an insignificant fraction of the rhodopsin. Recording the electrical responses of the photoreceptors and horizontal cells indicates that they dark-adapt almost instantaneously, but the *b*-wave and responses of the ganglion cells (the fibers of which form the optic nerve) need approximately 10 minutes to reach maximum sensitivity (Figure 5, top). Clearly *visual* adaptation will reflect this slowly adapting system and not the status of the receptors (or horizontal cells).

Applying the same light-adapting protocol to the study of human dark adaptation—first with a normal observer and then in Oguchi's disease—leads to a remarkably revealing result. Since the network mechanism governs the course of dark adaptation when trivial amounts of rhodopsin are bleached, the normal eye recovers to maximum sensitivity in about 10 minutes—just as we found in the skate. But in Oguchi's disease, the weak light-adapting field causes thresholds to remain elevated for at least 30 minutes (Figure 5, bottom). The profound long-lasting effect on visual sensitivity induced by exposure to a dim light suggests that this condition results from an abnormality in the processes that control network adaptation.

We are still a long way from a satisfactory understanding of these and other disorders of the retina. However, with the help of the skate and other marine organisms, we are beginning to make some inroads in our quest for the cellular and ionic bases of the essential processes of vision.

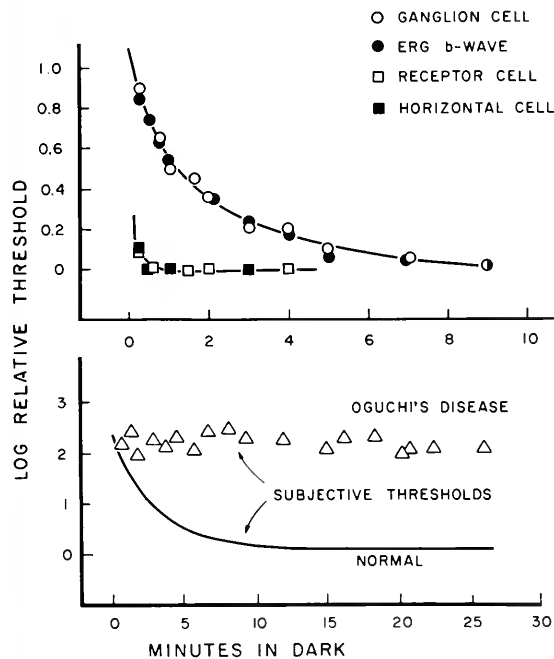


Figure 5. (Top) Dark-adaptation curves recorded from different elements in the skate retina after exposure of the eye to weak light. Under such conditions, the receptors and horizontal cells dark-adapt significantly faster than do the b-*wave and ganglion cells, indicating the presence of a "network" adaptive mechanism within the retina. (Bottom) Dark adaptation following exposure to a weak light of a patient suffering from Oguchi's disease (triangles). The recovery of sensitivity is so slow that it is not detectable on the time scale of the graph. The normal dark-adaptation curve after such an exposure (line) is complete within about 10 minutes.*

John E. Dowling is professor of biology at Harvard University, Cambridge, Massachusetts. Harris Ripps is professor of ophthalmology and physiology at New York University School of Medicine.

Except where noted, line drawings are by Patricia A. Sheppard.

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Sperm-Egg Interactions

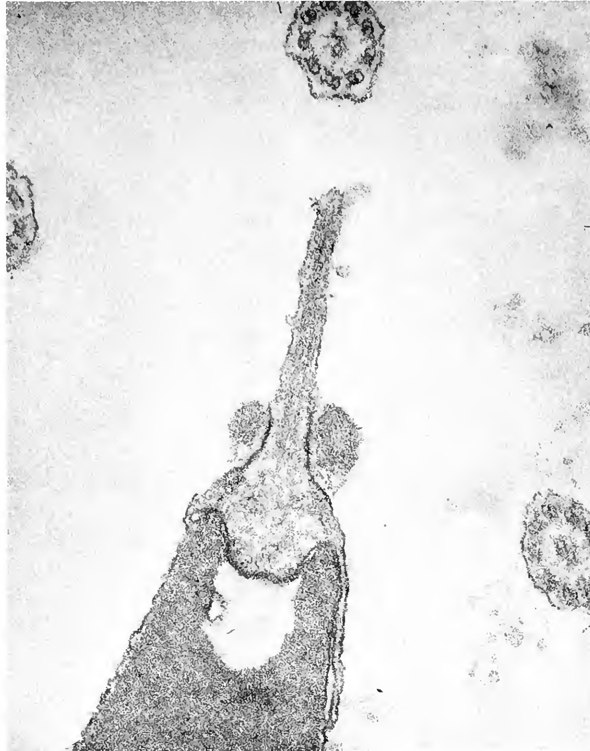

Figure 1. Electron micrograph showing the acrosomal process of sea urchin sperm (39,000X). The filament attaches to the egg surface and becomes the first portion of the sperm to fuse with the egg. (Franklin Collins, Scripps Institution of Oceanography)

by David Epel

Fertilization and the beginning of development have always captured the imagination and posed intriguing questions for researchers. Two cells are involved and the pattern is always the same. One, the egg, the largest cell in the body, is nonmotile; it contains all of the information and nutrients necessary for embryonic development but has only one-half of the organism's genome, or genetic material. The other cell, often the smallest in the body, is the sperm; it is motile and carries the other half of the genome. If these two cells interact successfully and fertilization occurs, the development of a new individual will begin. If a successful contact does not occur, the two cells will die within hours or, at the most, a few days. Living organisms expend a large amount of energy making sure that these two cells will meet each other and that fertilization will take place. That is, a great deal of physiology and behavior is directed toward successful reproduction.

How to study the process of fertilization is another problem. It is obviously difficult to do so in mammals, where fertilization and development are internal, and few eggs are produced during a reproductive cycle. However, there are certain organisms that produce many eggs to be fertilized outside the body, and these systems can be easily

studied. In such organisms, which include many marine forms, the eggs are left to fend for themselves, with the result that few eggs reach maturity. Typically, the eggs are fertilized externally in the ocean, where they develop freely, often into swimming intermediate forms called larvae. This motile phase has the advantage of allowing wide dispersal of the species within the sea, as well as exploitation of a particularly good year. The most familiar terrestrial example of this kind of life cycle is the metamorphosing insects. Occasional "plagues" of caterpillars or locusts are simply the manifestations of "good" years.

The marine organisms that have proven most suitable for the study of fertilization are echinoderms (especially sea urchins, sand dollars, and starfish), molluscs (particularly clams), and some annelids. In sea urchins, for example, the breeding season can last from three to six months, during which a female can produce between four million and four hundred million eggs. Especially important, the eggs and sperm can be easily removed from the organisms and fertilized in vitro. One simply suspends the eggs in sea water and adds a small amount of sperm while stirring. Within seconds the eggs are fertilized synchronously, and one can then study the events occurring in an egg population.

This sort of work on echinoderm development ended centuries of speculation about the role of semen in procreation. In 1875 the biologist Fol, using starfish eggs and sperm, first observed the process of fertilization under the microscope. Since then, much of what is known about embryonic development has been learned from similar studies of marine eggs and sperm. Indeed, most of the important early work was carried out at two marine stations, the famous Stazione Zoologique in Naples and the equally famous Marine Biological Laboratory in Woods Hole, Massachusetts. In fact, the first unique contributions of American biology were in development and genetics and were concerned with the embryonic development of marine organisms. This research laid the foundation for our current understanding of development in all forms and was also important to our understanding of animal evolution.

Today the eggs of marine organisms are particularly useful in determining the molecular mechanisms of early development, especially the initiation of development at fertilization. Many studies have shown that the blueprint for the first phases of development is set down during egg formation, or oogenesis. The egg can be viewed as a taut spring, ready to be activated. Everything is there; the sperm is really unnecessary in that any number of artificial stimuli can initiate development. Furthermore, one can activate development in the presence of potent metabolic inhibitors. For example, one can inhibit protein synthesis and the egg will still develop as far as first cell division. Thus, fertilization is strictly the activation of a program established during oogenesis.

Three major steps of this program, as determined from studies of sea urchin eggs, are recognition of the egg by the sperm, regulation of sperm entry by the egg, and metabolic activation of the egg.

Recognition of the Egg

The sperm and egg can be considered as cells that are primed to fuse with each other. Yet when the sperm is released at a considerable distance from the egg, what is to prevent it from fusing with just any cell or object it meets? For example, if one places some sea urchin sperm on a microscope slide, many of the sperm will attempt to "fertilize" the glass slide; they will attach to the surface as if the slide were an egg. Similarly, one can mix mammalian sperm with the normal somatic cells of the animal, and some of the sperm will attach to them. It seems as though organisms would want to make sure that the sperm save itself for the real thing, the egg.

Studies of sea urchins and annelids suggest how the sperm accomplishes this specific recognition. The eggs of these organisms are surrounded by gelatinous coats that apparently call forth exposure of the sperm's "entry card." When the sperm comes into contact with these coats, it undergoes a remarkable change that results in the exposure of a new sperm membrane. In the invertebrates this membrane is attached to an extension of the cell that forms in front of the sperm. This extension, referred to as the acrosomal process or acrosomal filament, can be seen in Figure 1. The filament attaches to the egg in a specific fashion, the two membranes of egg and sperm fuse, and the sperm enters the egg. Similar membrane changes take place in mammals, except that an acrosomal filament does not form. In both cases, however, the new membrane that ultimately fuses with the egg is exposed only when the sperm is near the egg.

Regulation of Sperm Entry

As discussed above, the egg and sperm each contain one-half of the total genetic material of the organism, and fertilization restores the genome to its normal complement. When more than one sperm enters the egg—an event called polyspermy—the number of chromosomes increases by 50 percent and the genetic

continuity of the organism is lost. Because it is imperative that polyspermy be prevented, all species have formidable mechanisms for keeping more than one sperm from entering the egg or fusing with the egg nucleus. If polyspermy does occur, the embryo soon dies or is aborted.

The magnitude of the problem can be seen in Figure 2, which shows the large number of sperm that can attach to the surface of a sea urchin egg (here, 15 seconds after insemination). Figure 3 shows the attachment of a single sperm to the surface of an unfertilized sea urchin egg at two different magnifications. Quantitative work has indicated that as many as 2000 sperm can bind to an egg, yet only one will enter.

Recent studies of fertilization in sea urchins and molluscs have revealed at least two mechanisms that block polyspermy. First, not all areas of the egg's surface appear receptive to penetration, so only a few of the attached sperm can enter the egg. Second, after a sperm has fused with the egg, the egg secretes several proteolytic enzymes, which destroy the sperm-binding sites on the vitelline layer, thus releasing the supernumerary sperm. If eggs are fertilized in the presence of protease (enzyme) inhibitors, more sperm enter the egg and polyspermy takes place.

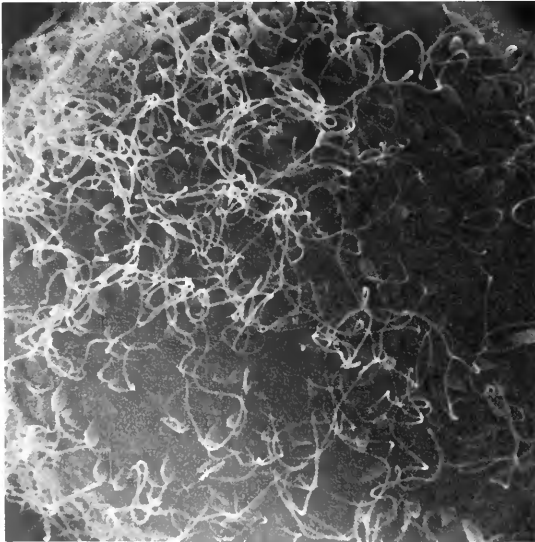

Figure 2. Scanning electron micrograph of the Strongylocentrotus purpuratus *egg showing the large number of sperm that have attached to the vitelline layer within 15 seconds of insemination. Magnification is 1540X. (Mia Tegner, Scripps Institution of Oceanography. From M. J. Tegner and D. Epel, "Sea urchin sperm-egg interactions studied with the scanning electron microscope,"* Science, *vol. 179, 16 February 1973, p. 685. Copyright 1973 by the American Association for the Advancement of Science.)*

A conceptual problem with the second mechanism is that the proteases are secreted 20 to 25 seconds after insemination. By that time hundreds, even thousands, of sperm could be bound to the egg; still only one will enter. Some biologists have therefore proposed an earlier, more rapid change on the egg's surface that prevents more sperm from entering. Another possibility is that there is no early change but that the egg's plasma membrane modulates sperm entry. Recent work in our La Jolla laboratory indicates that agents perturbing the membrane can affect the block to polyspermy, which suggests the importance of this modulating mechanism.

Nevertheless, there must be several types of blocks to polyspermy because some organisms have a strong prevention mechanism that does not involve the secretion of substances to the exterior. A good example of this is seen in the surf clam, *Spisula,* whose egg quickly (a matter of several seconds) loses sperm receptivity. Investigations of this blocking mechanism suggest that it involves a rapid change in the plasma membrane, possibly through contractile proteins in the egg cortex.

The only other system that has been extensively examined is that of the hamster egg; as in the sea urchin, the secretion of proteases may be involved.

Metabolic Activation of the Egg

As already noted, the egg has a short lifetime. If fertilization takes place, reactions are initiated that will result in the development of a new individual. Questions about these reactions–their nature, how they occur, how they initiate development–have long held the attention of developmental biologists. Recent studies of sea urchin eggs have shown that there is a definite sequence of events after fertilization. The activation process seems to break down into two temporally distinct phases: There is a constellation of changes during the first 60 seconds of development, followed by a "dark" period of about 4 minutes during which nothing much happens; then a large number of changes begin at 5 minutes after insemination. The early changes include alterations of the ionic content of the egg, especially an increase in total intracellular calcium; among the late changes are significant increases in permeability of the egg, especially to potassium ion and various organic substances such as amino acids and nucleotides.

A few of the changes that occur after fertilization in the egg of the West Coast sea urchin, *Strongylocentrotus purpuratus,* are shown in Figure 4. There is actually an almost embarrassing number of

Figure 3. Sperm attaching to the surface of an unfertilized sea urchin egg. Above: 9900X; below: 15,100X. (Mia Tegner, Scripps Institution of Oceanography)

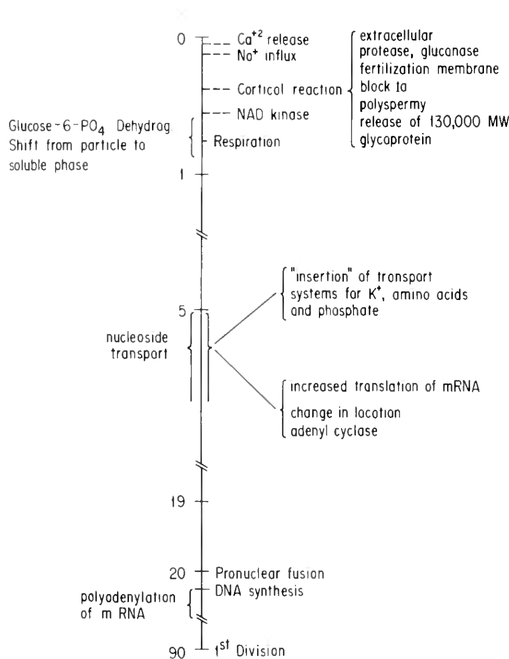

Figure 4. The program for the early development of Strongylocentrotus purpuratus *eggs at 17°C showing the nature and timing of some of the changes. Numbers refer to minutes elapsed since insemination. Fertilization changes occur in sequence, beginning with the release of intracellular calcium (Ca^{+2}).*

changes, and a major problem has been to determine whether they are related to each other in a chain reaction, or whether they occur independently. Recent analyses, using various agents that activate only partial development of the egg, have revealed that the early changes appear to be related in a chain reaction. The primary trigger seems to be the release of calcium, apparently from intracellular stores. Thus one can artificially activate eggs with drugs that are known to specifically promote the transfer of calcium across membranes. These drugs work with every egg that has been tried—marine invertebrate as well as amphibian and mammalian. How calcium acts to initiate development is still unclear. It appears to be involved directly in the secretory phase, but how it activates the later phases of embryonic development is unknown.

The later phases, which are not activated in a chain reaction, seem related to the release of a suppressor protein from the cell surface. Normally, when the egg undergoes the secretory phase, the membrane is reorganized and the suppressor protein is released. The loss of this protein then arouses the egg to begin embryonic development. That these proteins are suppressive has come from our observation that adding them to activated eggs suppresses the eggs' metabolism back to the unfertilized state. These results assume greater significance in light of the theory that a similar mechanism is involved in the transformation of normal somatic cells to the cancerous state.

Biomedical Applications

Fertilization studies of marine organisms have greatly contributed to the basic knowledge of sperm-egg interactions. In addition, this work has proved relevant to cancer research and fertility regulation.

Many investigations suggest that the metabolic transformation of eggs at fertilization resembles the transformation of normal cells to the cancerous state. Thus the egg's changing from the dormant to the actively dividing state may provide important experimental clues to understanding how normal cells become malignant. An advantage to using the sea urchin system is the ease with which this "transformation" can be accomplished at will by adding sperm or activating agents.

Because many of the phenomena of fertilization are common to all organisms, the easily studied marine species can provide information applicable to other higher forms. A greater understanding of fertilization could provide new and novel means of fertility regulation, possible targets being the three major steps discussed in this article. Considering activation of the egg, for example, contraceptive mechanisms could be designed to precociously activate ovulated eggs so that they cannot be fertilized or develop. More specifically, calcium-transporting drugs do not activate eggs properly, and the eggs are aborted at an early stage. Further basic research on fertilization promises more approaches to fertility regulation.

David Epel is professor of biology at Scripps Institution of Oceanography, University of California at San Diego. He is also co-director of the embryology course at the Marine Biological Laboratory, Woods Hole.

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Microtubules

by R. E. Stephens

Sea urchin eggs and sperm tails, scallop and oyster gills, and oocytes from bizarre-looking polychaete worms are unlikely tools for medically related basic research, but they are all sources of the ubiquitous cellular organelles known as microtubules. Though seemingly an esoteric subject, microtubules are the object of considerable current interest due to their involvement in a multitude of life processes. Measuring only about 0.00002 millimeters in diameter and of quite variable length, these rigid, hollow, cylindrical structures passively serve to determine cell shape or form, and actively participate in the processes of cell division, sperm motility, ciliary movement, and the transport of materials within cells.

Microtubules are intimately involved in the normal functions of all higher organisms, from conception until death, initially as instruments of cell division and mediators of the morphogenetic movements of differentiating cells. Later, in the adult organism, microtubules form lung cilia and sperm flagella—the vibrating "mechanochemical transducers" that are so vital to respiration and to reproduction, respectively. During nerve outgrowth and later in the functioning nerve axon, bundles of microtubules lend strength and rigidity to these fine cell extensions and serve to guide and perhaps propel cell constituents and nutrients from the cell body down to the far-distant nerve endings. Microtubules have also been implicated in the less-monumental task of aiding or directing the transport of cellular products to and through the cell membrane in the secretion of such things as plasma proteins and hormones. Thus fertilization, early development, nerve growth and maintenance, glandular function, and the action of respiratory cilia are all dependent in some manner on microtubules.

The sea provides a wide diversity of organisms and a unique abundance of materials with which to explore the fundamental role of these organelles in the various life processes and, one hopes, to better understand the basic mechanisms of these processes in both normal and abnormal cells and tissues. Since a thorough discussion of the subject—just covering information gleaned from marine studies alone—would fill many volumes, five closely related topics are dealt with here and are illustrative of the great variety of experimental approaches now in use.

Cell Division

Vital to the life cycle of all multicellular organisms is the highly controlled and orderly process of cell division, or mitosis. Chromosomes, carrying duplicate copies of the genetic material, must be equally apportioned between daughter cells at each division. The cell accomplishes this task with a device known as the *mitotic apparatus*, a structure consisting of two asters, or "star bursts," of microtubules between which the chromosomes are positioned and attached. The formation and function of the mitotic apparatus are illustrated in Figure 1, where a dividing sea urchin egg has been photographed in polarized light in order to make the mitotic apparatus or spindle visible. Such an optical system renders microtubules either bright or dark, depending on their position in the field, and allows one to observe the spindle fibers, the bundles of parallel microtubules that attach to and pull the chromosomes. This sensitive optical technique, utilizing polarized light to directly observe the mitotic apparatus of living cells throughout division, was developed by S. Inoué, now at the University of Pennsylvania, working primarily with marine eggs both at Woods Hole, Massachusetts, and at Friday Harbor, Washington.

As the photographs reveal, microtubules in the mitotic apparatus are clearly not static structures. Inoué and his co-workers have shown that the microtubules are in a constant state of flux with subunit material from the surrounding cytoplasm and that equilibrium shifts first toward

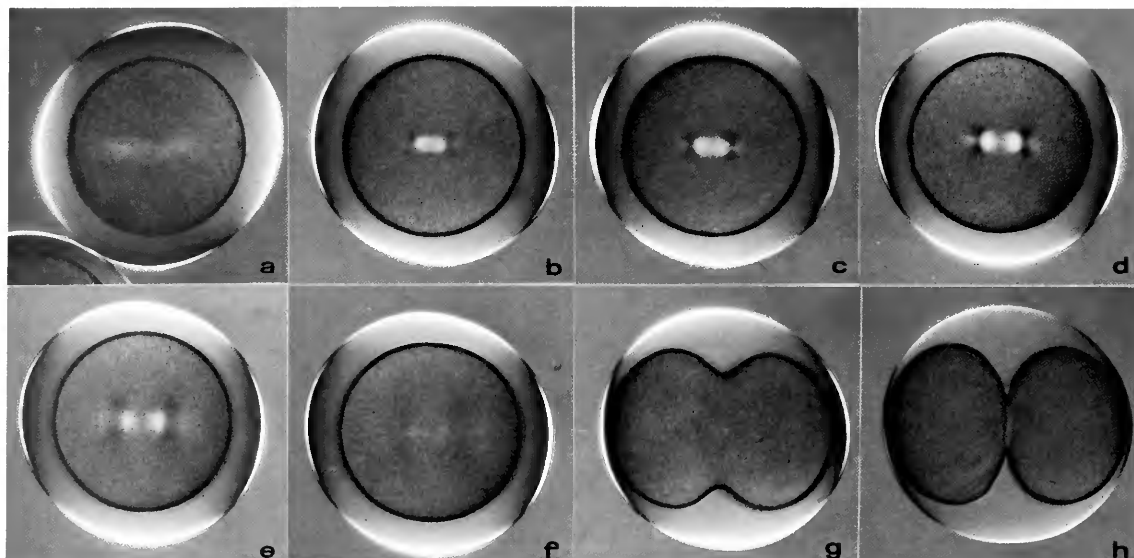

Figure 1. Dividing egg of the sea urchin Lytechinus, *photographed in polarized light, showing the progressive formation, function, and disappearance of the mitotic spindle, followed by cleavage. (E. D. Salmon, MBL, Woods Hole)*

and then away from microtubule formation as the mitotic apparatus is assembled and then disassembled. This shifting or "dynamic" equilibrium between microtubules and their subunits is involved in the growth of spindle fibers and also may be implicated in the active movement of the chromosomes themselves, initially to position the chromosomes for separation and later to regulate or direct their transport to the poles.

Throughout mitosis, bundles of microtubules in the spindle converge on the center, or pole, of each aster and also on a single point, the kinetochore, of each chromosome. These focal points, or orienting centers, are critical to the functioning of the mitotic apparatus in that they give directionality to the system. Chromosomes lacking kinetochores (perhaps resulting from radiation damage or from the action of chemical mutagens) remain unseparated, one cell does not receive its full complement of chromosomes, and a genetic abnormality may result. The exact nature of these microtubule orienting centers is unclear, but their mode of action is a central question in cell division studies, as is the nature of the dynamic equilibrium and its control, both spatially at the kinetochores and poles, and temporally throughout the cell cycle.

One approach to studying the nature of the mitotic spindle is to perturb its equilibrium and see what happens. Edward Salmon of the Marine Biological Laboratory, Woods Hole, has been doing just that, using high hydrostatic pressures in the range of 3000-10,000 psi to dramatically shift the spindle assembly process. He has developed a miniaturized pressure chamber and has made it compatible with a variety of microscope optical systems to allow continuous monitoring of single living cells while they are under pressure. Using oocytes of the tube-dwelling polychaete worm *Chaetopterus,* chosen because these cells contain spindles that are naturally arrested at metaphase while awaiting fertilization, he has found that application of high pressures simply causes the mitotic apparatus to dissolve abruptly, leaving the chromosomes where they were, while the use of more moderate pressures causes shortening of the spindle fibers and a poleward movement of the chromosomes (Figure 2). Like high hydrostatic pressure, low temperature also causes microtubules to dissolve; similar results can be obtained by rapid versus slow cooling of these same cells. A gradual shift in the spindle fiber equilibrium can transport chromosomes, but is this how the cell does it? At this point, no one knows for sure. One thing is quite clear, however, and that is that microtubule integrity is somehow required for the normal, orderly separation of chromosomes.

Salmon's pressure studies raise a very interesting ecological question, namely, how have deep-sea organisms adapted to permit cell division to occur under the high hydrostatic pressures and low temperatures so characteristic of the sea bottom? Why are their spindles stable to these conditions while those of littoral organisms are not? Further work with living cells under simulated environments should help to answer this question and may shed some light on the processes regulating mitotic

spindles in general.

The study of mitosis in single cells offers the obvious advantage of dealing with an intact and active system, but such a situation is not easily amenable to biochemical analysis. Sea urchin eggs can be obtained and fertilized in gram quantities, consisting of literally millions of cells all developing in synchrony. This should be contrasted with mammalian eggs, where each female typically produces only one egg at any given time. Quantity and synchrony are the primary reasons that marine eggs have served as model systems for so many studies into the nature of cell division (see page 34).

Mass quantities of simultaneously fertilized sea urchin eggs can be placed in a chemical medium that will cause the release and stabilization of the mitotic spindles at whatever stage of the mitotic cycle the experimenter desires. A decade ago R. Kane, now director of the University of Hawaii's Kewalo Marine Laboratory, devised such a method whereby cells are lysed or burst in a carefully buffered solution of a dilute alcohol or glycol to yield isolated spindles whose protein components could be easily solubilized and analyzed. An example of such an isolated spindle is shown in Figure 3, photographed both in polarized light to reveal the well-ordered spindle fibers and in phase-contrast to illustrate mass distribution and the chromosomes.

Such isolated spindles cannot function on their own, but recent studies carried out at Woods Hole by two pairs of workers, J. Rosenbaum and L. Rebhun using spindles from surf clam eggs and G. Borisy and S. Inoué using spindles from polychaete eggs, found that the addition of microtubule protein derived from mammalian brain would "add on" to the existing spindle microtubules to produce

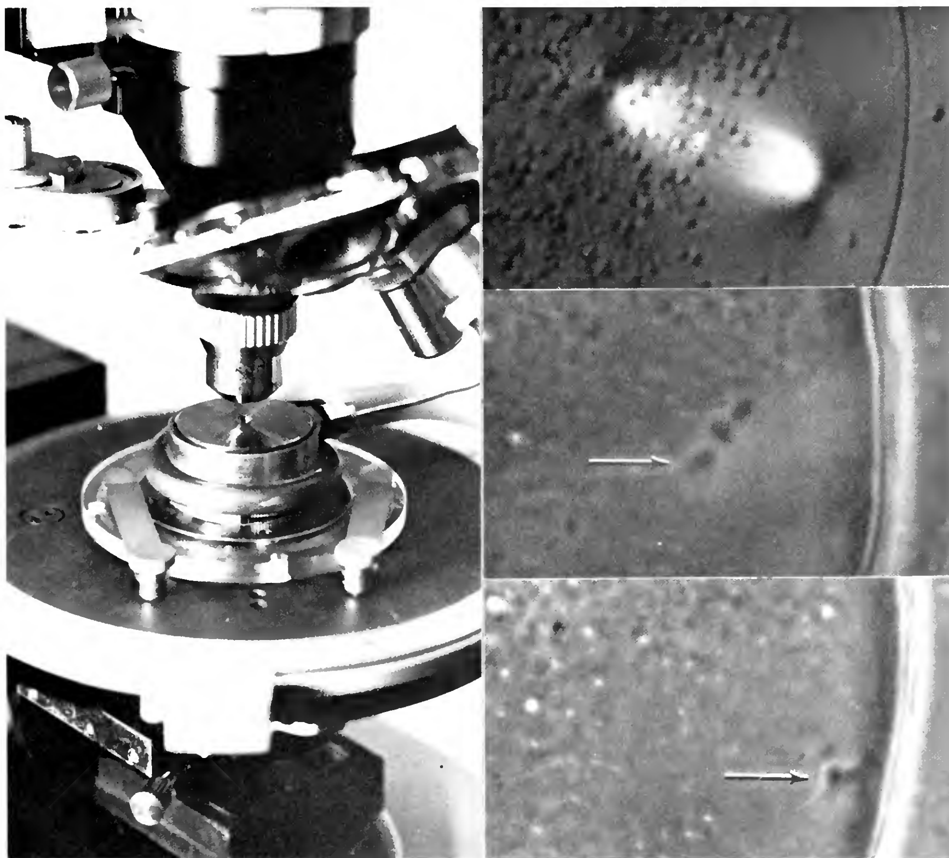

Figure 2. Miniaturized microscope pressure chamber (left) with a living Chaetopterus *oocyte at atmospheric pressure (top) and after application of high (center) and moderate (bottom) hydrostatic pressures. The arrow indicates the position of the chromosomes. (E. D. Salmon, MBL, Woods Hole)*

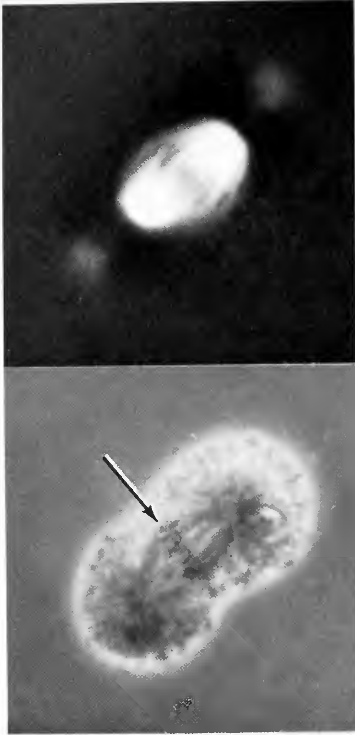

Figure 3. Mitotic spindle isolated from the cold-water sea urchin Strongylocentrotus, *seen in polarized light (top) and in phase-contrast (center), and drawn schematically (bottom). The arrow indicates the position of the chromosomes.*

"hybrid" spindles that could respond to temperature and to calcium ions, just as do those in the living cell. Providing the isolated spindle with microtubule protein subunits, albeit from a different species, evidently restored the equilibrium conditions characteristic of the intact cell.

The isolated mitotic apparatus illustrated in Figure 3 was obtained from the sea urchin *Strongylocentrotus droebachiensis*, a beast that has two dubious claims to fame: the longest binomial name in the animal kingdom, and the ability to breed in freezing waters. Circumpolar in distribution, this urchin has a breeding season that coincides with the winter's lowest temperatures, and, in spite of the cold-sensitivity generally observed for other intertidal or terrestrial organisms, the eggs of this species produce functioning spindles even below $0^{\circ}C$. In addition, the eggs are able to regulate the amount of microtubule protein subunits available for spindle formation in accord with the environmental temperature. We have been using these two apparent anomalies to study low-temperature spindle assembly and also to investigate the environmental regulation of the assembly process, the aim being to determine how the cell controls or modulates spindle formation according to external conditions. Isolation of the spindle by Kane's method and separation of the microtubule protein subunit "pool" from the egg cytoplasm before and during mitosis permit a biochemical comparison of microtubule protein and other factors at various stages of mitotic apparatus assembly or under differing environmental conditions.

Once the chromosomes have been separated by the mitotic apparatus, a "purse-string" known as a *contractile ring* pinches the cell in two (Figure 1, g-h). First described by T. Schroeder of the University of Washington's Friday Harbor Laboratories, this fine, filamentous ring exists only briefly, forming immediately prior to cleavage and disappearing as cleavage is completed. The exact positioning of this ring beneath the cell membrane is a direct consequence of the position of the asters earlier in mitosis. Using dividing eggs of the sand dollar, R. Rappaport, working at the Mount Desert Island Marine Biological Laboratory in Maine, has extensively studied this curious process in which the mitotic apparatus specifies the cleavage plane for the entire cell. It does so at the time when the chromosomes have lined up awaiting separation (Figure 1, d-e). The mitotic apparatus can be moved about the cell with a microneedle to induce furrows in the "wrong" place, but repositioning it later in the cell cycle has no effect. Also, moving the spindle too far from the cell surface can prevent furrow formation. Rappaport's work clearly shows that some sort of interplay between the microtubules of the asters and the region beneath the cell surface can determine precisely where a cell will cleave in two. Like the mobilization of the spindle and the movement of chromosomes, the exact mechanism for this specifying or inducing process remains a mystery.

Early Development

Mimicking in every basic respect the early development of human embryos, the developing sea urchin egg has served for many years as a model system for embryologists and biochemists alike. The ease of observation, the simplicity of experimental manipulation, and the large quantities of synchronous cells that can be obtained make this organism unique, unsurpassed in its usefulness as an embryological tool for large-scale studies.

The basic patterns of nucleic acid and protein synthesis during early development have been determined in the sea urchin embryo chiefly through efforts led by P. Gross and R. Raff at Woods Hole and MIT. Since the mitotic apparatus appears and disappears at each division throughout the embryo's life, one major question concerned the origin and fate of the microtubule protein, known as *tubulin*, both during the cell cycle and throughout embryogenesis. To make a very long story short by synthesizing the results of these and many other workers, the protein tubulin was found to preexist in large amounts in the unfertilized egg, and it is maintained in a "steady-state" condition by constant, balanced synthesis and degradation during development. Unlike the mitotic apparatus that it forms, tubulin does not cycle in amount. Rather, small quantities are apparently recruited from this steady-state pool, mobilized to form a spindle, and then disassembled after the spindle has served its purpose. Again, the notions of equilibrium and pool modulation crop up, but now from strictly biochemical studies.

The mitotic spindle is not the only object that the embryo constructs of microtubules. After a fixed number of divisions, each cell over much of the embryo's surface simultaneously grows one cilium, a hairlike structure that propels the embryonic sea urchin through the water (Figure 4). Also made of tubulin, but apparently of a slightly different

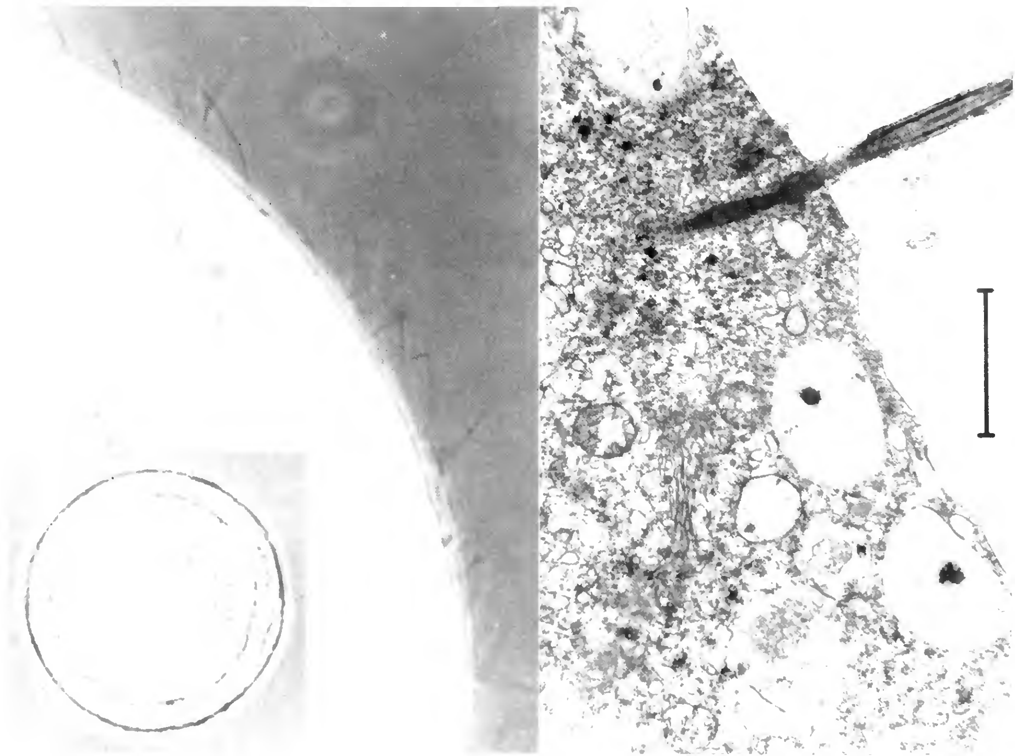

Figure 4. A sea urchin embryo at the blastula stage (inset) is covered with hairlike cilia, seen here in phase-contrast on the living embryo (left) and as a thin-section in the electron microscope (right). Scale bar = 1 micron.

variety than that of the mitotic spindle, the microtubules of cilia are involved in generating their characteristic whiplike motion. Arranged in a cylinder as nine "doublet" arm-bearing tubules surrounding a pair of individual tubules (the so-called "9+2" structure), ciliary and flagellar microtubules are held together by complex radial and circumferential linkages. Cilia and flagella seem to have identical "9+2" structures, but differ in how they beat. A lengthwise section of a cilium is seen in Figure 4, while a cross-section of a flagellum is seen in Figure 5.

As if on cue, the microtubules of embryonic cilia are all assembled simultaneously and rapidly. Is the synthesis of the ciliary tubulin the controlling step in this first true morphological event in embryonic differentiation? Our own recent work indicates that the answer is a qualified no. Just as Raff, Gross, and co-workers found for the total tubulin pool in the dividing cell or developing embryo, the bulk of the tubulin specifically destined to become incorporated into cilia later in development is also found in a steady-state pool. Some may be synthesized as the cilia begin to grow, but the "missing ingredients" that the embryo makes when it wants to construct cilia appear to be the linkage proteins that assemble with the microtubules to hold them in their "9+2" pattern. Serving perhaps as molecular nuts and bolts, struts and braces, these "architectural" proteins are intimately involved in the assembly process. Is a similar mechanism at work in the assembly and function of the mitotic apparatus? Are catalytic amounts of key ingredients missing, the controlled synthesis of which permits microtubule assembly and function in cell division? The study of the control and assembly of embryonic cilia may tell us more about how other microtubule systems function and also how the "programmed" synthesis of proteins leads to developmental events.

The Sperm Flagellum

The snakelike swimming motion of the sperm flagellum propels the sperm head toward its ultimate target, the egg. This primitive and fundamental form of cell movement has been the subject of several decades of research, only recently culminating in an explanation of its mechanism. This description was derived primarily from studies on sea urchin sperm carried out by Ian and Barbara Gibbons of the University of Hawaii's Kewalo Marine Laboratory.

Like cilia, the sperm tail contains a "9+2" arrangement of microtubules surrounded by a membrane. It moves in an undulatory fashion, propagating a sinusoidal wave from the head toward the tip of the tail. The Gibbons' discovered that the membrane could be gently removed by detergent treatment, causing the motion to stop, an effect resulting from the fact that the energy source for the motion–adenosine triphosphate (ATP)–had drained away. Re-addition of ATP caused normal movement to resume *if* the conditions were right. Such membraneless sperm enabled the researchers to determine optimal conditions for movement, simply by bathing the sperm with appropriate solutions and adding ATP, in essence duplicating the intracellular milieu (Figure 5).

This line of reasoning produced evidence which indicated that sperm motility was a direct function of an ATP-splitting enzyme called *dynein,* originally discovered by I. Gibbons in the protozoan ciliate *Tetrahymena.* This enzyme formed the paired arms characteristically found on each outer microtubule doublet in the "9+2" structure (Figure 5, arrow). These two arms have slightly different solubility properties, and when the Gibbons' extracted just one member of the pair from the membraneless sperm and then added ATP, the flagellum beat precisely half as fast. Both arms are clearly needed for movement, but how do they work?

When the Gibbons' induced normal movement in the sperm with high ATP levels and then rapidly diluted the preparations, the sperm were "frozen" in position, with the undulating waveform in a state analogous to rigor mortis in muscle. When they observed the "9+2" structure of these "rigor" sperm in the electron microscope, they found that the arms from one microtubule doublet were grasping the adjacent microtubule doublet, all around the circumference of the structure (Figure 5).

They concluded that dynein arms grasped, crawled, and then disengaged in a coordinated fashion to cause local relative sliding of one microtubule doublet over the next adjacent one. The architectural proteins of the "9+2" structure were thought to confine or constrain this local sliding to convert translational movement (sliding of microtubules) into bending movement (wave propagation).

An ingenious test of this hypothesis was performed by K. Summers, at the time a student of I. Gibbons. He first treated sperm flagella with a digestive enzyme at concentrations sufficiently low that the architectural elements of the "9+2" structure

were selectively disrupted but the dynein arms and the microtubules went unscathed. Summers then added ATP and observed not the usual swimming motion but, rather, a dramatic sliding apart of the structure.

Dynein arms are of more than just academic interest, for recently B. A. Afzelius and co-workers in Sweden studied two brothers suffering from a form of sterility and discovered that their sperm, though normal in all other respects, lacked dynein arms and were consequently immotile. Thus at least one example of human sterility has a clear-cut molecular basis, in this case at the level of the sperm's propulsion mechanism.

The disease cystic fibrosis has an interesting though indirect connection with the normal movement of cilia in molluscan gills. Barbara Bowman and her colleagues at the University of Texas Medical Branch at Galveston have devised a simple clinical assay for this human genetic disease, involving the application of a suspected patient's serum to oyster gills. Though no obvious symptoms may be present, even the serum from carriers of the disease will cause the gill cilia to stop beating. The exact reason for this curious phenomenon is not known, but it is suspected that some characteristic protein factor in the patient's serum interacts with the membrane of the cilium and somehow interferes with the control mechanism for normal beating motion. This control is thought to involve the regulated flow of calcium ions, which, in turn, regulate the interaction of dynein and microtubules.

Relative sliding of one microtubule past another, mediated through a dynein arm or cross-bridge, clearly serves as the basis for movement in both cilia and flagella, the only real difference being in the type of waveform produced. Many workers feel that this same kind of mechanism may function in other microtubule systems as well. For example, microtubules attached to chromosomes may actively slide over microtubules emanating from asters, thus pulling chromosomes toward the poles during mitosis. Or particles in nerve axons or other cell extensions may be propelled or kicked along between parallel microtubules by means of rapidly vibrating dynein arms. These ideas are somewhat controversial and as yet unproven, but the mechanism already shown to power cilia and flagella at least poses a testable hypothesis for other forms of cell movement involving microtubules.

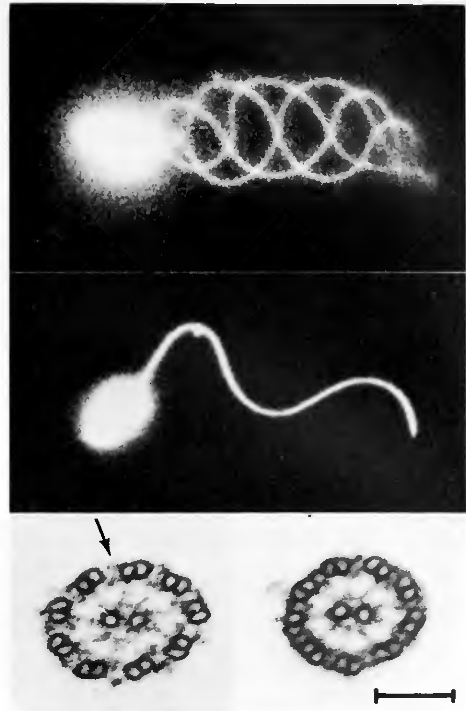

Figure 5. A membraneless sperm reactivated by the addition of ATP (top dark-field strobe photo) may be frozen in a kind of rigor mortis by simple dilution (center photo). The electron microscope reveals that, in cross-section, the dynein arms (arrow, lower left) bridge the double microtubules of the "9+2" structure together during movement (lower right). Scale bar = 0.1 micron. (I. R. Gibbons, University of Hawaii)

Microtubule Homology

Dimensionally, all microtubules appear to be the same, even down to the partial and whole tubules that form the doublets in cilia and flagella. They all have diameters of about 220 Å, and their walls are made up of 40 Å roughly spherical protein subunits, arrayed in a shallow spiral. The microtubule might be likened to a hollow cylinder built out of tennis table balls, with 13 balls forming the circumference (Figure 7). Considering the apparent structural identity, why do various microtubules have different stabilities and different functions in different cells? Why are mitotic microtubules, and those in the axons of nerve cells as well, in a constantly changing equilibrium, and why do they exist as simple cylinders while microtubules in cilia and flagella are generally permanent structures, existing as pairs or doublets, and able to specifically bind dynein arms and other accessory items? One hypothesis that we are currently pursuing is that the protein tubulin, which builds each morphological or functional microtubule

type, is itself slightly different at the primary structural level. That is, there may be slight differences in the order or sequence of individual amino acids that form the protein.

In collaboration with C. Fulton and R. Kane, we produced antibodies against sea urchin flagella microtubule doublet protein. This antibody preparation was used to quantitatively distinguish among the tubulins derived from the mitotic apparatus, embryonic cilia, or individual halves of flagellar doublet tubules. Our results indicated that all of these tubulins shared common antigenic determinants but that each also contained certain unique ones. Put another way, these tubulins were very closely related in terms of overall amino acid sequence but must have had subtle differences in local regions of that sequence.

Another more direct approach that is now being used is to subject tubulin derived from various sources to a process called "tryptic fingerprinting." The enzyme trypsin specifically breaks the tubulin polypeptide chains wherever the amino acid lysine or arginine occurs in the sequence. The resulting fragments (peptides) are deposited as a small spot on one corner of a glass plate coated with silica gel, chromatographed in one direction with an organic solvent, and then subjected to an electric field in a perpendicular direction. The resulting "maps" or "fingerprints" of tryptic peptides, now separated by both charge and relative solubility, can be compared from one tubulin type to another in order to determine how closely related the proteins really are and whether there are small localized differences. Our results so far indicate that the tubulins derived from the two tubule halves in flagella microtubule doublets are indeed composed of closely related but clearly different sets of polypeptide chains, just as the immunological data had hinted.

Other findings also indicate possible chemical differences in structurally identical microtubules. For example, the "9+2" tubules of bay scallop gill cilia have quite different solubility properties when compared with their counterparts in sperm flagella in the same organism. Also, genetic evidence derived from the green alga *Chlamydomonas* indicates that certain flagellar mutants may be missing the pair of microtubules at the center of the "9+2" structure or one member of the outer, normally doublet tubules, but appear to be otherwise intact. Both of these cases suggest that all of the tubules cannot be composed of the same identical protein.

Certain neurological diseases, such as Alzheimer's and related conditions, are characterized by a vast proliferation of filamentous material within nerve cell bodies or their axons, resulting in a blockage of normal flow of materials within such affected cells. Considerable evidence points to the possibility that the filamentous material is a modified or variant tubulin, somehow no longer able to form normal microtubules (also called neurotubules). This deduction was based on the fact that certain agents which can mimic the diseases can also cause normal tubulin to assemble into different forms, often filamentous spirals, resembling those found in the diseased state. Thus Alzheimer's disease and its relatives may be due to the presence of genetically distinct tubulin or to some chemically modified tubulin that will not form normal neurotubules.

Biochemical studies of possible tubulin variation should lead to a better understanding of mitotic, ciliary, and flagellar microtubules and of neurotubules in normal and diseased states and to their differing roles in the cell. If the tubulin from different microtubules turns out to be a different gene product in each case, we are left with the rather perplexing dilemma of how the dividing and developing cell can sort these proteins out when building different kinds of structures with characteristic microtubules.

High-Resolution Structural Studies

Any characteristic polypeptide chain subtleties in tubulin molecules should be reflected in the fine details of microtubule substructure, giving a sort of "molecular specificity" to the tubule that it forms. Is there some structural uniqueness in the microtubules of cilia and flagella that specifies arm attachment, doublet formation, or pairing, in contrast to mitotic or neurotubules where no such obvious specificity is found?

Probing down to the atomic level, x-ray diffraction can reveal the three-dimensional arrangement of the protein polypeptide chains within the wall of the microtubule. Such a study is now underway at Brandeis University's Rosenstiel Basic Medical Sciences Research Center, where C. Cohen and her collaborators are investigating the substructure of the sea urchin flagella doublet microtubules and mammalian brain neurotubules.

Pure preparations of these microtubules can be sedimented in an ultracentrifuge and then drawn into quartz capillaries. A capillary containing the highly concentrated and aligned microtubules is then placed in an x-ray beam, and, after a suitable exposure time, a characteristic diffraction pattern is obtained on a photographic plate (Figure 6). This pattern results from diffraction of the x-ray beam by the three-dimensional arrangement of protein molecules within the cylindrical microtubule. Analysis of the diffraction spot positions and intensities is performed by computer in order to deduce the original spatial arrangement of the protein's polypeptide chains.

Results thus far correlate fairly well with several earlier electron microscopic studies on different materials. These revealed the gross features of the microtubule surface and the positioning of the tubulin molecules within the cylindrical wall, but certain more-detailed features, specifically the asymmetric shape and position of the tubulin building blocks, are unique to the x-ray analysis. Unlike the electron microscopic approach, which employs negative-staining techniques involving

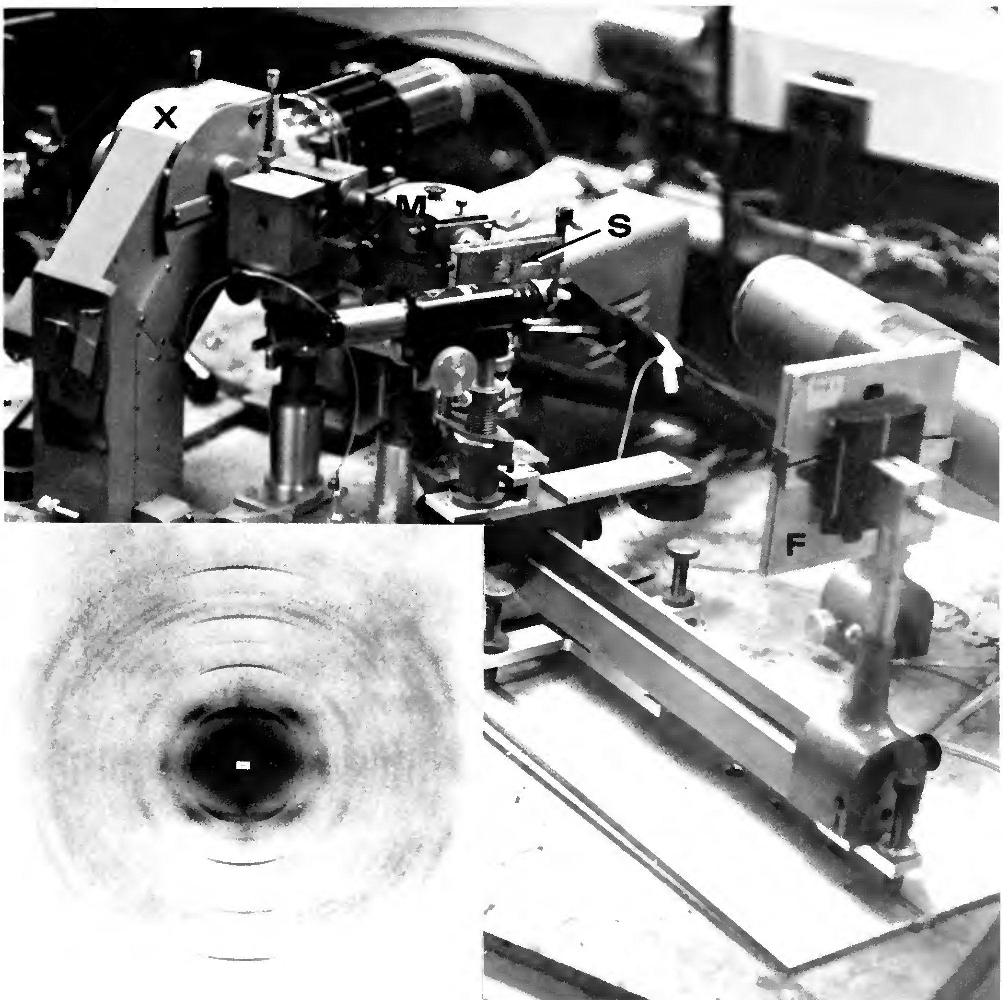

Figure 6. An x-ray diffractometer, showing the source of x-rays (X); the monochromator (M), which focuses the x-ray beam; the specimen (S); and the film carrier (F) on which the diffraction pattern (inset) forms.

heavy-metal salts and is limited to dried samples, the x-ray methodology is applicable to wet samples under a variety of physiological conditions.

At this point, analysis at very high resolution is incomplete, but preliminary results indicate that x-ray diffraction can reveal the characteristic shape of the molecular building blocks of the microtubule (Figure 7). This approach certainly holds great promise for yielding basic information about the three-dimensional substructure of various microtubules and should help to decipher the reasons for the varied functions of microtubules in living cells.

Conclusion

The full circle of life—from egg and sperm, to embryo, to adult, and back again to egg and sperm—is at each finite step dependent upon a complex interplay of factors dictated by an orderly reading of the genetic information. One intracellular structure, the microtubule, is central to this continuum of events due to its elusive and ever-changing role in cell division and cell movement. Normal development and normal physiological function seem to depend heavily on the proper operation of various processes that use microtubules. Sperm motility, mitosis, and ciliary action in respiration are direct and obvious examples, while nerve growth and maintenance or various secretory processes are less direct and less well documented. Thus far, one reproductive disorder and one nervous disease have been traced to some malfunction of systems directly dependent upon microtubules. In both cases this connection could never have been made were it not for the fact that the "normal" systems had already been so well characterized through basic research.

Most major findings about microtubule form and function have come from studies using marine material, performed primarily at marine facilities. In this case the researcher's equipment consists not of the familiar collecting gear, formaldehyde, and museum jars so typical of marine labs of old, but rather the sophisticated tools of the biochemist or biophysicist. This invasion of the marine realm by the modern cellular and developmental biologist has been a fruitful one and promises to be even more so in the future, for the abundance and diversity of experimental material and experimental questions provide a seemingly endless challenge.

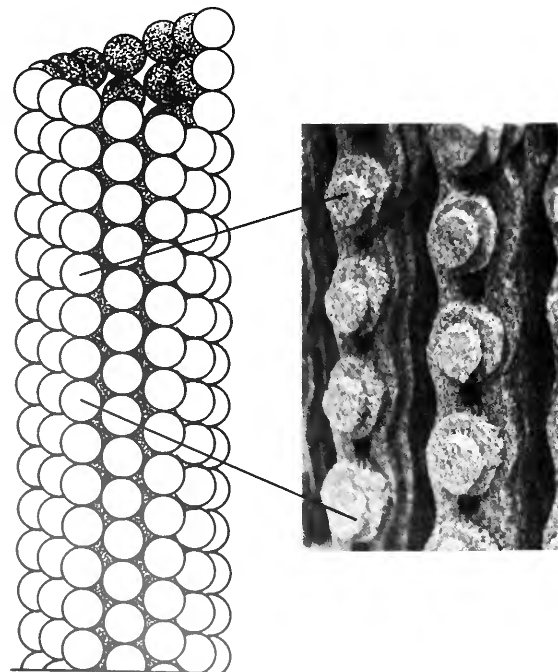

Figure 7. A medium-resolution model for the substructure of a portion of the microtubule wall (right). The repeating contours represent the relative distribution of protein molecules to form longitudinal filaments, two of which are shown here. In the complete microtubule (left), thirteen such filaments, each comprised of repeating "beads" (tubulin molecules), form a cylinder. (E. Mandelkow, Brandeis University)

R. E. Stephens is jointly a resident investigator at the Marine Biological Laboratory, Woods Hole, and an associate professor of biology at Brandeis University, Waltham, Massachusetts.

Suggested Readings

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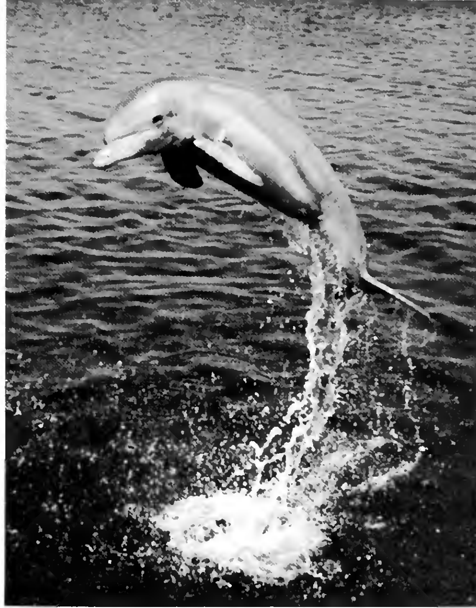
Diving Mammals and Biomedical Research



The bottlenose porpoise, Tursiops truncatus, *is the most common cetacean in captivity and the one most frequently used in biomedical research. This animal, a male named Tuffy, participated in several open-ocean diving experiments.*

by Sam H. Ridgway

Diving is essential for marine mammals. Since most sea life is below the surface, large mammals must be able to dive and remain submerged for relatively long periods in order to feed. The animal must be able to hold its breath, withstand the effects of pressure (about one atmosphere for each 10 meters of ocean depth), and keep warm in a relatively cold environment (water transports heat from a submerged body at least 20 times faster than does air). Because the animal must be a good swimmer, it has a spindle-shaped body that is hydrodynamically efficient, as well as powerful muscles of propulsion associated with flukes (tail) or fore or hind flippers (limbs). Finally, in order to navigate, avoid obstructions or predators, and find food, an ocean mammal must have a sensory system that functions well under varying sea conditions—for example, the porpoise's excellent sonar, or echolocation, that allows it to find its way in dark or very turbid waters.

It is these capabilities—the structural and functional adaptations—that have been the thrust of biomedical investigations of marine mammals. Recently, more attention has been given to the conservation of these animals, and, in addition to research possibly having applications to man, there have been studies concerning animal diseases, nutrition, and husbandry.

Marine mammals are by no means ideal laboratory animals. They are large and often difficult to acquire. For swimming they need a

Figure 1. California sea lion, famous for circus tricks and balancing acts helped by its long and mobile whiskers.

pool with a large pump that continually draws water from the sea or that recirculates the water through a filter to maintain sanitation. Special water treatment, such as chlorination, is sometimes necessary. As for diet, a small sea lion or seal will eat 3-5 kilograms, and a bottlenose porpoise 7-10 kilograms, of fish or squid each day. Thus, the expense of keeping just one animal can be considerable.

Marine mammals belong to three orders: the Cetacea (porpoises, dolphins, and whales); the Pinnipedia (seals, sea lions, and walrus); and the Sirenia (manitees and dugongs). The sea otter, a member of the order Carnivora, and the polar bear are also considered to be marine mammals. Most biomedical research on diving has involved the smaller cetaceans and pinnipeds, especially the California sea lion, the gray, common, elephant, and Weddell seals, and the bottlenose porpoise.

California Sea Lion

The California sea lion, *Zalophus californianus*, commonly seen in trained "seal" acts, has small external ears, or pinna, and large propulsive fore flippers on which it can stand or run about on land (Figure 1). This animal is probably most famous for its ability to balance a ball on the tip of its nose—a feat made easier by the fact that the sea lion has long (6-15 centimeters) mobile whiskers that can wrap around the ball and help to hold it in place. These whiskers appear to be excellent tactile organs and are employed in finding food and avoiding obstacles. When a sea lion is blindfolded, it extends the whiskers before starting to swim.

A sea lion in our laboratory was trained to hold its breath just under the surface of the water for as long as 8 minutes, and I would estimate that the maximum submersion time for large males of this species is at least twice as long. Roland Raffler of the Naval Undersea Center trained two young sea lions to dive untethered in the open sea to depths as great as 250 meters.

Gray Seal

An inhabitant of the northern North Atlantic, the gray seal (*Halichoerus grypus*) is fairly common around the British Isles, Norway, the Soviet Union, Iceland, Greenland, and Canada. In some areas, it maintains breathing holes in the arctic ice. The gray seal is a phocid or true seal. It does not have pinna, swims primarily with the rear flippers, and is not nearly so mobile on land as is the sea lion. In the late 1930s, P. F. Scholander used gray seals in some of his pioneering experiments on respiratory physiology of diving mammals. I have trained a two-year-old gray seal to remain submerged for as long as 8 minutes. When I closed off the breathing hole in its tank, the same seal made a dive of 18 minutes without ill effect. B. L. Scronce of the Naval Undersea Center is presently training three of these animals to dive in the open ocean. Thus far they have reached a depth of 100 meters.

In order to compare brain waves and heart rates (by means of electroencephalograms and electrocardiograms, respectively) during voluntary and forced dives, gray seals were trained to dive on command in a large covered glass tank equipped with a breathing hole (Figure 2). When the breathing hole was blocked, the seal was forced to dive. Just before the hole was closed, a buzzer was sounded to warn the seal. Upon hearing the signal, the animal took a hurried breath, expelled it as a large bubble under the glass inside the tank, then rushed back for more breaths before the hole was blocked. After 2 or 3 minutes of submersion, the seal returned to the bubble and with great "nasal dexterity" rebreathed the air, leaving only a tiny bubble containing no more than 2 or 3 milliliters of air. Later the seal exhaled a liter or so of air and left it under the glass for a couple of minutes, then breathed it again. Because the seal's heart rate increased when it rebreathed air, stopcocks had to be installed in the ceiling of the tank to vent off the animal's cache during dives. After this had been done, the seal stopped the behavior.

It was not until this behavior of exhaling and then rebreathing air had been observed repeatedly in several different seals that its possible significance was realized. In northern latitudes the gray seal spends a part of every year swimming and diving under arctic ice while maintaining breathing holes. If a large air bubble were exhaled under ice, carbon dioxide would rapidly diffuse from the bubble

because it is about 40 times more soluble in sea water than is oxygen. The animal could therefore get rid of some carbon dioxide and, at the same time, "store" air in several locations before making a long exploratory dive under the ice.

The gray seal weans at approximately three weeks of age, and since the seals used in the experiment described above were only about four weeks old when they were collected off Iceland, it was very unlikely that they had been exposed to ice. Thus, their behavior could not have been learned by observation of other seals. For this behavior to be innate, it must be important to the survival of the species. It would be interesting to find out if and how seals living under the ice use such behavior.

The Common Seal

The common seal, or harbor seal (*Phoca vitulina*), is widely distributed in the upper half of the Northern Hemisphere (Figure 3). Its diving depth is not known, but it is capable of relatively long submersion times—probably more than 20 minutes. This seal was used extensively by R. J. Harrison and J. D. Tomlinson in London to elucidate the functional significance of some anatomic features, such as the caval sphincter, which can close down, limiting blood flow returning to the heart, and to

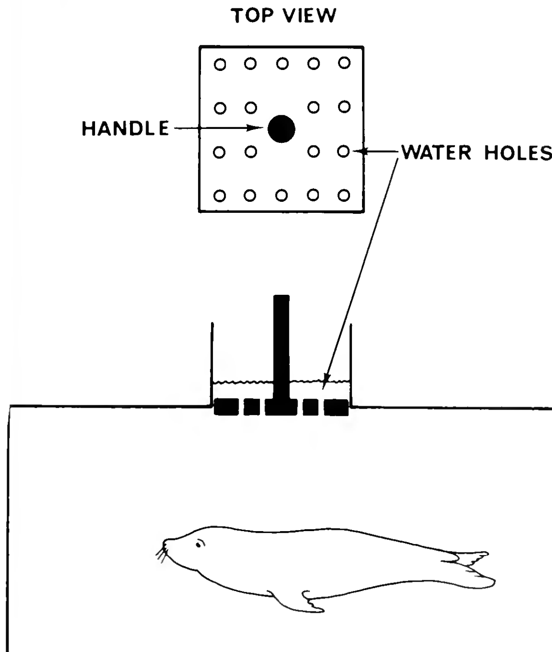


Figure 2. The setup for forced unrestrained dives by gray seals. After a submersion, the device blocking the breathing hole is removed and the seal is allowed to breathe. The upper figure is a top view of the device used to block the breathing hole.

Figure 3. A young elephant seal (left) and a common seal (right).

establish that bradycardia (slow heart rate) during a dive was maintained via the vagus nerve. H. V. Murdoch, E. Robin, and their associates used common seals from Maine in a series of experiments that answered a number of basic questions about respiratory and circulatory physiology. Lawrence Irving and J. Hart employed common seals to describe some mechanisms of thermoregulation.

Elephant Seal

Largest of pinnipeds is the elephant seal, *Mirounga angustirostris* (Figure 3). Males have reached 6.5 meters in length and 4 tons in weight. This seal, generally considered to be a good diver, can remain submerged for 45 minutes or more and probably descends to great depth. A small seal (100 kilograms) in our laboratory made trained dives of 13 minutes' duration and was once observed, apparently asleep, submerged on the bottom of the tank for 23 minutes. Elephant seals have an extremely large blood volume, reaching 20 percent of body weight in young animals—two or three times as much blood in proportion to body weight as has man or dog.

Weddell Seal

Although seldom kept in a laboratory, the Weddell seal (*Leptonychotes weddelli*) of the Antarctic has been a very useful subject for diving studies. These animals live on or under the antarctic ice for most of the year. In diving, they must return to the surface to breathe and thus depend on breaks in the ice, caused by wind or currents, which they keep open by gnawing at the ice.

Investigators such as R. Elsner and G. L. Kooyman of Scripps Institution of Oceanography have caught Weddell seals and transported them

across the ice to a point well removed from any breathing holes. They attach recording gear to an animal and release it through a newly bored hole, perhaps enclosed by a laboratory hut. When the seal returns to the hole to breathe, the scientists recapture it and retrieve data on the dive. Because of this method of taking the laboratory to the field, the Weddell seal has become one of the most thoroughly studied of all diving mammals. Dives of more than one hour's duration and of 600 meters' depth have been recorded.

Bottlenose Porpoise

The bottlenose porpoise, *Tursiops truncatus,* is widely distributed in temperate and tropical waters. Because of its great adaptability, *Tursiops* has been used in most research on cetaceans.

In 1969 J. Kanwisher, B. L. Scronce, and I had a splendid opportunity to test the diving capability of a trained bottlenose porpoise in the open sea. The animal was quartered in a small floating pen in the Pacific Ocean about 200 meters from the Marine Bioscience Facility at Point Mugu, California. For diving tests, the porpoise was called from its pen to swim beside a small boat to a test buoy located in deep water about 8 kilometers offshore.

The porpoise usually positioned itself on the stern wave of the boat to get something of a free ride by "surfing" most of the way. Sometimes, when there were large waves at the dive site, the porpoise entertained himself between dives by riding the waves. (It is this playful wave-riding behavior that allows us to capture some species of cetaceans in deep waters.)

Our porpoise, a male named Tuffy, was trained to dive and press a switch located at the end of a heavy electrical cable 308 meters long. When a high-frequency signal was turned on at a control box aboard the boat, Tuffy was required to dive and press the switch turning off the sound. Immediately, a low-frequency tone signaled the porpoise to return to the surface and exhale into an inverted water-filled funnel 50 centimeters below the surface (Figures 4 and 5). (The average dive to 300 meters took just over 3 minutes round-trip.) The porpoise was also taught to breath hold just under the surface, in response to a signal, and to exhale into the funnel on command.

Tuffy had been trained to participate in the U.S. Navy Sealab II tests in 1965, delivering items to and carrying lines between divers on the sea floor. We took advantage of Tuffy's experience to have him swim rapidly back and forth between two divers on the bottom at a depth of 20 meters.

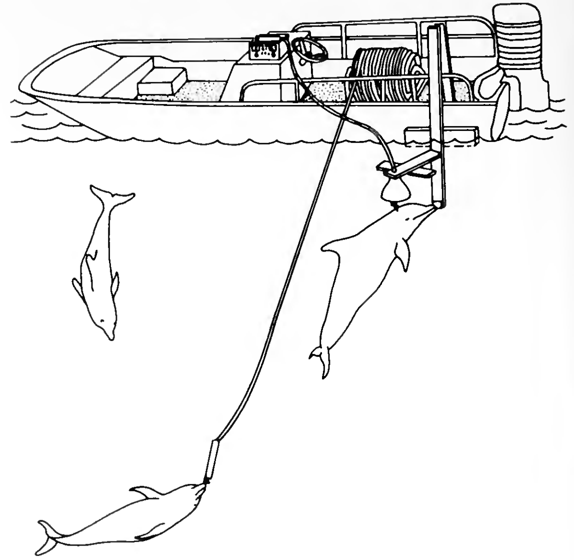


Figure 4. The procedure for deep-diving tests in the open ocean. Upon hearing a high-frequency signal, the porpoise dives to the test switch and turns it off. When a low-frequency sound begins, he returns to the surface and exhales into the funnel.

After 2 or 3 minutes, the low-frequency buzzer was sounded from the boat to signal the porpoise to return to the surface and exhale into the funnel.

With these various methods, expired air was collected from deep dives, surface breath holds, and shallow dives that involved considerable exercise (swimming back and forth between divers). The analyses for oxygen and carbon dioxide after dives

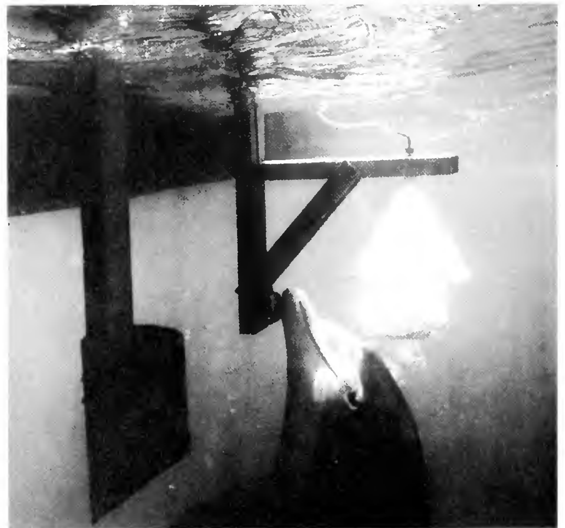


Figure 5. Porpoise exhaling into the funnel after completing a deep dive.

and breath holds requiring the same periods of time (up to 4.5 minutes) showed that the most oxygen was taken from the lung air when Tuffy was swimming back and forth at shallow depths. Less oxygen was used on breath holds near the surface, and much less oxygen was required at depths greater than 100 meters.

Although prolonged breath holding is essential for deep diving, this ability alone does not enable the animal to go to great depths. Structural adaptations, such as thoracic collapse (Figure 6), allow the air to be compressed away from the alveoli (air cells) of the lung during the increasing pressure of a dive. In addition, nitrogen is prevented from going into solution in the blood in significant quantities, and the animal avoids any problem of decompression sickness (bends). Our data, derived from a cooperating experimental porpoise, diving while completely free in the open ocean, substantiated the hypothesis of thoracic and alveolar collapse first introduced over 30 years ago.

Figure 6. Porpoise pushing the diving test switch at a depth of 300 meters. Thoracic collapse is evident, especially in the area behind the left flipper. Photograph was taken with an underwater camera that was triggered when the porpoise pressed the test switch.

Let us consider some of the factors that enable marine mammals to breath hold for prolonged periods. As a porpoise dives, the body starts to make adjustments to conserve oxygen. The heart beats more slowly, reaching about one-third to one-fifth the predive rate in porpoises. At the same time, blood vessels that supply much of the body start to constrict, saving the store of blood for organs such as the heart and brain that need it most. Since water at depth is usually much colder, the constriction of peripheral blood vessels also tends to conserve body heat by keeping blood away from cooling surface tissues, which, in turn, lowers their metabolism and oxygen requirement.

In comparison to land mammals, most marine species have increased oxygen transport and storage capabilities. The quantity of muscle myoglobin (the pigment that stores oxygen in the muscles) is much larger, and the total blood volume is greater, especially in those species that are better divers. Although the red blood cells of most porpoises are slightly smaller in diameter than human red blood cells, the better divers have more cells and considerably more hemoglobin. All these factors combine to increase oxygen transport and storage ability.

Even so, this is not enough oxygen to maintain aerobic metabolism during the longer dives that some marine mammals are known to make. Many tissues, especially the muscles, must rely on anaerobic metabolism, that is, metabolism that does not require oxygen.

Data obtained from our diving experiments with Tuffy in the open ocean indicate that during the final half of a deep dive there was just barely enough oxygen to maintain the heart. So what of the brain? Since man's brain is responsible for as much as 20 to 25 percent of the total body oxygen consumption, and since the bottlenose porpoise also has a large and convoluted brain (Figure 7), we suggested that toward the end of a deep dive a nearly complete anaerobicity exists throughout the animal, including, at least partially, the brain. At the time, in 1969, this appeared a highly unlikely idea to many. However, the following year Elsner and his co-workers at Scripps published evidence of cerebral tolerance to asphyxia in Weddell seals, and in 1971 D. Kerem, also at Scripps, demonstrated anaerobic metabolism in the brain of the harbor seal during the late stages of a submersion. Since then, L. Simon, E. Robin, and co-workers at Stanford University have shown that the level of pyruvate kinase, an enzyme that has been closely linked with anaerobic metabolism, is higher in Weddell seals than in common seals or sea lions. In these three species, there is increased pyruvate kinase in the brain and the heart with increasing maximal diving times. The idea of anaerobic metabolism in the brain of diving marine mammals is certainly becoming more tenable.

Bradycardia

Over one hundred years ago, Paul Pert, the great French physiologist, described the phenomenon of

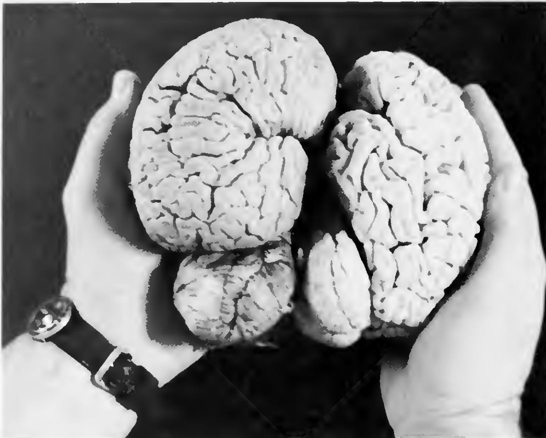


Figure 7. Brain of a bottlenose porpoise (left) compared with a human brain.

"reduced frequency of heart contractions" in submerged ducks. This condition has since been termed diving bradycardia, and it is the most familiar cardiovascular response in diving aquatic animals. In ducks and seals the response can be eliminated by sectioning the vagus nerve; in ducks, bradycardia can also be eliminated by sectioning the ophthalmic branch of the trigeminal nerve. Many investigators have therefore speculated that marine mammals have sensors about the head or snout that trigger bradycardia when the head is submerged.

Most early researchers used various methods of forcing the animal to dive. It is now recognized that restraint and fright may have been an important component of the physiological data reported from many experiments on marine mammals. For this reason more recent investigators have emphasized training the animal to cooperate in the experiment and using nonrestrictive means, such as implanted telemetry devices, to obtain physiological measurements.

To learn more about diving bradycardia, I studied development of the phenomenon under differing conditions in seals and sea lions. Radiotelemetry devices were implanted in the animals so that they could be studied over long periods (a year or more) without restraining them. A seal was trained to submerge and put his nose on an underwater light; he was to hold it there until the light was turned off and a whistle was sounded indicating that he had performed correctly and would be rewarded with a fish from an underwater feeder.

When the seal first submerged, the heart rate dropped from 100 beats per minute (BPM) or more down to around 10 BPM for a few seconds, then increased to 20-40 BPM where it remained until the light went off and the whistle sounded indicating the end of the dive. At the sound of the whistle, the heart rate immediately went up, well before the seal resurfaced. Sometimes there would be another decrease in heart rate while the seal remained underwater to eat the fish, but as soon as the animal reached the surface and took its first breath, the heart rate increased to 120-180 BPM.

When a seal was forced to submerge by having its breathing hole blocked, the heart rate immediately fell to as low as 3 BPM, remained under 15 BPM for the entire dive, and increased dramatically with the first breath after the dive.

Since it seemed that the seal could adjust bradycardia to fit the circumstances of the dive, D. Carder, W. Clark, and I decided to see if we could condition a sea lion to lower its heart rate. In order to eliminate any possible effect of immersion, we chose to work with the sea lion out of the water. A sea lion's heartbeat normally cycles with respiration, increasing to 100-140 BPM during breaths and decreasing to about 60-90 BPM between breaths. These normal episodes were reinforced by sounding a command tone when the heart slowed and then giving a second tone (or bridging stimulus) when the rate slowed for at least three successive beats, followed by a fish reward. At first the sea lion was required to lower its heart rate only to 80 or 90 BPM, but this demand was gradually reduced to 10 BPM within 20 seconds after the command tone was sounded.

Thus the sea lion can slow its heart rate either directly or through some other muscular adjustment and exhibits an impressive degree of voluntary bradycardia. Vasoconstriction and other components of the "diving reflex" may also be under voluntary control.

Precise knowledge of the mode of control of autonomic responses in the sea lion might shed light on certain human medical conditions, such as sudden infant death syndrome. Recent investigations have suggested that apnea, abnormal heart rhythm, and other aspects of the human "diving reflex" may be involved in sudden infant death syndrome.

Diet and Metabolism

The diet of marine mammals is normally high in protein and fat. Such a diet is helpful in increasing the metabolism and allowing the animal to keep warm. Very little carbohydrate is consumed since most fish contain only a trace (1 percent or so) and squid has only a little more.

When I gave a porpoise a glucose tolerance test, the results were similar to those of a human diabetic. There was no rise in blood insulin. Gloria Patton of the Naval Undersea Center found

that insulin did rise when a porpoise was given amino acids. The animal's blood sugar level, which is normally higher than that of man, is produced from amino acids through the process of gluconeogenesis, in which the other pancreatic islet hormone—glucagon—is quite important.

The fats contained in a marine mammal's diet are mostly of the highly unsaturated type, which deteriorate very rapidly in storage. When formulating a fish-substitute ration, it was therefore necessary to use non-fish products such as saturated animal fats. Some porpoises have been fed saturated fat for three years, with no increases in cholesterol level in the blood.

Diving Mammals and the Bends

Of course, marine mammals are breath-hold divers. They do not breathe compressed gas as scuba and hard-hat divers do. For a long time this was thought to be sufficient explanation of why marine mammals do not experience the bends when making numerous dives at sea, but more recently there have been reports of decompression sickness and bendslike conditions in human breath-hold divers.

As mentioned earlier, we have a good idea how bends are prevented in marine mammals during deep dives—over 100 meters or so. However, when many successive dives are made to lesser depths, as most feeding dives by seals and porpoises undoubtedly are, there should be a distinct probability of bends—unless the animals have some resistance to the condition. Our trained porpoise Tuffy made 48 dives to depths of from 40 to 260 meters in a single 4-hour session without any discernible ill effect.

Porpoises have slowed blood-clotting times and are deficient in clotting factor 12, or Hageman factor. This may mean that porpoises have a decreased propensity for intravascular clotting, which may be a component of bends resistance since intravascular coagulation has been shown to be a major factor in severe cases of bends. Disseminated intravascular coagulation also occurs in human shock cases.

Among the circulatory changes during prolonged dives is an increase in blood acidity. In man, slow-flowing acid blood is highly coagulable. It seems likely that diving mammals have evolved blood factors that resist or inhibit such potentially damaging situations.

James McCormick of Bowman Gray School of Medicine has demonstrated that guinea pigs treated with heparin show a reduced incidence of deafness after decompression (deafness commonly occurs in decompression sickness). In addition, whale heparin is reported to have a very high anticoagulant activity. McCormick and I both suspect that porpoises may have resistance to bends because there is less tendency for emboli (gas bubbles and clots) to form in the blood due to a more potent heparin, an absence of Hageman factor, or other biochemical adaptations involved in the coagulation mechanism.

Conclusions

No other group of mammals offers so many interesting challenges to the physiologist as those that live in the sea. Through studies of these animals may come answers to such medical problems as human diving diseases, shock, sudden infant death syndrome, diabetes, and stress metabolism. By investigating the adaptations of marine mammals to water immersion, we may even learn more about the ways in which man can adapt to prolonged weightlessness during space flights.

Throughout history enlightened men have had a high regard for marine mammals, especially porpoises. Because of our admiration and great empathy for these splendid animals, we must proceed very carefully in imposing our will upon them, even in the most humane experiments. Research involving trained animals in the open sea, where an animal is free to swim away and leave the experiments forever if it so desires, have given me great confidence. Although a few have chosen to desert, the great majority of marine mammals have returned, even after a week or more of freedom.

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Figures 4-6 from S. H. Ridgway, B. L. Scronce, and J. Kanwisher, "Respiration and deep diving in the bottlenose porpoise," *Science*, vol. 166, 26 December 1969, pp. 1651-54. Copyright 1969 by the American Association for the Advancement of Science.

Suggested Readings

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Metchnikoff Revisited: From Rose Thorns and Starfish to Gout and the Dogfish

by Gerald Weissmann and Sylvia Hoffstein

Marine organisms constitute a singular biological resource for the exploration of basic mechanisms underlying human disease. Indeed, many processes common to higher organisms can be studied to greater advantage in lower marine forms because of the great variety—with respect to shape, size, and accessibility—of the cells and organelles (parts of cells) that these creatures have developed in response to the selective pressures of their environment. Consequently, it is often possible to analyze *general* phenomena of cell biology in experimental systems unique to such diverse species as echinoderms (sea urchins), bipinnaria (starfish), molluscs (clams), and elasmobranchs (dogfish, sharks, skates).

Perhaps the most venerable example of the utility of marine forms for analysis of disease is afforded by the experiments of Metchnikoff, who, in 1883, proposed the theory of phagocytosis on the basis of his detailed observations of starfish larvae. (Phagocytosis is the process whereby cells eat and digest foreign materials such as disease-causing microbes.) Since much of the work in our laboratory, from 1960-75 (*1-5*), has been directed at elaborating the general observations of Metchnikoff (albeit, one hopes, with the aid of more modern insights), it seems worthwhile to present Metchnikoff's own description (*6*) of the first intersection between phagocytosis and inflammation:

> One fine day, my family had gone to the circus to see some particularly well-trained performing apes. I remained alone with my microscope, in order to watch living, motile cells of a transparent starfish larva, when I was suddenly seized by an idea:
>
> "The same cells must also serve in the defense of the organism against foreign invaders?"
>
> I became excited. With great steps I paced up and down the room [the living room of Metchnikoff's house by the straits of Messina, which he had set up as a marine biological laboratory], and hurried finally to the sea-shore, in order to collect my thoughts. Were my supposition to be true, I told myself, then a thorn which is stuck into a starfish larva, which has neither blood vessels nor a nervous system, would soon become surrounded by motile cells, exactly as one observes in a man who has run a splinter into his finger. No sooner said, then done! Our apartment had a little garden, in which, a few days earlier, we had converted a tangerine-tree into a Christmas tree for the children; from this garden I plucked some rose-thorns and immediately inserted them under the skin of the beautiful starfish larvae which were as transparent as water.
>
> Because of my excitement, I naturally could not sleep, filled with anticipation of the results of the experiment, which, in the earliest hours of daylight I was able to verify as completely successful! This experiment was the foundation for my theory of phagocytosis to which I dedicated the next 25 years of my life.

The motile cells of the starfish larvae were soon termed phagocytes, and the relationship between phagocytosis and inflammation became gradually clearer. Before Metchnikoff, it had been assumed that blood vessels or the nervous system were involved in inflammation; or even that *fever* itself played a role. It was Metchnikoff's insight into the utility of a marine organism that lacked a circulatory or nervous system—and that could not regulate its own temperature—that placed phagocytosis at the center of the inflammatory response. It is not appropriate here to detail the *fin de siècle* battle between humoral and cellular theories of host defenses, a struggle in which the sides were determined not only by intellectual, but also by national affinities. Suffice it to recall that the Nobel Prize of 1908 was shared by Metchnikoff, by then representing the French, or cellular, school of immunology, and by Ehrlich, the leader of the German, or humoral, school. By

1908 a kind of détente had been reached. It was appreciated that the inflammatory response observed after phagocytosis, by cells of an immune host, to foreign invaders was due to the uptake of antibody-coated particles (mainly microbes) by phagocytic cells. The digestive ferments (enzymes) of these cells were diverted, by accident as it were, to an attack as injurious to the host's tissues as to the invading microorganism.

As an introduction to the next episode, which culminated in the description of lysosomes as those organelles responsible for the direct relationship between phagocytosis and inflammation, we can again quote from Metchnikoff (*6*):

> The leucocytes [white blood cells], having arrived at the spot where the intruders are found, seize them after the manner of the *Amoebae* and within their bodies subject them to intracellular digestion. This digestion takes place in the vacuoles in which usually is a weakly acid fluid which contains digestive ferments (cytases); of these a very considerable number are now recognized . . . The cytases must also be grouped with the soluble ferments which are not thrown off by the phagocytes so long as these remain intact. Immediately these cells are injured, however, they allow a part of their cytases to escape.

Lysosomal Enzymes and Inflammation

Relatively little new information as to the relationship between phagocytosis and inflammation emerged in the fifty or so years after Metchnikoff's formulation. But in 1958, at the Marine Biological Laboratory in Woods Hole, Massachusetts, Christian deDuve announced to the Society of General Physiologists (7) the discovery of a new organelle—the intracellular repository of Metchnikoff's cytases—the lysosome. It was quickly recognized that phagocytic cells were particularly rich in lysosomes; indeed the characteristic granules of our most numerous and active white cells, the polymorphonuclear leukocytes, are now considered to constitute a paradigm for lysosomes. Moreover, early in the framing of this paradigm, deDuve had suggested that lysosomes served the cell as a kind of "suicide sac," causing death of the cell in which they were contained when the cell was injured in various ways.

Therefore, it is not surprising that between 1958 and 1970, it was considered likely that inflammation resulted from injury to the leukocytes, which "allowed part of their cytases to escape." Indeed, we had been able to show, with enzymes obtained from lysosomes of leukocytes, that these enzymes, or cytases, were capable of provoking inflammation when injected into various experimental animals (*1-5*). However, controversy raged (or smoldered, to be exact) as to whether primary injury to lysosomes could also lead to death of the cell in which the lysosomes were harbored, or whether these organelles simply contributed their lytic ferments to the post-mortem cleanup of debris after lethal injury to the plasma membrane, the mitochondria, or other cellular constituents. Indeed, our own work had cast doubt on the "suicide sac" hypothesis of lysosomal enzyme release.

It turns out that when polymorphonuclear leukocytes ingest particles such as bacteria or antigen/antibody complexes, they extrude a portion of their lysosomal enzymes by a mechanism we have called *regurgitation during feeding* (Figure 1). As the phagocytic vacuole containing an ingested particle is joined by the leukocyte's lysosomes at its internal border, the vacuole remains open at the plasma membrane, permitting an accidental regurgitation of enzymes (*1-5*). In Metchnikoff's terms, the "cytases escape" *not* because of cell injury, but because there is a direct, secretory pathway between portions of the cell's digestive tract (the lysosomes) and the extracellular space. This sequence of events has been deduced both from electron microscopic studies and from the biochemical demonstration that phagocytosing cells selectively release lysosomal enzymes (for example, beta-glucuronidase, cathepsins, lysozyme) but not cytoplasmic enzymes (for example, lactate dehydrogenase) into the surrounding medium.

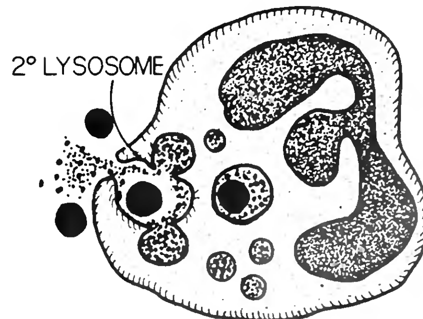

Figure 1. Escape of lysosomal enzymes from phagocyte, as foreign material is ingested, results from incomplete closure of phagocytic vacuole. This is joined by primary lysosome (coarse stippling) to form secondary lysosome, from which the inflammatory material escapes inadvertently.

Gout: Interactions of MSU with Leukocytes

Since the early 1960s, clinicians have been fairly certain that acute gout is caused by the sudden deposition, in and around joints, of crystalline monosodium urate. The pain and swelling of knees, toes, and ankles appear to result from the presence of small cyrstals, visible by light microscopy, within joint tissues and leukocytes of the joint fluid. Therefore, the interaction of urate crystals with leukocytes had been studied by many investigators in order to understand how this sort of crystal-induced inflammation is launched. (An old hypothesis was that the needlelike crystals physically punctured the cell.) It soon became clear that the crystals were ingested by human polymorphonuclear leukocytes. Immediately after uptake, very little happened; but 30-45 minutes after ingestion, the cells die.

What became an issue (*8-10*) was the mechanism whereby the gouty crystal (monosodium urate, or MSU) kills the cell (after this sort of latent period). When human polymorphonuclear leukocytes are exposed to artificially produced crystals of MSU in vitro, they release into surrounding fluids *both* lysosomal enzymes and cytoplasmic enzymes (*5*). Thus, the regurgitation-during-feeding mechanism, characterized by *selective* extrusion of lysosomal enzymes, was certainly not at play. Two possibilities remained: either the crystals acted directly on the plasma membrane so as to injure the cell from without; or the crystals were ingested and taken into lysosomes, finally to rupture the lysosome, and thereby the cell's integrity, from within. This latter mechanism has been called *perforation from within.*

Wallingford and McCarty (*8*) had found that crystals of MSU could directly rupture human red cells, provided there was no serum surrounding the red cell plasma membrane. Indeed, they presented evidence that crystalline MSU formed hydrogen bonds with the membranes of red blood cells and suggested that the crystals might also form such bonds with the internal membranes of the leukocyte lysosome, causing the organelle to rupture and to perform its true role as a "suicide sac."

Upon hearing this hypothesis, we were struck by the idea that its critical testing might lead not only to molecular explanations of acute gout, but also to a test of the "suicide sac" hypothesis of deDuve: that lysosomes, if permitted to leak their cytases into the cell sap, would kill the cell itself. We were able to demonstrate (*11*) that isolated lysosomes (from rabbit and human) could, indeed, be ruptured by crystals of MSU in vitro, provided the experiments were done in media that did not prevent hydrogen bonding, a specific kind of chemical interaction between crystals and membranes. And other needle-shaped crystals that did not form hydrogen bonds were incapable of producing membrane damage, thus disposing of the pin-prick theory. Moreover, when we prepared artificial lipid membranes (liposomes), crystals of MSU disrupted these structures as readily as they did natural membranes; again, only in media that did not constrain hydrogen bonds.

But critical to the proof of the dynamics of this sequence (ingestion, fusion of lysosomes with the phagocytic vacuole, hydrogen-bonding of crystal to the membrane, rupture of the secondary lysosome, and perforation from within) was its direct observation in living cells. And that required use of nonhuman material, because the lysosomes of human leukocytes can barely be seen by light microscopy. The utility of marine organisms for biomedical research became obvious again.

Introducing the Dogfish to Gout

We were fortunate to discover that the phagocytes of the dogfish, *Mustelus canis,* possess lysosomes large enough (0.7-0.8 micron) to permit cinemicroscopic testing of our perforation-from-within hypothesis. So we prepared white cells (buffy coat) from this species, which is abundant around Woods Hole (*12*), as we had human cells.

First, we studied the cells biochemically, as we had human cells. When dogfish white cells were incubated with urate crystals, both cytoplasmic enzymes and lysosomal enzymes were released, the latter somewhat more slowly than the former. This sequence would be expected if either hypothesis were correct—if the cells that had ingested the urate crystals were killed after perforation-from-within or if the crystals induced primary injury to the cell's plasma membrane, as in ordinary cell death induced by detergents.

How could we prove that it was the *ingestion* of urate crystals that mediated cell death and enzyme release? The answer came from carefully observing the behavior of cells exposed to urate. Using light microscopy we saw that half of all cells that had ingested urate crystals died within 30 minutes. Within an hour 87 percent were dead. In sharp contrast, only 8 percent of white cells that had *not* ingested crystals were killed during the same time period.

Close examination of individual cells over the entire sequence from ingestion to death revealed that the ingestion phase was very rapid—less than 3 minutes. After the crystals were taken

up, the cells continued to move. In about 15 minutes, the crystals in their phagocytic vacuoles began to fuse with primary lysosomes to form secondary lysosomes that gradually lost their granular appearance and became, within 45 minutes, pale, crystal-filled "lakes." Not long thereafter, by the end of the first hour, cell death was evident. Crystals were ejected, to be taken up by living cells nearby.

These observations were fully confirmed, in greater detail, by electron microscopy (Figures 2 and 3).

In order to provide a more crucial test of the hypothesis that ingestion was required for urate-induced injury, we treated cells with cytochalasin B, an agent known to prevent ingestion, but which does not prevent either surface contact or enzyme release. Both biochemical and electron microscopic observations confirm that cell-crystal interactions result in fusion of swollen lysosomal membranes with the plasma membrane, with lysosomal contents being released inside, and eventually outside, the cell (Figure 4).

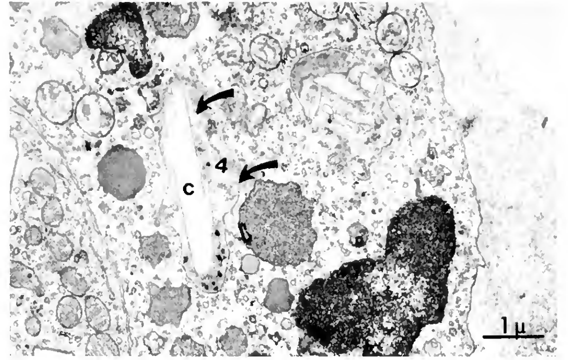

Figure 3. The critical stage (#4) in which the membrane of the lysosome is first perturbed, and then ruptured (arrows) after interaction of monosodium urate crystal with dogfish phagocyte. Electron micrograph taken 60 minutes after ingestion. Close inspection of secondary lysosomes in cells that appeared morphologically intact showed occasional membrane discontinuities. Images were even seen in which the broken ends of the membrane curled up on themselves. Prolonged incubation for demonstration of lysosomal enzyme activity showed reaction product in primary and secondary lysosomes with some traces in the cytoplasm adjacent to secondary lysosomes as well. Such images are most likely due to the release of lysosomal enzymes from broken organelles; artifacts caused by diffusion of reaction product were excluded by control observations. No such extralysosomal deposits of reaction product were seen in early (0-15 minutes) material.

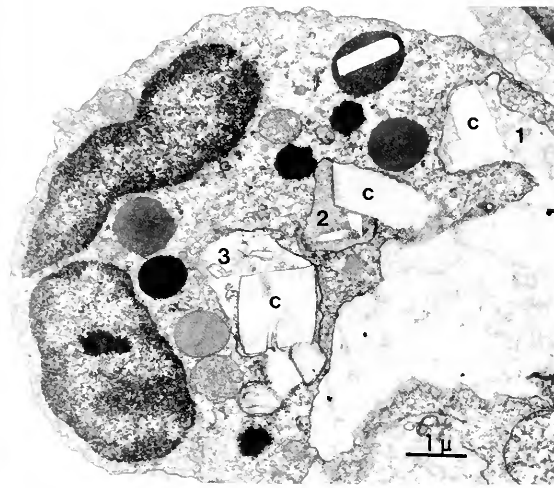

Figure 2. Electron micrograph illustrating the first three stages of interaction between crystals (C) of monosodium urate (MSU) and dogfish phagocytes. Numbers (#) refer to stages in the process shown in diagrammatic form in Figure 4. Buffy-coat suspensions were fixed for electron microscopy at various intervals after exposure to MSU crystals. In all specimens, crystals (C) could be seen inside the forming secondary lysosomes (#1, #2), the appearance of which suggested that the flexible lysosomal membrane accommodated itself to the shape of the crystal. In specimens fixed more than 15 minutes after exposure, some of the crystal-containing granules were swollen when compared to adjacent granules, and their contents appeared more flocculent and less electron-dense (#3).

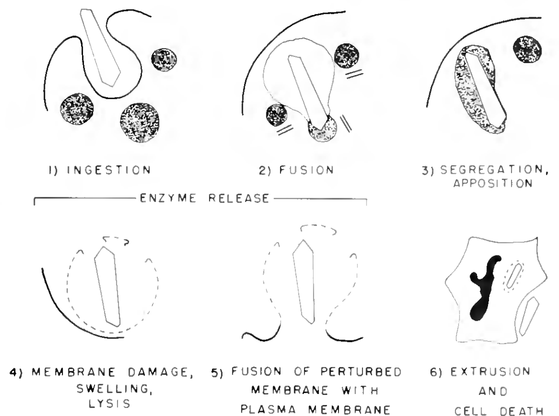

Figure 4. Schematic representation whereby the dogfish phagocyte is killed by its encounter with monosodium urate (MSU), mimicking events in human gouty arthritis. Numbers 1-4 of sequence are depicted in Figures 2 and 3.

Summary

Our studies (*11, 12*) with dogfish phagocytes indicate that the molecular basis of gouty inflammation can be succinctly stated in terms that would have pleased Metchnikoff and that support (in this one pathologic instance) deDuve's "suicide sac" hypothesis.

1. Polymorphonuclear leukocytes ingest monosodium urate crystals.

2. The lysosomes (Metchnikoff: vacuoles with weakly acid reaction) of the cell merge with the phagocytic vacuole. Among the contents of lysosomes (Metchnikoff: cytases) are enzymes that digest the inhibitory plasma proteins that coat the crystal, thereby permitting hydrogen bonds to form between crystal and membrane. The organelle swells osmotically.

3. The organelle bursts, releasing lytic enzymes (cytases) into the cytosol, and destroying the cell.

4. The contents of the leukocyte are emptied out into joint tissue, and the inflammation of acute gout results.

The use of marine organisms has only begun to elucidate mechanisms of human disease such as in the model discussed above. Thus, for example, we have also been able to use the dogfish phagocytes as a model of cells having a genetic deficiency of a lysosomal enzyme and to replace this enzyme by means of genetic engineering. It has been possible to force the dogfish cell into taking up the missing enzyme by wrapping the material to be introduced in an artificial lipid layer coated with antibody. Acting as if this kind of biological spansule were a foreign invader, the dogfish cells were persuaded to ingest the missing enzyme, thereby reconstituting their own genetic deficiency. This model is relevant to genetic engineering in many human genetic diseases resulting from absence of a specific lysosomal enzyme.

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