Unexpected Progress in Photoreception

A recent gathering of photobiologists has replaced their major guiding hypothesis of the past 15 years by a new one

One of the most insidious and nefarious properties of scientific models is their tendency to take over, and sometimes supplant, reality.

—ERWIN CHARGAFF

For almost 15 years virtually all biologists studying the means by which vertebrate photoreceptor cells transduce a light stimulus into an electrical response have been in the thrall of the "calcium hypothesis." In its basic formulation, which did not change much throughout its reign, the hypothesis suggested that the effect of light is to release calcium from storage within the photoreceptor cell, which then interacts with ion channels in the membrane, generating the electrical response.

The principal conclusion of a recent Dahlem conference in West Berlin* is that the calcium hypothesis as originally formulated is almost certainly wrong. Cyclic GMP has assumed the status as most favored candidate for the elusive phototransduction messenger, an idea whose longevity matches that of the calcium hypothesis but, until now, has never equaled its popularity. It is possible that cyclic GMP exerts its effect by direct interaction with its target rather than via a series of enzymic intermediates, which would be a first for this ubiquitous internal messenger.

A major question that any solution to the transduction problem has to encompass is the astonishing amplification that is built into the system. The absorption of a single photon by a single photopigment—rhodopsin—in a dark-adapted receptor can shut off 2 to 4 percent of the conductance through the membrane, which implies that several hundred ion channels have been closed, presumably by the release of many times that number of messenger molecules. The biochemistry of photoreceptor cyclic GMP that has been elucidated over recent years does indeed offer such an amplification mechanism (see box on page 501).

Transduction is, however, just one of a pair of basic puzzles presented by photoreception, the second one being light adaptation. How do cells that can respond to a single photon—albeit minimally and rather slowly—also respond to light levels five log units higher? An idea that began to emerge from the Dahlem meeting was that, perhaps this is the role that calcium performs. Perhaps calcium modulates the response of the cyclic GMP system to ever increasing levels of illumination? Persuasive to some, this idea also had a goodly number of critics.

It is of course inaccurate to speak of "the" vertebrate photoreceptor, as there are two types, the names of which betray their gross anatomy: cones, which mediate color vision and high light sensitivity; and rods, which respond to low light stimulation. As difficult as the biology of rods has proved to be to understand, that of cones is even more so. Cones, as one participant at the Dahlem meeting said, are a virtual complete mystery. Most detailed work on vertebrate photoreception is done on rods, particularly those of amphibians, which are largest and therefore most accessible.

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Rods are curious cells: they seem to do everything the wrong way around. Unlike most reactive cells, which depolarize on stimulation (moving from an internal voltage of −70 millivolts (mV) to transiently positive), rods hyperpolarize (their membrane potential shifting from −30 mV to −70 mV). Depolarization is caused by a rapid influx of sodium ions, hyperpolarization by a temporary interruption of such a flow. It is as if rods are constantly switched on when they are not being stimulated: a current is flowing in the dark called, not surprisingly, the dark current. On illumination, they are switched off.

Their internal biochemistry reflects this too. Deric Bownds of the University of Wisconsin likens it to the excited state of muscle or other hormone-sensitive cells: high levels of calcium, high levels of cyclic nucleotide, both of which (probably) decrease on stimulation.

In addition to being unusual in their electrophysiology and biochemistry, rods have proved to be exceptionally difficult to study. The principle reason is that even the slightest manipulation appears to alter their function in some way, which makes interpretation of observations exceedingly precarious. Moreover, accurate measurement of internal levels of some of the key components—including calcium and cyclic GMP—have so far proved impossible. Hence, the calcium hypothesis accumulated a lot of supportive circumstantial evidence but nothing that could be taken as definitive. This, its plausibility and the force of personality of its chief proponent, William Hagins of the National Institutes of Health, are the reason the hypothesis endured for so long.

When the calcium hypothesis first came onto the scene—in 1971—it seemed a perfect answer to the frustrations that photobiologists then faced. For years the prime focus of interest had, understandably enough, been the rhodopsin, which constitutes so much of the rod's molecular structure (see box on page 502). Indeed, so focused on this photopigment was people's attention that preparation of rod material for study often meant washing the rods until virtually nothing but rhodopsin remained.

George Wald of Harvard University had shown in the late 1930's that the pigment was composed of a protein called opsin combined with 11-*cis* retinaldehyde, a derivative of vitamin A. Later work by Wald and others showed that illumination isomerized the 11-*cis* chromophore to the all-*trans* form, which caused the pigment to bleach through a series of intermediates at a decreasing pace. As bleaching involved a major reconfiguration of the protein pigment, it appeared conceivable that this might be responsible for initiating the electrical response. Exactly how, was unclear. Especially unclear was how the amplification process might occur.

So, when the calcium hypothesis came along it filled a real conceptual void and seemed particularly attractive because of its apparent analogy with muscle action.

*"The molecular mechanisms of photoreception," 25–30 November 1984, West Berlin. Proceedings of the meeting will be published in 1985 and can be obtained from Dahlem Konferenzen, Wallostrasse 19, D-1000 Berlin 33, Federal Republic of Germany.

In both there were convenient stores of calcium: disks in photoreceptor cells and lacunae in the sarcoplasmic reticulum of muscle. In both, stimulation releases the calcium, which initiates the response. Calcium, after all, is a common ion channel controller and was therefore a logical candidate for this role in the photoreceptor. Or so it seemed.

Hagins demonstrated that exposure of rods to high concentrations of calcium caused a similar response to the light-induced signal, which was taken quite reasonably to imply that once it passed into the photoreceptor through the ion channels, the raised intracellular calcium shut the channels down, causing hyperpolarization in the usual manner. And later work, including some quite recent results, appeared to show that when rods are illuminated under normal conditions there is a flux of calcium out of the cell. The interpretation here was that light boosted intracellular calcium, some of which flooded out of the cell.

In fact, as was revealed at the Dahlem meeting, there are now strong questions about both the putative increase in intracellular calcium on illumination and the interpretation of the experimental calcium-induced light response.

Nevertheless, in the absence of these latest results, the calcium hypothesis looked promising, especially as it fitted logically with the idea that on illumination a bleached rhodopsin molecule might punch a hole in the disk membrane, or more subtly act as a calcium ion carrier. However, in spite of being a transmembrane protein bearing all the molecular architecture of a transport system, rhodopsin has never been shown to so function, either individually or in aggregates. But this was negative evidence, and there were possibilities of other channels in the disks, including one gated by cyclic GMP. And in recent times Hagins and his colleagues have been developing the idea that calcium and cyclic GMP might interact in the transduction process but with calcium playing more or less its traditional role.

When the organizers worked out their planning for the Dahlem conference a little more than a year ago the calcium hypothesis looked as strong as ever. The meeting was expected to be a further sophistication of the model. Several new results seemed to confirm this, including the publication in May of data from a new technique for measuring calcium release from rods. However, other results had begun to accumulate in those months running up to the conference, which collectively raised important question marks.

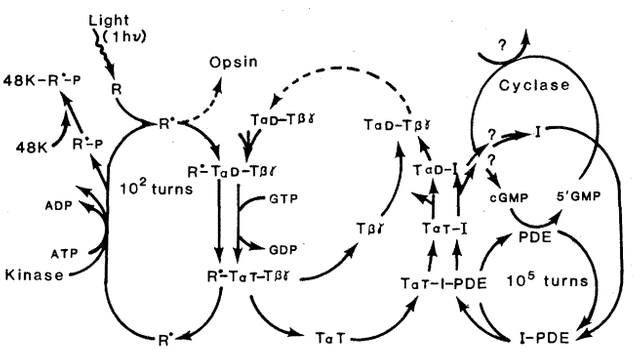
For instance, Benjamin Kaupp, and collaborators in Bownds laboratory, showed that intracellular levels of calcium could be substantially reduced (by treatment with an ionophore) without abolishing the cell's ability to respond to light. In experiments begun in mid-August, Trevor Lamb and colleagues at the University of Cambridge, England, used a different technique for mopping up intracellular calcium, and got similar results. If the cell can shut down its ion channels in the virtual absence of calcium, the calcium hypothesis must be in doubt.

Another Cambridge group, in Alan Hodgkin's laboratory, managed to infuse calcium into dark-adapted rods and show that although the dark current is shut down, as in the light response, the reaction time is slow, at least several seconds. Moreover, if light is shone during this slow calcium-induced shutdown, the cell responds immediately to cut off the

An Amplifying Cascade

During the past half decade or so biochemists have tracked down some—probably not all—of the components in an enzymic cascade that is triggered by light-activated rhodopsin and leads to rapid hydrolysis of cyclic GMP, with a tremendous built-in amplification.

Once bleached, a rhodopsin molecule can make a productive interaction with another membrane-bound protein, known variously as GTPase, G protein, Transducin, or, more simply, T protein. T protein is composed of three subunits, Tβ and Tγ, which appear to be restricted to movement within the plane of the membrane (usually written Tβγ), and Tα, which might well be able to rattle around within the interdisk space. In their inactive form, the three subunits are aggregated together, and a molecule of GDP is bound to the Tα unit. On interaction with activated rhodopsin, R*, the GDP is exchanged for a GTP, and (probably) the Tα dissociates from its partners. This Tα then kicks the membrane-bound phosphodiesterase (PDE) into action (possibly by removing an inhibitory subunit, I), which hydrolyzes cyclic GMP. Details of the switch-off mechanism are still unclear, but at some point the GTP on the Tα is converted to GDP, which signals the release of the inhibitory unit, thus shutting down the diesterase. The extreme fluidity of the vertebrate rod membrane appears to be important in the multiple interactions between R*, T protein, and PDE, even though some of the action is in the interdisk space. It is interesting to



Activated rhodopsin leads to cyclic GMP hydrolysis via an amplifying cascade.

note, therefore, that rhodopsin mobility in many invertebrate disks is highly restricted.

The shutdown of activated rhodopsin is also unresolved, although it appears to involve multiple phosphorylations at the C terminus by a soluble kinase, possibly combined with the subsequent binding of another soluble protein, known as 48K. Each bleached rhodopsin can switch on 10^2 T proteins, which in turn activates 10^3 PDE's—giving an amplification of roughly 10^5.

Illumination of rhodopsin also leads to a boost in guanyl cyclase activity, the overall result of which is an increase in the flux of the cyclic nucleotide, rather than a precipitous decline in level. How the cyclase is activated—whether via a drop in the intracellular calcium or by R* directly—remains unclear. The search for the missing components goes on.—**R.L.**

dark current. King-Wai Yau and Kei Nakatani at the University of Texas, Galveston, obtained similar observations. The doubt here is, if calcium is the key that locks the ion channel, why does it do it so slowly under these conditions, and why does a light flash override it?

Further problems for the calcium hypothesis comes from Geoffrey Gold's laboratory at Yale, once a source of support. Having reported some years ago that, consistent with the calcium hypothesis, calcium flows from the light-stimulated rod, Gold now finds with more refined measurement that the time course of calcium flux changes does not fit with the light-induced electrical response. Specifically, he observes a flow of calcium into the rods during the recovery stage following light stimulation, a period when the channels are still partially closed. This would not be expected if internally elevated calcium were indeed blocking the channels. He infers that the intracellular calcium is reduced, not increased, on illumination.

Yau has come to the same conclusion using an electrophysiological approach. The interpretation is that under dark conditions there is a two-way flow of calcium across the membrane: a pumping out via a sodium/calcium exchange mechanism and a leakage in via the light sensitive channels. On exposure to light the rod shuts down the light-sensitive channels, which stops the inward leakage of calcium but not the outward pumping. The result is a transient elevation of calcium levels outside the rod and an internal reduction. As the channels begin to open the inward leakage resumes, following the concentration gradient to lowered intracellular levels. If true, this clearly is inconsistent with the traditional calcium hypothesis.

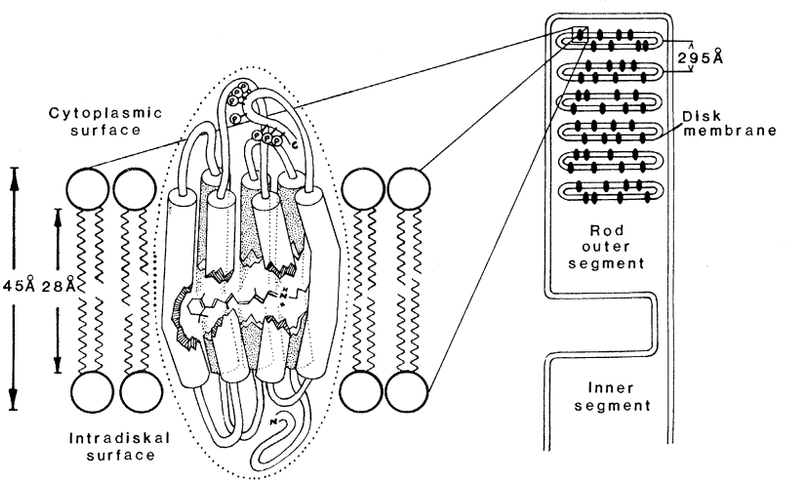
The most dramatic and most telling results of all, which were revealed late in the conference proceedings, concern observations with plasma membrane "patches," done by Yau and independently by a Russian group, which was not present. By placing a pipette on the surface of a rod, sealing, and then pulling, a small patch of membrane adheres across the bottom of the pipette, which can then be exposed to chosen chemicals and the membrane response measured. Both teams find that exposure of the inner surface of the membrane to calcium does not radically affect the response of the ion channels whereas very low levels of cyclic GMP rapidly opens them. In addition to the startling implications for the nature of the phototransduction mechanism, this is the first demonstration of the direct effect of the cyclic nucleotide on its target. Cyclic GMP usually works by activating kinases, which then phosphorylate target proteins.

The scenario for phototransduction would therefore be that the channels are maintained in an open position by high levels of cyclic GMP in the dark and that exposure to light initiates a rapid hydrolysis of the nucleotide, which causes the channels to close. The problem here is that although the culmination of work in several laboratories over the past half decade or so has demonstrate a very

A Paradoxical Molecule

The ubiquitous photopigment rhodopsin is a paradoxical molecule: it bears all the molecular features of a transmembrane transport protein, and yet it appears to function as a hormone, in initiating an enzymic cascade leading to cyclic GMP hydrolysis.

Amino acid sequencing, and most recently DNA sequencing, of bovine rhodopsin has revealed it to be composed of a series of α-helices linked by "loops." The protein, which has a molecular weight of 41,000, appears to pass back and forth across the membrane with the α-helices bunched closely together, leaving the C terminal end on the cytoplasmic face of the disk and the N terminal in the intradisk space. Although all attempts to demonstrate that rhodopsin might be a transport molecule have produced negative results, John Findlay, of the University of Leeds, England, pointed out firmly that this multihelical stacking within the membrane is typical of transport proteins and, so far at least, has not been seen in nontransporters: the transport possibility must therefore remain open. It is interesting that bacteriorhodopsin, the purple pigment of certain halobacteria, has the same



Scheme of a rhodopsin molecule within a disk membrane.

overall configuration (but not amino acid sequence) as bovine rhodopsin and is a light-activated transmembrane proton pump. Rhodopsin may, of course, be the evolutionary descendant of a transport protein, which has since been modified and lost that function.

While the retinaldehyde chromophore is buried deep within the membrane portion of the pigment, the sites that interact with T protein, which initiates the cyclic GMP cascade, and the kinase, which phosphorylates the bleached pigment, are in the cytoplasmic portion of the molecule. These sites are exposed only after bleaching: the intact photopigment itself is a remarkably inert molecule. So far, no function has been ascribed to the intradisk segments of the protein chain. This is tantalizing because in preliminary comparisons of bovine and *Drosophila* rhodopsin, Meredithe Applebury, of Purdue University, has observed that one of the few conserved regions between the two pigments is the intradisk loop between helices 4 and 5. This section has some features that would equip it as a calcium or magnesium binding site, albeit an imperfect one.—**R.L.**

specific, light-initiated hydrolysis of cyclic GMP (see box on page 501), it has proved very difficult to demonstrate a reduction of the cyclic nucleotide at physiological light conditions.

One clear possibility here is that, of the quite large mass of cyclic GMP in rods, only a very small amount is free and available for binding with the channels. Changes in this component might not be detectable by current methods. Another aspect of this is that, as Nelson Goldberg of the University of Minnesota illustrated quite forcefully, light stimulation also induces increased guanyl cyclase activity, which resynthesizes the cyclic GMP. The result is a light-initiated increase in turnover.

For some biochemists, the idea of a change in cyclic GMP flux as a gate controller is attractive, presumably via an energy-linked process. For others, the accelerated machinery provides a neat mechanism for inducing a spike increase in cyclic GMP activity, specifically if hydrolysis is turned on just slightly before synthesis. The fact that, as has been demonstrated with the patch experiments, direct application of cyclic GMP to the channels can turn them on in the apparent absence of much of the rest of the cell's biochemical machinery tends to support the change in activity rather than change in flux idea.

However, some biochemists, including Bownds, wonder whether cyclic GMP is indeed the whole story. They wonder whether there are yet more components to be discovered, parallel pathways to be uncovered. For instance, one candidate recently considered as a potential messenger in photoreceptors is inositol triphosphate. Results with this compound so far are tantalizing, if somewhat conflicting.

Meanwhile, calcium appears to be in search of a job. Fortunately there is one readily at hand. One repeated observation is that reduced levels of calcium decrease the light sensitivity of rods. Combine this with the possibility that one effect of light is to reduce intracellular calcium, and with the demonstration that reduction of calcium modulates the metabolic machinery for the turnover of cyclic GMP, and the potential emerges that calcium might be the mediator of light adaptation. Whether calcium modulation of cyclic GMP turnover can account for the range of physiological light sensitivity remains to be seen.

The Dahlem meeting certainly produced a surprise for most participants: it is not often that a research field turns around quite so dramatically.

—ROGER LEWIN

AIDS Virus Genomes

The molecular characterization of the virus that causes AIDS (acquired immune deficiency syndrome) continues to move at a rapid clip. Four groups have now determined the complete nucleotide sequences of the genetic material of viruses that have been linked to the disease.

Paul Luciw of Chiron Research Laboratories in Emeryville, California, Jay Levy of the University of California, School of Medicine, in San Francisco, and their colleagues report the sequence for the virus they have christened "AIDS-associated retrovirus" (ARV) in this issue of *Science* (p. 484). A second group, including Lee Ratner, Flossie Wong-Staal, and Robert Gallo of the National Cancer Institute, Mark Pearson of E. I. du Pont de Nemours and Company in Wilmington, and William Haseltine of Harvard's Dana-Farber Cancer Institute, report sequences for two isolates of the virus designated "human T-cell lymphotropic virus-III" (HTLV-III) in the 24 January issue of *Nature*. A group from Genentech, Inc., in San Francisco, is also publishing AIDS virus sequences in an upcoming *Nature* issue. And the fourth group, from Luc Montagnier's laboratory at the Pasteur Institute in Paris, published the sequence for the virus they have called "lymphadenopathy-associated virus" (LAV) in the January issue of *Cell*. Despite the different names given these viruses, there is now general agreement that they are variants of the same virus.

One question that the sequence data can help answer concerns how closely the AIDS virus resembles other retroviruses, especially HTLV-I and -II. All four groups find that there are substantial differences between the genome of HTLV-III and the HTLV-I and -II genomes, although some short segments show resemblances. "ARV is no more closely related to HTLV-I and -II than to other retroviruses," Luciw concludes. Simon Wain-Hobson of the Pasteur Institute presented similar findings for LAV at the recent "HTLV Symposium," which was sponsored by the NCI and held in Bethesda, Maryland, on 6 and 7 December. However, Gallo notes that there are other points of similarity between HTLV-III and the other two HTLV's that would favor including them in the same viral category.

Another important question, one that bears upon the possible mode of action of the AIDS virus as well as its relation to HTLV-I and -II, concerns whether its genome contains a "long-open reading frame" (LOR region) near its right-hand end similar to those in the HTLV-I and -II genomes. The products of the HTLV-I and -II LOR regions increase viral and possibly cellular gene expression, according to Haseltine, and this activity may be what causes the malignant transformation of infected cells. Haseltine, Wong-Staal, and their colleagues also have evidence that HTLV-III produces an analogous factor.

The organization of the ARV genome does not show a LOR region comparable to that of HTLV-I and -II, Luciw says, although it does have a protein-coding sequence about half the size of LOR in an analogous location. He notes that the organization of the ARV genome is very similar to what Wain-Hobson proposes for LAV. The Gallo group has a different interpretation of the coding sequence arrangement in the right-hand region of the HTLV-III genome. Although they do not see a separate LOR region there, their results indicate that the long *env* gene has a dual function. Depending on how the messenger RNA product of the gene is spliced, it can produce either the protein that forms the viral envelope or a protein of size comparable to the HTLV-I and -II LOR products.

Finally, restriction mapping of the genomes of various AIDS virus isolates has already shown that these may vary in structure, a result that is being confirmed by the sequence analyses. For example, the sequence of the long terminal repeat of HTLV-III, which was determined by the Gallo–Wong-Staal group and also appears in this issue (p. 538), differs in about 5 percent of its nucleotides from the long terminal repeat of ARV. Sequence analysis may help to identify viral gene segments that are highly conserved, and thus important for viral activities. Moreover, the corresponding peptides might eventually prove useful for producing reagents to detect infection by the virus or a vaccine to protect against AIDS.—JEAN L. MARX