A Tale of Two Pigments

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Recent discoveries on photoreceptors for both red and blue light compel a reexamination of certain older work, including some of my own. New data from molecular biology have removed the uncertainty in the interpretation of absorption and action spectra to define pigment systems active in photobiological phenomena.

In 1968, while on sabbatical at the Department of Biophysics at King’s College in London, I made use of their Universal Microspectrophotometer (UMSP-1, Zeiss, Göttingen, Germany) in an attempt to define the intracellular locale of phytochrome (Galston, 1968). I selected apical regions of etiolated oat (Avena sativa) coleoptiles and pea (Pisum sativum) epicotyls, known to have abundant phytochrome, exposing the plant material briefly to ambient white light during subsequent manipulations. I prepared either hand-cut sections, three to four cells thick, from fresh tissue, or thinner (15–20-µm) sections from frozen material with a cryostat-microtome. After being mounted in either glycerol, water, or paraffin oil, the material with a cryostat-microtome. After being mounted in either glycerol, water, or paraffin oil, the sections were scanned with a 0.5- to 1.0-µm diameter spot to obtain an automatically recorded absorption spectrum of each of the various parts of the cell. The system obviously worked well because the absorption spectra of slightly green chloroplasts showed characteristic chlorophyll and carotenoid peaks, and repeated scans coincided closely (see Fig. 1 in Galston, 1968).

For observations on phytochrome, I exposed the sections alternately to 5 min of either actinic 650-nm (red [R]) or 750-nm (far-red [FR]) light, and took a new absorption spectrum at the same location after each actinic exposure. With a 0.5-µm-diameter scanning spot, I noted distinct localized spectral shifts following the actinic R and FR treatments. These shifts appeared only in isolated areas of the nucleus, especially near the nuclear membrane, but not elsewhere in the cell. Most of the observed spectral changes occurred at or in the vicinity of the expected wavelengths for phytochrome transformation, but some did not. Feeling somewhat unsure of the significance of some of the data because of deviations from phytochrome’s known peaks, I mailed my results to Sterling Hendricks (U.S. Department of Agriculture, Beltsville, MD; now deceased), the discoverer of phytochrome, and solicited his opinion. To my pleasant surprise, his response was so strongly positive that he volunteered to (and did) submit my manuscript to the Proceedings of the National Academy of Science. At his suggestion, the paper includes the raw spectrophotometric data from the recording. Hendricks even mentioned that an “unusual” R-/FR-induced reversible shift in the 580 to 620 nm region that I had noted for a glycerol-mounted section (see Fig. 4 in Galston, 1968) confirmed his previous observations with glycerol-mounted dried films of phytochrome in gelatin.

Bolstered by this support from Hendricks, I concluded that my findings were evidence for the occurrence of phytochrome in the nucleus. I hypothesized that my data strengthened “the view that the pigment interacts in some way with genetic material, although the localization at or near the nuclear membrane may indicate control of passage of materials between nucleus and cytoplasm.”

To my knowledge, there has never been a refutation or even a repetition of these experimental findings. Nevertheless, as Linda Sage has observed in her account of the history of phytochrome research (Sage, 1992), “skepticism greeted this interpretation, because there appeared to be too little phytochrome to generate such an absorbance change.” For example, Spruit (1972), discussing the feasibility of phytochrome microspectrophotometry, concluded that “.... it appears doubtful whether local phytochrome concentrations inside the cell can be found sufficient to obtain spectrophotometric readings, comparable in sensitivity with such readings made on bulk samples.” To add to the skepticism, immunolocalization evidence obtained by Pratt et al. (Coleman and Pratt, 1974; Pratt and Coleman, 1974) revealed phytochrome only in the cytoplasm, not in plastids, mitochondria, or nuclei.

With regard to the spectrophotometric argument, I pointed out orally at meetings that the situation would be altered if, in fact, the majority of the phytochrome were aggregated as particulate material in the nucleus, forming an optically dense body that would give a stronger absorption signal. At the time, this suggestion was discounted because there was no evidence for the presence of phytochrome in the nucleus. However, only a little later, Quail et al. (1973) reported that cytoplasmic phytochrome, localized in the centrifugal supernatant fraction after prolonged dark or FR, became pelletable after exposure to R. This suggested that the location and status of...
phytochrome in the cell might depend on prior illumination and perhaps other conditions.

Within the last several months, this situation has become considerably clearer. Quail et al. (Martinez-Garcia et al., 2000) have shown that cytoplasmic phytochromes A and B bind to a nuclear transcription factor after R irradiation, thus moving to the nucleus after photoactivation. This binding of phytochromes to promoters turns on the expression of light-activated genes. Others (Kircher et al., 1999; Yamaguchi et al., 1999) have presented similar evidence for light-driven movement of phytochrome from cytoplasm to nucleus, leading Nagatani (2000) to summarize the situation as follows in a Science Perspective: "phytochromes perceive a light stimulus, move into the nucleus, interact with PIF3 which is bound to the G-box motif of a light activated gene, and switch the gene on."

Of course, it is not clear that the signals I detected resulted from such nuclear phytochrome complexes, but at least any theoretical objections to accepting that they might have represented such signals have now vanished. It all depends on the details of the experiment. We now know that phytochrome in dark-grown seedlings resides largely in the cytoplasm and moves to the nucleus only after irradiation with red light transforms it to the FR absorbing form of phytochrome (PFR). This movement takes some time, and in the case of the predominant phytochrome A, is accompanied by a loss of photoreversibility. Thus, for my results to be meaningfully connected to phytochrome, the conditions of prior irradiation of the tissue and the time interval involved before measurement of photoreversibility were critical. If I got these conditions right, it could only have been due to chance, because of course at the time I was ignorant of these relevant parameters. I can only say that preparation of tissue sample took place under the subdued light of the laboratory incandescent bulb, and required 10 to 15 min before the sample could be placed into the beam of the spectrophotometer. As reported above, the sample was then exposed for 5 min to actinic red light of unknown fluence rate before the first absorption spectrum was taken, then to 5 min of actinic far-red of unknown fluence before the second spectrum was recorded. If these parameters were "correct," then the detected reversibility could have been due to phytochrome A. This situation probably reinforces an old rule; i.e. when theory and data are in conflict, one should usually trust the data and alter one’s theoretical interpretation.

Our understanding of pigment localization has been even further transformed by very recent observations from the laboratory of Steve Kay (Mas et al., 2000). Recalling that many plant responses depend on interactions between multiple photoreceptors, Kay and his colleagues have found that cooperation between phyB and cry2 in control of flowering, circadian rhythms, and hypocotyl elongation in Arabidopsis depends upon their joint presence in nuclear "speckles" that are formed in a light-dependent fashion. Not only does phyB come down with cry2 in co-immunoprecipitation experiments, but the two pigments are able to transfer energy between them by a quantum-mechanical process of resonance energy transfer. Such a non-radiative mechanism can occur only if the two pigments are closely appressed, so that photoexcitation of one pigment can lead to fluorescence of the other. The authors conclude: “Together, these results demonstrate the light-dependent colocalization of phyB and cry2 in specific nuclear speckles.” What Kay et al. are describing might thus be characterized as a higher plant “eyespot.” The function of this pigment complex may well involve the binding of Ca^{2+} to a protein recently found by Guo et al. (2001) to be enriched at the periphery of the nucleus, near the nuclear envelope. The similarity to my description of the spectrophotometric localization of phytochrome is striking.

Some of my other earlier spectrophotometric data are also brought forward by these observations. Recently, the Cashmore (Ahmad and Cashmore, 1993; Cashmore et al., 1999) and Briggs (Christie et al., 1998; Briggs and Huala, 1999) laboratories have used molecular biological techniques to establish that the blue light photoreceptors cryptochrome and phototropin, respectively, are flavoproteins. This confirms a suggestion I made more than a half century ago (Galston, 1949, 1950) partially on the basis of spectrophotometric evidence. At that time, virtually all informed opinions on phototropic receptors favored carotenoids, rather than flavins, as the relevant photoreceptors. When I discovered that photoactivated riboflavin could cause the oxidative destruction of indoleacetic acid (Galston, 1949), I suggested that this mechanism might be responsible for the well-known asymmetry in auxin distribution in unilaterally illuminated coleoptiles. This proposal had to be discarded in view of Briggs’ quantitative support (Briggs et al., 1957) of Went’s suggestion (Went, 1928) that there was no change in total diffusible auxin during phototropic curvature. This indicated that lateral auxin translocation, rather than auxin destruction, was responsible for the asymmetries in auxin and growth patterns, and led to a rejection of the significance of the riboflavin-indole-3-acetic acid reaction and thus of riboflavin’s involvement in phototropism. However, I had also pointed out (Galston, 1950) that photoactivated flavins could catalyze oxidation of several amino acids like His and Trp, as well as peptides, enzymes, and even bacteriophages containing these amino acids. Thus, it was a mistake to discard the flavin hypothesis of photoreception on the basis of auxin data alone. I also pointed out (Galston, 1959) that action spectrum data from the blue region of the spectrum could not be used to discriminate between flavins and carotenoids. So, in
this instance as well, modern genetic and molecular
techniques have validated hypotheses derived from
spectral data that could not resolve an old problem
concerning photoreceptors.

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