8 Action Spectroscopy in Biology

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8.1 Introduction

Action spectroscopy is a method for finding out what the initial step is in a photobiological or photochemical process. More exactly, the method serves to identify the kind of molecule absorbing the active light.

The basic principle of the method is the following: The more light that is absorbed, the greater its effect on the material systems under study. By comparing the effects of light having different wavelengths, a measure is obtained of the relative absorption at different wavelengths by the molecule directly affected by the light. This can then be compared to absorption spectra of various compounds. If everything works out, one can identify the compound absorbing the active light in the photoprocess under study.

A hypothetical example: molecule A, which is present in an organism, has the absorption spectrum shown in Fig. 8.1. Absorption of light by A causes a certain effect, say formation of anthocyanin in a plant. If a certain anthocyanin synthesis is obtained by irradiating for *t* minutes with *N* photons per m^2 and second of wavelength λ_1 (or λ_3), it ought to suffice with half as many photons of wavelength λ_2, since such light is absorbed twice as strongly. Or, conversely: if it is experimentally found that the two lights have the action described, this is an indication that molecule A is mediating the light effect. With only two wavelengths investigated, the conclusion is still very uncertain. If the agreement between efficiency of the light and the absorptive power of A is extended over a wider spectral region, the conclusion will be more firmly founded.

Figure 8.2 shows the result of a fictive experiment involving the pigment with the absorption spectrum in Fig. 8.1. The effect of various exposures to light of wavelengths l_1 and l_2 has been measured. The effect of the irradiation is plotted along the vertical axis versus some quantification of light on the horizontal axis (this can be either photon fluence or photon fluence rate or photon irradiance, depending on what is being studied).

Note that if we compare the effects of a certain amount of light or certain irradiance (such as that indicated by 1 or 2 in Fig. 8.2) for the two wavelengths, these effects do not (except in very special cases) have the same ratio (2) as the corresponding absorption coefficients in Fig. 8.1. Thus, we cannot construct an action spectrum just by comparing effects of a fixed exposure or irradiance. We must construct curves such as those in Fig. 8.2, so we can see how much light is needed for a specific effect to follow, which we choose as standard action.

The rest of this chapter will be more historical in character than other chapters. My experience is that action spectroscopy is better understood by studying several real examples of its use than theory alone. In addition, the papers cited here include some that can stand as good examples for young scientists. In some cases they reflect real scientific

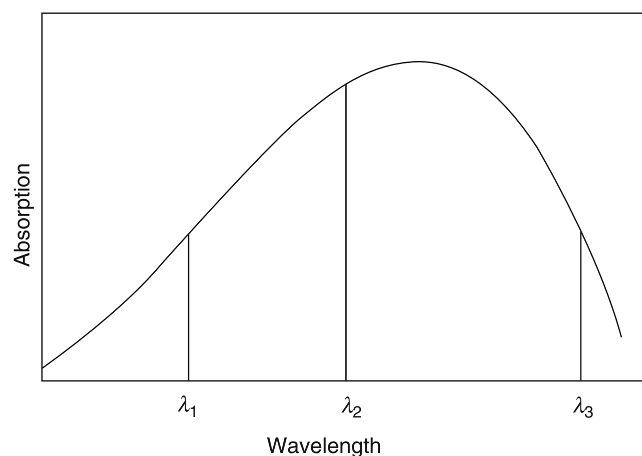

Fig. 8.1 The concept of action spectrum (see text)

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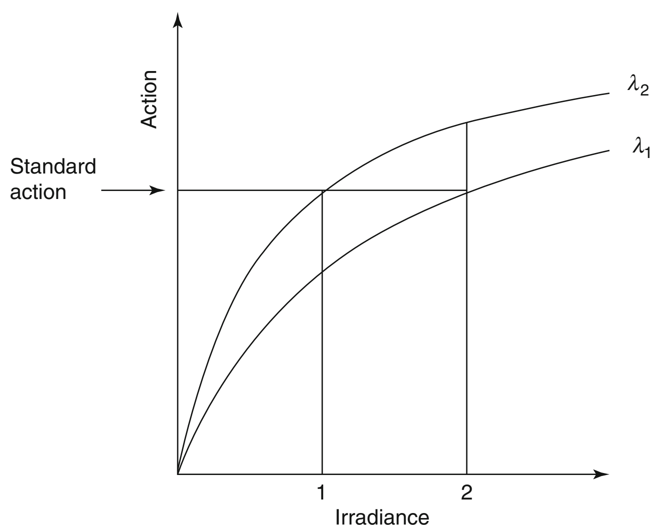

Fig. 8.2 The effect of different exposures of a sample (same system as in Fig. 8.1) on light of wavelengths λ_1 and λ_2. The effect or action of the light is plotted on the *vertical axis*. We compare in particular how much light is needed to achieve a certain action, which we choose as a standard action. We can see that twice as much is needed at wavelength λ_1 as at λ_2. From this we can deduce that light of wavelength λ_1 is absorbed by the active pigment only half as efficiently as light of wavelength λ_2

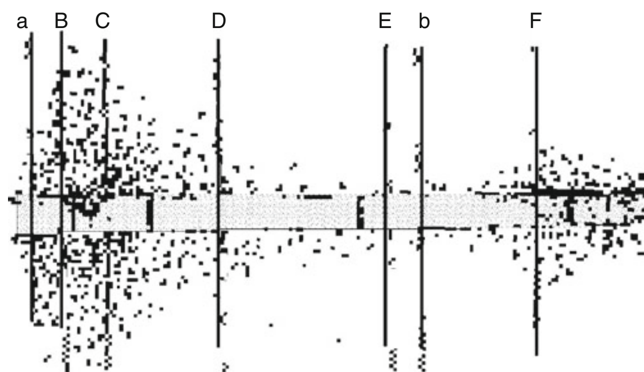

Fig. 8.3 A piece of a filamentous green alga (*Cladophora*, of which two whole cells and parts of two more cells are seen) with swimming bacteria in the sunlight spectrum projected in a microscope. The letters indicate the Fraunhofer lines in the solar spectrum, which are used for wavelength calibration: a = 718 nm, B = 687 nm, C = 656 nm, D = 589 nm, E = 527 nm, b = 518 nm, F = 486 nm. The accumulation of bacteria is greatest in the red region around 680 nm and in the blue region below 486 nm. These regions correspond to the main absorption bands of chlorophyll (From Engelmann (1882a))

ingenuity, and despite the rapid development of science, they have withstood the ravages of time remarkably well.

8.2 The Oldest History: Investigation of Photosynthesis by Means of Action Spectroscopy

Action spectroscopy may have its roots in Young's and von Helmholtz's theories about color vision. The first one, to my knowledge, to directly use action spectroscopy was T. W. Engelmann (1882a, b, 1884). He projected, under the microscope, spectra onto different algae and assayed the amounts of oxygen formed as a consequence of photosynthesis taking place in the algae. He estimated the relative amounts of oxygen by watching the accumulation of oxygen-loving (aerotactic) bacteria (Fig. 8.3).

Engelmann compared the oxygen-forming efficiency of different lights by reducing the light until the swimming movements of the algae stopped due to oxygen deficiency. In this way he could ascertain that in green algae it is chlorophyll that absorbs the light active in photosynthesis, while other pigments also participate in other kinds of algae. Figure 8.4 shows some of his comparisons between absorption spectra and action spectra. The chlorophyll present in red algae (as evident from their absorption spectra) does not show up in the action spectrum for their oxygen production. The cause of this surprising fact was not revealed until after World War II when Duysens, Emerson, and others discovered that two different photochemical systems cooperate in plant photosynthesis.

In addition to being an important step in the development of action spectroscopy and also in the history of photosynthesis research, Engelmann's experiments are important as early examples of a very sensitive "bioassay" of a chemical compound. The method of measuring oxygen by means of bacteria was so unconventional and the stated sensitivity in relation to other methods available at the time so remarkable that Engelmann was challenging the scientific authorities of his time. The algologist Pringsheim (1886) in Berlin as well as the Russian photosynthesis expert Timiriazeff (1885) found reason to criticize him using very harsh words.

Engelmann drew the correct conclusion that, in addition to chlorophyll, other pigments (colored substances) are able to absorb light and make it available to the photosynthesis process. He also understood (Engelmann 1882b) that there are other pigments in plant cells, which do not participate in photosynthesis but, on the contrary, "shadow" or "screen" the photosynthetically active pigments.

Engelmann's student Gaudikov studied chromatic adaptation (a designation in today's language, more consistent with the usual meanings of adaptation and acclimation, would be chromatic acclimation) in red algae and cyanobacteria, i.e. their acclimation to light of different colors. However, action spectra for this process were not determined until the 1960s by the Japanese Fujita and Hattori (1962) and in the 1970s, with greater precision, by the Americans J. Scheibe, S. Diakoff, and T. C. Vogelmann (see Diakoff and Scheibe 1973; Vogelmann and Scheibe 1978).

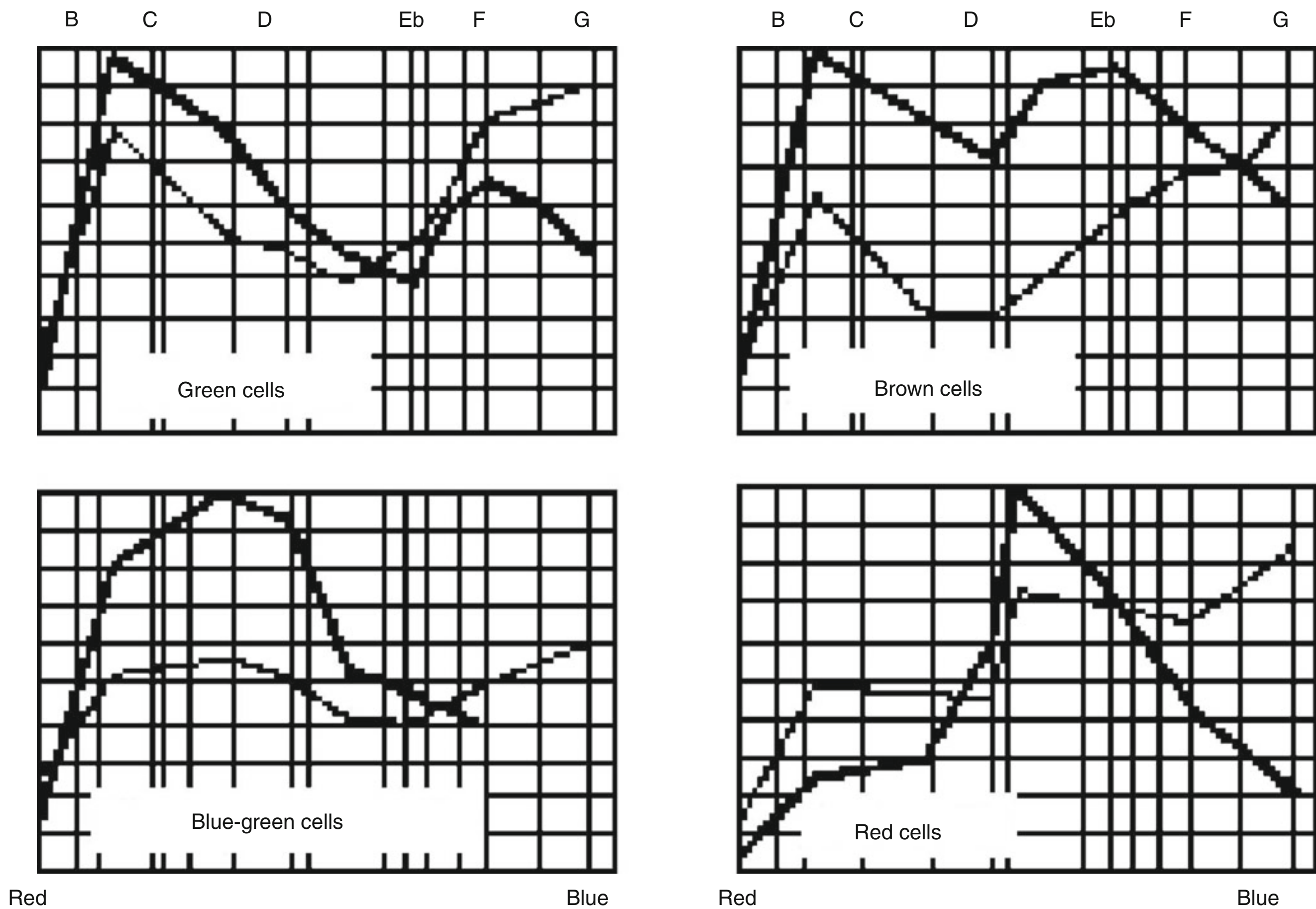

Fig. 8.4 Absorption spectra for algal cells (percent of incident light not penetrating the cells, *thin lines*) and action spectra for photosynthesis (*thick lines*). The high efficiency of light around 520 nm for photosynthesis by brown algae is due to light capture by the carotenoid fucoxanthin, the high efficiency around 620 nm in blue green algae to light capture by phycocyanin, and the high activity around 560 nm in red algae to light capture by phycoerythrin (From Engelmann (1884))

As for action spectra of photosynthesis, a few investigations were carried out during the intervening years, but real progress beyond Engelmann's results did not take place until the 1940s. For details of this development, the reader is referred to Haxo (1960). An early attempt was made also by Levring (1947) in Sweden to determine action spectra for photosynthesis in various algae, but he used wide spectral regions isolated with filters.

In this connection it is interesting to see how different scientists emphasized different aspects: Engelmann discussed in detail his method, spectral bandwidth, but lumped the algae together under the headings "green cells," "red cells," etc. Levring was less critical with regard to method but careful to state the species used and published separate spectra for closely related species.

It was above all the spectra measured by the Americans Haxo and Blinks (1950) by a polarographic method for oxygen measurement that was to yield results valid to this day (Fig. 8.5). Because of them, it became possible to do very careful comparisons between action spectra for photosynthesis and absorption spectra for various pigments in plants. Per Halldal brought this method to Sweden and improved it further (see Björn et al. 2007).

8.3 Investigation of Respiration Using Action Spectroscopy

The great Otto Warburg and his constant coworker Erwin Negelein (who, by the way, also determined action spectra for photosynthesis) over many years studied how the respiration of yeast cells is inhibited by carbon monoxide and how this inhibition can be removed by light (Fig. 8.6). They developed action spectroscopy to an accurate quantitative

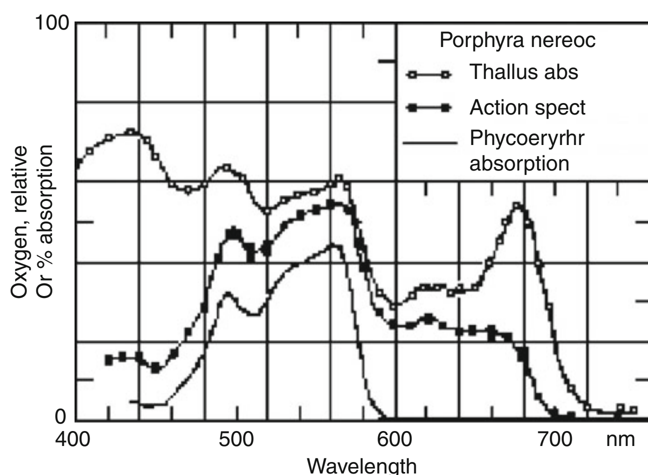

Fig. 8.5 The action spectrum for photosynthetic oxygen production in the red alga *Porphyra nereocystis* compared to the absorption spectrum of the same alga (Thallus abs.) and to the absorption spectrum of extracted phycoerythrin (From Haxo and Blinks (1950))

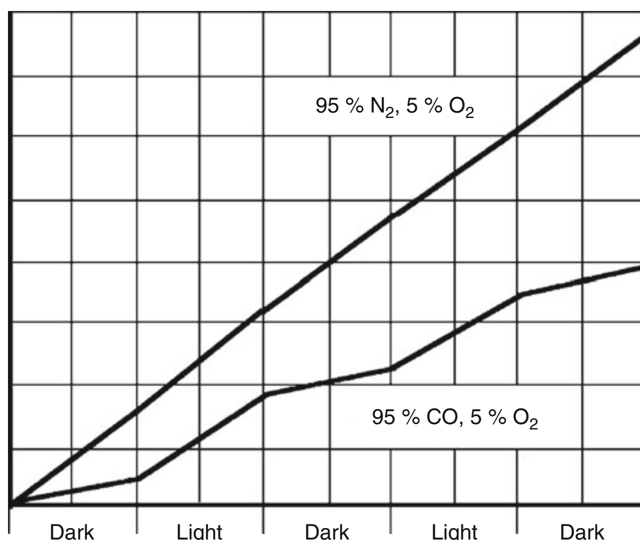

Fig. 8.6 The effect of light on oxygen uptake (respiration, *vertical axis*) in yeast in an atmosphere of 95 % nitrogen and 5 % oxygen (*straight line*) and in a mixture of 95 % carbon monoxide and 5 % oxygen. The horizontal coordinate is time, with alternating light and dark periods (From Warburg (1926))

method. As explicitly stated in one of their many papers, although they carried out the experiments together, it was Warburg who was the ingenious theoretician.

Their investigation led to the conclusion that the *Atmungsferment* (which we now call cytochrome *c* oxidase) is a protein to which iron-containing heme is bound and that the inactive complex formed with carbon monoxide is dissociated by light. The conclusion rests on the observation that the action spectrum for removing the inhibition of respiration by carbon monoxide agrees very well with the absorption spectrum for a complex between carbon monoxide and heme (Fig. 8.7). There is only a small shift in wavelength, which is explained by the binding to protein.

8.4 The DNA That Was Forgotten

At the beginning of the last century, Hertel (1905) in Jena had begun to study how microorganisms are affected by ultraviolet radiation. He managed to isolate nine different spectral lines from 210 to 558 nm and quantify the radiation using a thermopile. Considering the time, this was no small feat. Unfortunately, the evaluation of the biological effect was only semiquantitative. For constructing action spectra, he determined the irradiance that gave a just noticeable effect on the organism observable under the microscope. This effect could be stimulation of movement in *Paramecium* or contraction in rotifers. Unfortunately, he had no spectral line between 232 and 280 nm, and he therefore missed that region, which would later prove to be particularly interesting. Hertel's action spectra for ultraviolet damage to microorganisms are shown in Fig. 8.8. His experiments gave rise to a long-lived opinion that the deleterious action of ultraviolet radiation rises at an even rate toward shorter wavelength.

In the late 1920s, however, Gates (1928, 1930) found that the ability of ultraviolet radiation to kill bacteria varies with wavelength in the same way as does the ability of nucleic acid to absorb radiation (Fig. 8.9). This was the first indication of the fundamental importance of nucleic acid to life and a key experiment at the entrance to molecular biology.

At the same time as Gates' first report, another one was published, which was also on the road leading to the great revolution—Griffith's (1928) discovery of bacterial transformation. But it was not until after 1944 that the biological role of DNA became generally accepted by the demonstration of bacterial transformation by DNA (Avery et al. 1944).

One may wonder why Gates' experiments did not lead to a quicker development of DNA research. His work was in no way inferior to the transformation work. Perhaps an important reason was his way of publishing. In his first paper (1928) he does not show any convincing data. Despite this he clearly spelled out (although as we may think in retrospect, in a very cautious way) what was later proven to be essentially correct: "The close reciprocal correspondence between the curves of absorption of ultraviolet energy by these nuclear derivatives not only promotes the possibility that a single reaction is involved in the lethal action of ultraviolet light, but has a wider significance in pointing to these

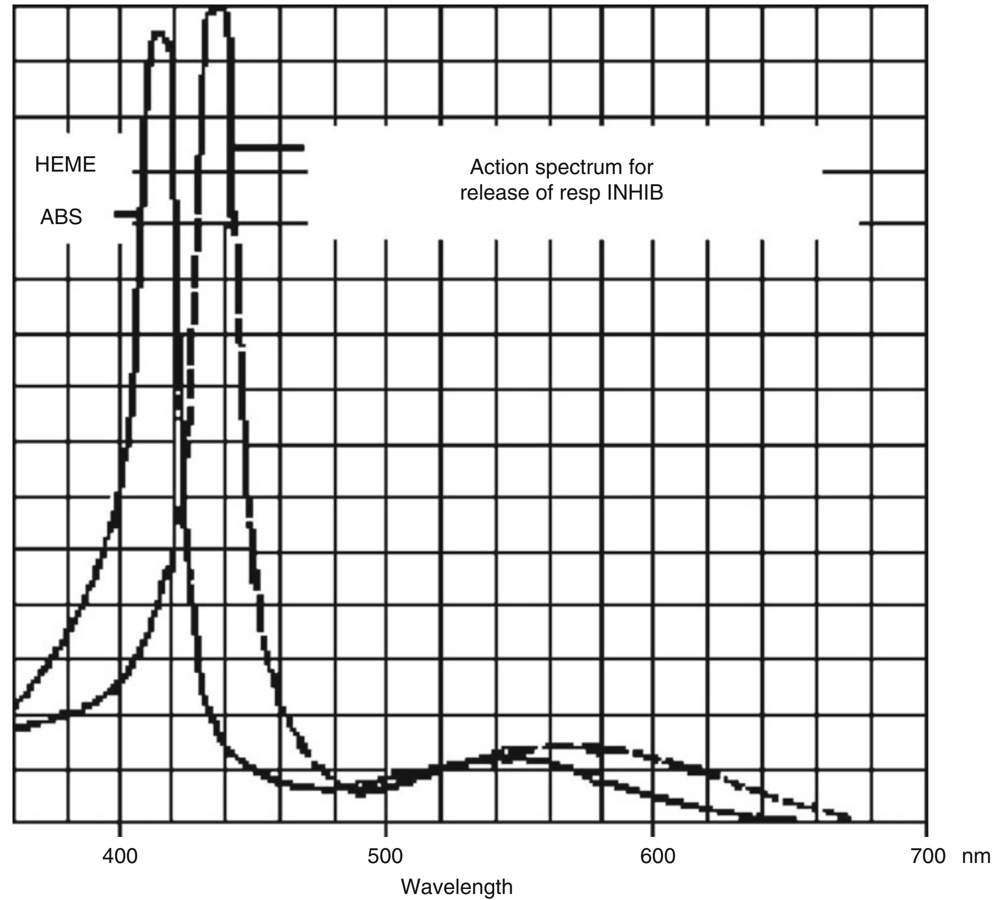

Fig. 8.7 Comparison between the absorption spectrum for heme and the action spectrum for release from CO inhibition of yeast respiration (From Warburg and Negelein (1929b); see also Warburg and Negelein (1929a))

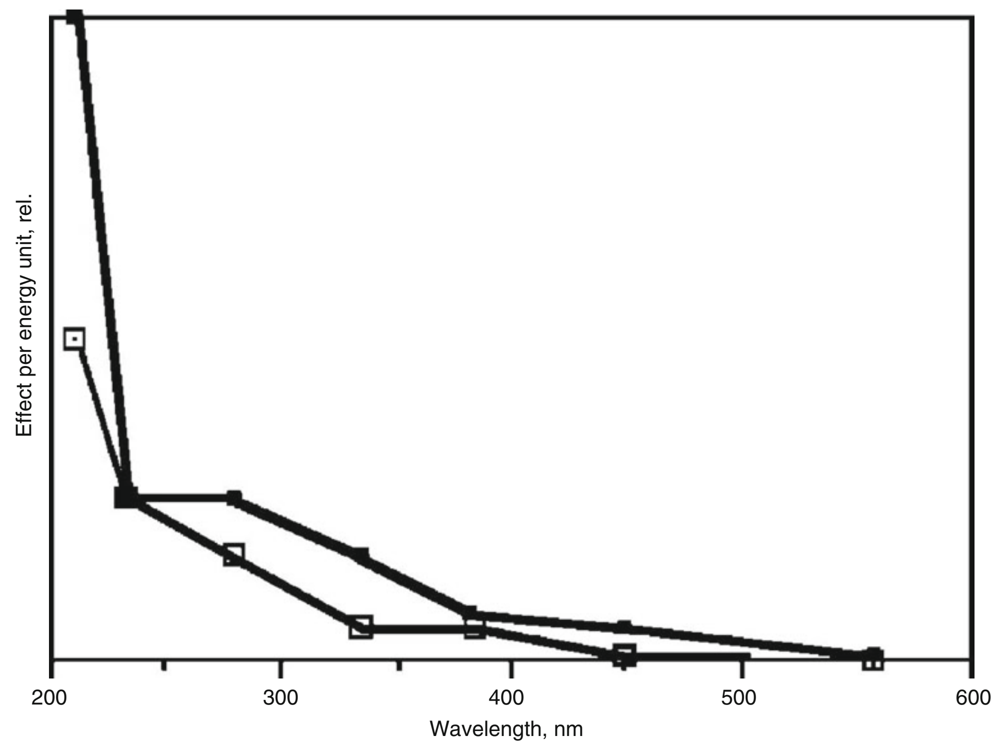

Fig. 8.8 Action spectra for induction of swimming movements in *Paramecium* (*top curve*) and for contraction in *Rotaria* (*curve with squares*) (Redrawn after Hertel (1905))

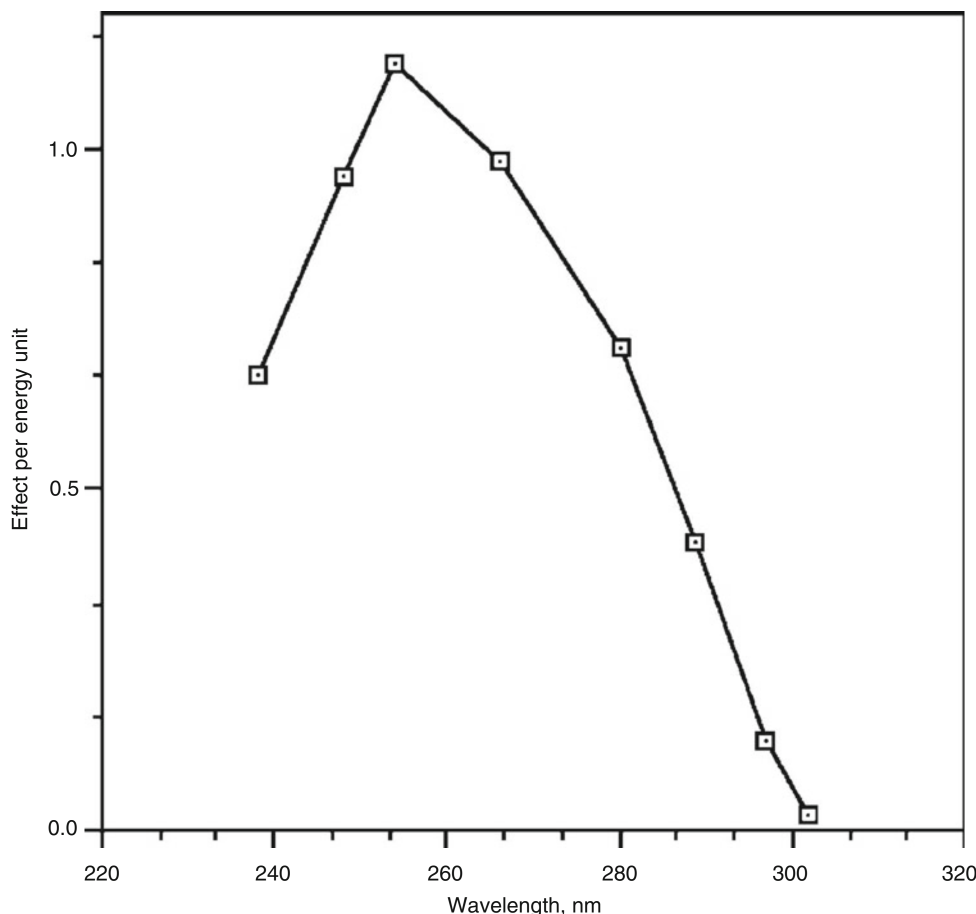

Fig. 8.9 Action spectrum for "killing" (inactivation of cell division) in the bacterium *Staphylococcus aureus*. The graph is similar to the absorption spectrum for DNA (Redrawn after Gates (1930))

substances as essential elements in growth and reproduction." After discussing some experiments done by others, which strengthened his views, he wrote: "Thus, while the relation of thymonucleic acid [i.e., DNA] to cell growth and reproduction remains a matter of conjecture, nevertheless the high concentration in the thymus gland and the coincidence of the evidence from these three independent series of experiments seem worthy of note, without further comment at present."

In the next paper Gates (1929) includes data for a bacterium, but the diagram is not drawn in such a way that it is easy to see the similarity to the DNA absorption spectrum, and DNA is mentioned neither in the discussion nor in the summary. In one more paper from 1930, the deleterious action of ultraviolet radiation is treated from another point of view. In one of his last publications, from 1931, Gates compares the action spectra for two bacterial species to the absorption spectra of the bacteria. About the critical substance, the destruction of which causes the death of the bacterial cells, Gates writes: "An examination of the evidence for its concentration in the cell nucleus, and the further search for evidence of its chemical character are reserved for the final paper of this series." Gates never got the opportunity to publish this final paper. He died on June 17, 1933. Although one paper was published posthumously, his followers obviously did not consider his ideas about DNA important, or even correct.

Contributing reasons to the fact that Gates' ideas never got the attention that their importance deserved were (1) that he was wrongly cited by later scientists (Hollaender and Claus 1936; e.g., "the maximum at 2499 A as reported by Gates"); (2), later scientists like Giese and Leighton (1935) went over to studying phenomena, e.g., swimming movements in *Paramecium*, which were very protein-dependent. Action spectra for such processes have maxima around 280 nm. This diverted the interest from nucleic acids to protein.

8.5 Plant Vision

One of the greatest triumphs of biological action spectroscopy is the discovery of the "vision pigment" of plants—phytochrome. However, at this point of the story, action spectroscopy is getting more complicated.

When the phytochrome saga opened, it was known that some effects of red light on plants could be canceled by exposing the plants to far-red light after the red. For instance,

some lettuce seeds do not germinate unless they are exposed to light after they have been allowed to take up water. Red light was found to be most efficient for this effect. Germination could be prevented by exposing the seeds to far-red light (720–740 nm) after the red.

Another example of red/far-red antagonism was the mode of growth of bean seedlings developing in darkness (Fig. 8.10). The tip of such a seedling is curved to a "plumular hook," but if the seedling receives just a minute of red light, the hook straightens out during subsequent growth (Fig. 8.10). Withrow et al. (1957) tackled the problem of quantifying the straightening effect of different kinds of light. For each of various fluences of light of different wavelengths, they measured by how many degrees the hooks of the bean plants were straightened out (Fig. 8.11).

They also quantified how efficient different kinds of lights were in counteracting the straightening effect of a previously administered saturating fluence of red light (Fig. 8.12). Based on the results, they were able to postulate the existence of a light-sensitive growth regulator, phytochrome. Phytochrome is formed in the plant in an inactive form (called P_r), which is transformable into the active form (P_{fr}) by red light.

The spectral curves that were obtained for the "straightening" and "bending" effects (Fig. 8.13) were postulated to correspond to absorption spectra for P_r and P_{fr}, respectively. It would never have been possible to isolate the phytochrome had not its "spectral signature" been determined beforehand in this way.

In Figs. 8.10 and 8.12, the lines for different wavelengths are not parallel. This means that the shape of the action spectra that are constructed from these lines will depend on the chosen level of action. That the curves are not parallel has to do with the fact that two photochemical reactions are involved to varying extents in all cases, i.e., the transformation of P_r to P_{fr} and the transformation of P_{fr} to P_r. The "most correct" shapes, i.e., those most closely corresponding to the absorption spectra of P_r and P_{fr} (Fig. 8.15), are obtained by investi-

Fig. 8.10 *Left*: Bean plants grown (*1*) in white light, (*2*) in darkness except for a few minutes of red light every day, and (*3*) in darkness except for a few minutes of far-red light every day

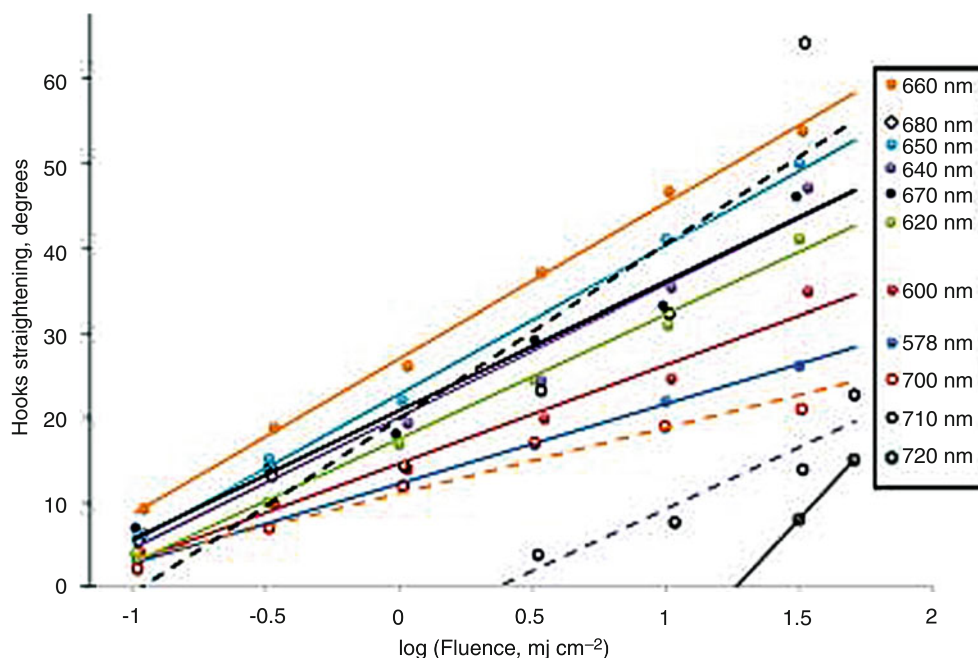

Fig. 8.11 The straightening effect on bean hooks of light of various wavelengths as a function of the energy fluence (Replotted from Withrow et al. (1957))

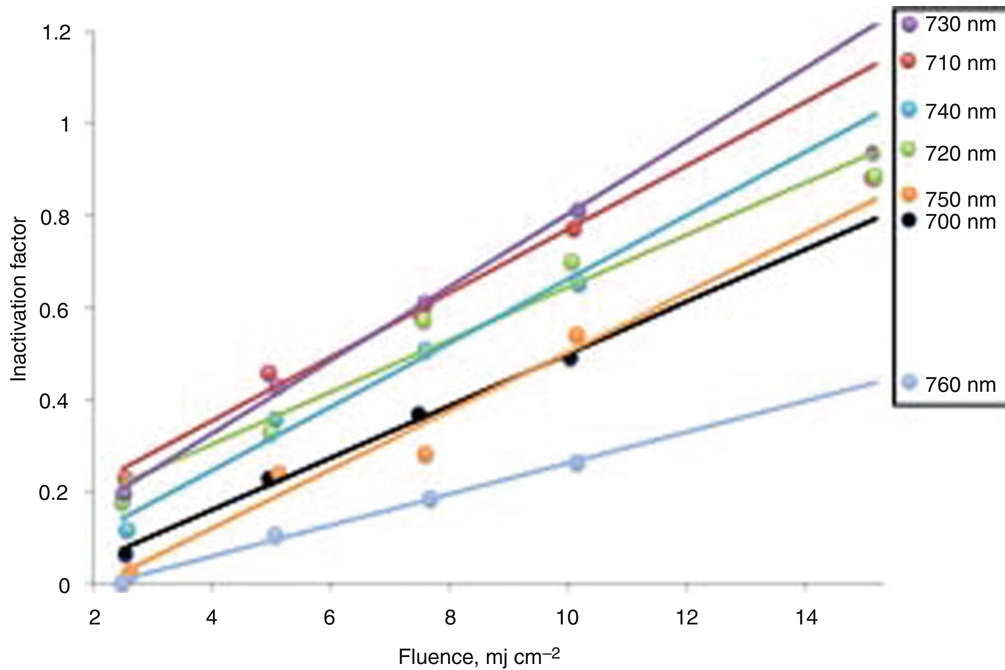

Fig. 8.12 Inactivation of red-induced hook straightening in bean plants by light of different wavelength as a function of fluence. In this case, a linear fluence scale was found to give better linearity of regression than the logarithmic scale in the previous graph (Redrawn from Withrow et al. (1957))

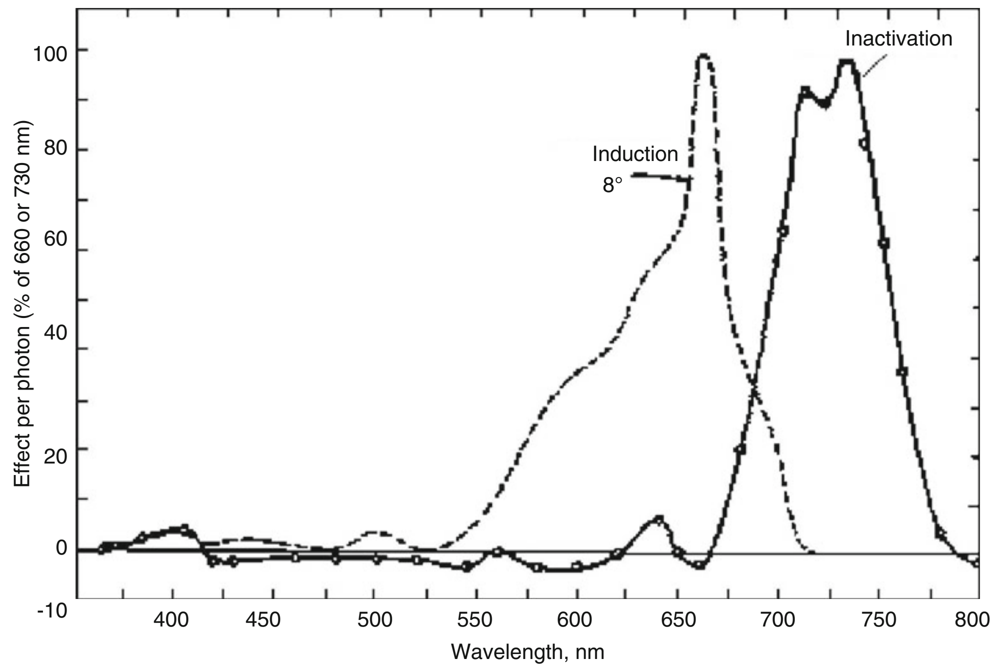

Fig. 8.13 Action spectra for straightening and for the inhibition of red-light-induced straightening of the bean hook, constructed from regression lines of the kind shown in Figs. 8.10 and 8.11 (Redrawn from Withrow et al. (1957))

gating the effects of very small fluences, resulting in small effects. Thus one has to find an appropriate compromise between this and the difficulty in measuring small effects accurately. In Fig. 8.14 a suitable level for recovering the action spectrum for $P_r ==> P_{fr}$ could be $P_{fr}/(P_r + P_{fr}) = 0.1$.

Let us see if we can make sense more in detail of the shapes and slopes of the graphs in Fig. 8.11. Consider the photoreversible system $P_r <==> P_{fr}$. It is now known that P_{fr} is the active form of phytochrome. Let us assume that monitored reaction (the straightening of the hook) is proportional to the amount of P_{fr}. This amount should be the difference between what is formed in the forward reaction $P_r ==> P_{fr}$ and that consumed in the back reaction $P_r <== P_{fr}$. The rate for each reaction is proportional to the product of fluence

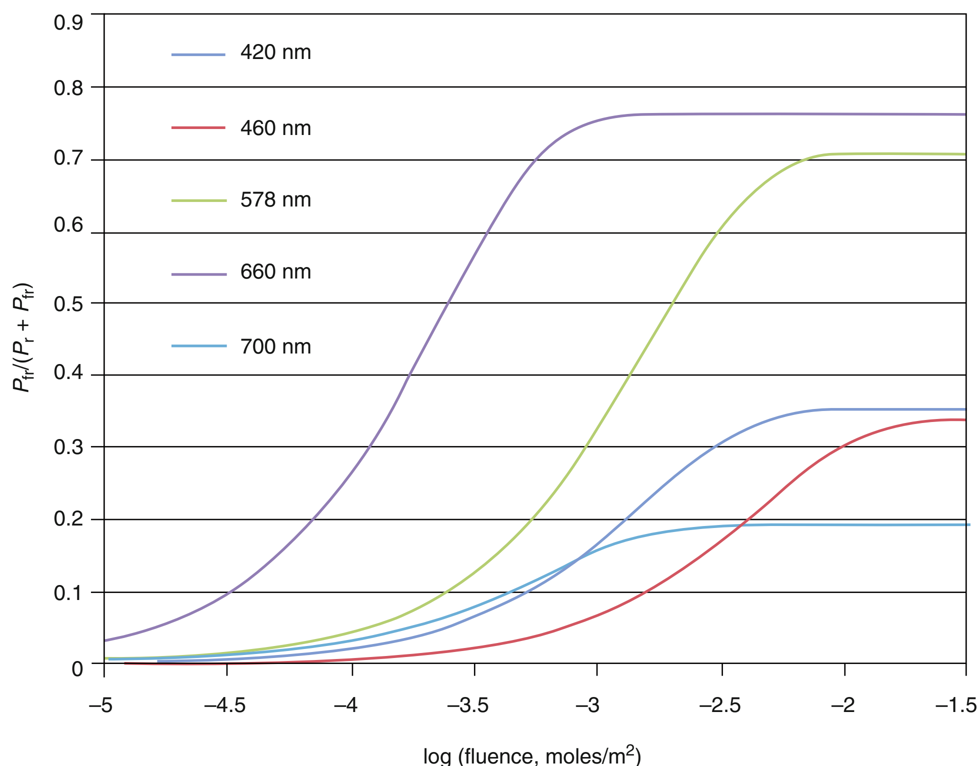

Fig. 8.14 Computed fraction of P_{fr} out of total phytochrome

rate and conversion cross section (the latter being the product of the absorption cross section and the quantum yield). So the net forward rate is $dP_{fr}/dt = I\times(P_r\times\sigma_r - P_{fr}\times\sigma_{fr}) = I\times((P_t - P_{fr})\times\sigma_r - P_{fr}\times\sigma_{fr}) = I\times(P_t\times\sigma_r - P_{fr}\times(\sigma_r+\sigma_{fr}))$. Here the sigmas stand for the conversion cross sections, the P for phytochrome concentration, r and fr for the red- and far-red-absorbing forms, and P_t for total phytochrome. We may for simplicity put P_t = 1 and express the other concentrations as fractions of this, which gives the simple form

$$dP_{fr} / dt = I\times\left(P_t\times\sigma_r - P_{fr}\times\left(\sigma_r+\sigma_{fr}\right)\right).$$

In integrated form this becomes

$$\sigma_r - (\sigma_r+\sigma_{fr})\times P_{fr} = \sigma_r\times\exp\left(-(\sigma_r+\sigma_{fr})\times I\times t\right).$$

or

$$P_{fr} = \left\{1-\exp\left[-(\sigma_r+\sigma_{fr})\times I\times t\right]\right\}/\left(1+\sigma_{fr}/\sigma_r\right).$$

In Fig. 8.14 we have plotted P_{fr} according to this expression as a function of $\log(I\times t)$, i.e., log (fluence).

The complications of this light-sensitive system are well demonstrated by the action spectrum determined by Hartmann (1967) for the inhibiting effect of prolonged and relatively strong illumination on the extension growth of lettuce hypocotyls (Fig. 8.16). At first glance it does not seem to have anything to do with the shapes of the absorption spectra of the two phytochrome forms. But Hartmann showed that this phenomenon could be explained by phytochrome being the mediator of the light action. However, in this case one has to take into account not only the photochemical reactions, but also the fact that the physiologically active form of phytochrome, P_{fr}, is unstable and disappears if there is no P_r present from which P_{fr} can be continually reformed. It is for this reason that light gives the greatest physiological effect, which causes only a small part of the phytochrome to be continuously converted to P_{fr}. Light of longer wavelength has no effect because too little P_{fr} is formed. Light of too short a wavelength has no effect because P_{fr} is formed too quickly, and all phytochrome disappears before it can act for a sufficient amount of time. The inhibition of the growth of the lettuce hypocotyls is an example of a so-called high-intensity reaction (HIR).

8.6 Protochlorophyllide Photoreduction to Chlorophyllide *a*

The present author and many other researchers have studied the action spectra for synthesis of chlorophyll and formation of chlorophyll. Important early contributions were made by J. H. C. Smith at the Carnegie Institution, T. N. Godnev and A. A. Shlyk in Belorussia, and H. Virgin

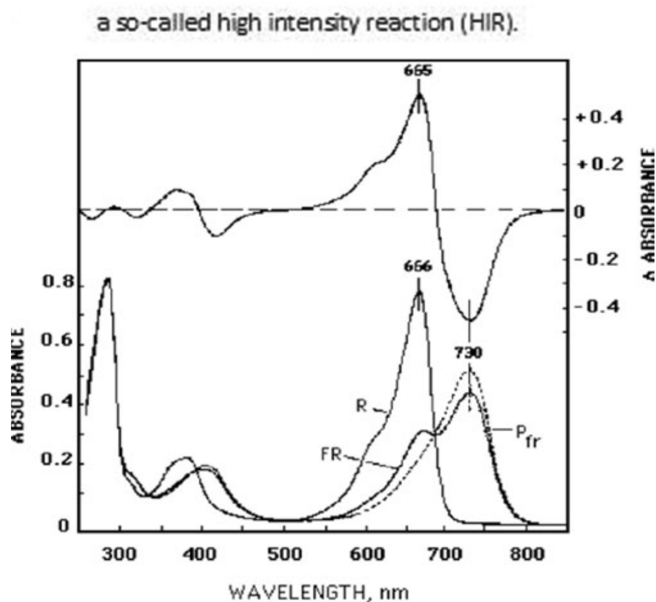

Fig. 8.15 *Top*: Absorption spectra of purified phytochrome from oats. The *curve marked R* is the measured absorption of far-red irradiated solution, containing almost only P_r. The *curve marked FR* is the measured absorption of red irradiated solution, containing mostly P_{fr} and some P_r. The *dotted curve marked* P_{fr} is the estimated spectrum of pure P_{fr}, which cannot be measured directly, since red light only partially converts P_r to P_{fr}. The *top curve* is a so-called difference spectrum showing the difference in absorption between far-red irradiated and red irradiated solutions. Since only the change due to irradiation shows up in this, almost the same difference spectrum can be obtained from plant tissue (After Vierstra and Quail (1983a, b)). *Bottom*: P_{fr} (*left*) and P_r (*right*) prepared from oat coleoptiles (Courtesy Gunvor Björn)

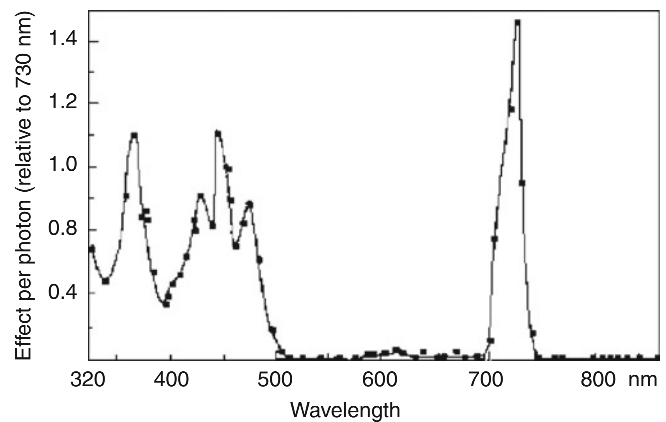

Fig. 8.16 Example of action spectrum for a high-intensity phytochrome reaction (HIR), the inhibition of lettuce hypocotyl longitudinal growth (Hartmann 1967). The sharp long-wavelength band is due to phytochrome, while a flavin-containing pigment is involved in the short-wavelength part

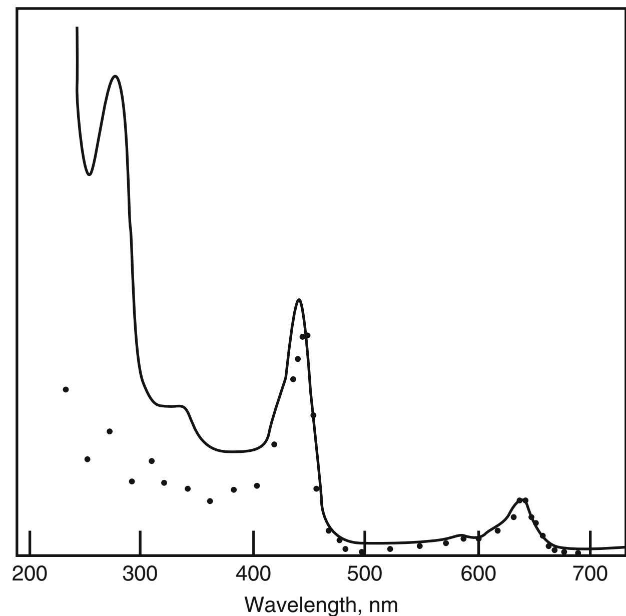

Fig. 8.17 The absorption spectrum of purified "protochlorophyll holochrome" (complex between protochlorophyllide, NADPH, and NADPH–protochlorophyllide photooxidoreductase) extracted from etiolated bean plants (Schopfer and Siegelman 1969) and the action spectrum for protochlorophyllide photoreduction to chlorophyllide *a* (Björn 1969a) (Redrawn from Björn (1969b))

in Sweden. Virgin's former students have continued this line of research in Göteborg (see Sundqvist and Björn 2007).

Chlorophyll formation is governed by several light-sensitive processes: conversion of protochlorophyllide to chlorophyllide with enzyme-bound protochlorophyllide as the light absorber, conversion of phytochrome, and action on the so-called blue light receptor.

One specific question in this context was whether radiation absorbed in the aromatic amino acids of the enzyme NADPH–protochlorophyllide photooxidoreductase would be able to cause the conversion of protochlorophyllide to chlorophyllide in the same way as does radiation absorbed in the protochlorophyllide itself. In Fig. 8.17 the absorption of the NADPH–protochlorophyllide photooxidoreductase complex with its substrates NADPH and protochlorophyllide is shown by the solid curve. In the visible region, the absorption is due to the protochlorophyllide. The high peak at 280 nm, on the other hand, is due to aromatic amino acids

in the protein. The action spectrum for protochlorophyllide photoreduction, shown by dots, has essentially the same features in the visible region but lacks the high peak at 280 nm. The conclusion is that energy absorbed in the aromatic amino acids cannot be used for photoreduction. There is also a small difference between absorption and action spectrum in the blue region (the so-called Soret peak of the spectra). This is probably because there are two fractions of protochlorophyll with slightly different spectra, of which only one can be converted by light (there may be no reductant, NADPH bound to the "inactive" complexes). There is also some blue-absorbing carotenoid contributing to the absorption spectrum.

8.7 Limitations of Action Spectroscopy: The Elusive Blue Light Receptor

Numerous "blue light phenomena" have been studied in plants and fungi: phototropism, chloroplast rearrangements, plastid differentiation, and nastic movements, to mention a few. The discussion about the possible nature of the molecule absorbing the active light in these processes has centered mainly on carotenoids and flavoproteins, because the action spectra are similar to absorption spectra of these compounds (which are so similar to each other and so variable with conditions and molecular details that a conclusion on action spectra alone seems impossible). As described in Chap. 11, it has been found that most blue light phenomena are mediated by flavoproteins but that some, e.g., stomatal movements, may be carotenoid-mediated. Because of the similarity of the absorption spectra for these two groups of compounds, action spectroscopy has not been able to distinguish between them. The questions have now mostly been solved, mainly by methods of molecular biology. However, action spectroscopy, after more than a hundred years, is a method still in use (e.g., Ziv et al 2007).

8.8 Another Use for Action Spectra

So far we have only discussed the use of action spectroscopy for identifying the molecules absorbing the light driving the various photoprocesses. I would like to point out one more important reason for determining action spectra. For this we shall go back to near the beginning of this chapter, to the damaging effect of ultraviolet radiation but look at it from another angle.

Over the past 35 years, there has been concern about the depletion of the stratospheric ozone layer (see Chap. 22). Such depletion results in increased levels of ultraviolet radiation at the surface of the Earth (unless other changes in the atmosphere were to compensate for the depletion in stratospheric ozone). Experiments have been carried out to forecast the biological effects of such changes in radiation. Ozone depletion has been simulated by exposing the organisms to be studied to artificial ultraviolet radiation. One problem has been that the artificial radiation cannot be given the same spectral composition as the additional solar radiation that would leak through a depleted ozone layer. Therefore, weighting functions have been needed to calculate how much artificial ultraviolet radiation that has to be administered to simulate certain ozone depletion, and for this, one has to determine action spectra for different ultraviolet effects. Initially, one relied on ordinary, "monochromatic" action spectra, determined as the other spectra in this chapter. However, since so many different ultraviolet effects with different action spectra are involved, it has turned out to be more realistic to determine "polychromatic action spectra." For this, one starts with a full spectrum and, for different samples, cuts away more and more of the short-wavelength part.

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