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Denaturation of Phytochrome

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The absorption spectra of phytochrome were found to be sensitive to the association between the chromophoric group and the protein moiety. The effects of protein denaturation were studied by measuring the absorption spectra of both forms of the photochromic pigment system. One form of phytochrome, P_{FR}, was much more susceptible to denaturation by urea, to attack by pronase and trypsin, and to sulfhydryl-reacting reagents than the other form, P_R. The results suggest that light-induced interconversions between P_R and P_{FR} involve changes of protein conformation as well as changes of the chromophoric group.

Action spectra of the effects of light on various aspects of plant growth and development (Borthwick and Hendricks, 1960) revealed the presence and action of a red-far-red photochromic pigment. The pigment, called phytochrome, was detected spectrophotometrically in intact plant tissue and was extracted as a soluble protein (Butler *et al.*, 1959). Procedures for purifying the chromoprotein have been described (Siegelman and Firer, 1964). The reversible photoconversion between the two forms of phytochrome[1] is retained in the purified solutions.

The best source of phytochrome has been dark-grown seedling tissue. The phytochrome extracted from different species of seedlings, however, showed differences in ease of purification and in resistance to denaturation. In particular, the phytochrome extracted from barley seedlings was less stable than that from maize or oats, and differences in the absorption spectra of barley phytochrome suggested that the chromoprotein might be altered by the purification procedures. These observations led to the studies of denaturation reported below. The term "denaturation" is used in a very general sense to indicate an unknown alteration of the protein.

The absorption spectra of both forms of phytochrome were found to depend upon the state of the protein. The absorption bands in the long-wavelength region of the spectrum, where other proteins do not absorb, provide a specific tag for phytochrome molecules and permit the phytochrome protein to be studied in the presence of large amounts of other proteins. In the present paper the absorption spectra have been used to indicate the progress and degree of denaturation. Complete denaturation was assumed when the red and far-red absorption bands of the chromophores disappeared. This operational assay for denaturation is thus limited to those parts of the protein which influence the chromophoric group.

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[1] Abbreviations used in this work: P_R, red-absorbing form of phytochrome; P_{FR}, far-red-absorbing form of phytochrome; EDTA, ethylenediaminetetraacetic acid.

$$P_R \underset{\text{far-red light}}{\overset{\text{red light}}{\rightleftharpoons}} P_{FR}$$

Materials and Methods

Phytochrome was purified through the Sephadex G-200 step according to the procedures described (Siegelman and Firer, 1964). Unless otherwise noted, the phytochrome was purified from dark-grown oat

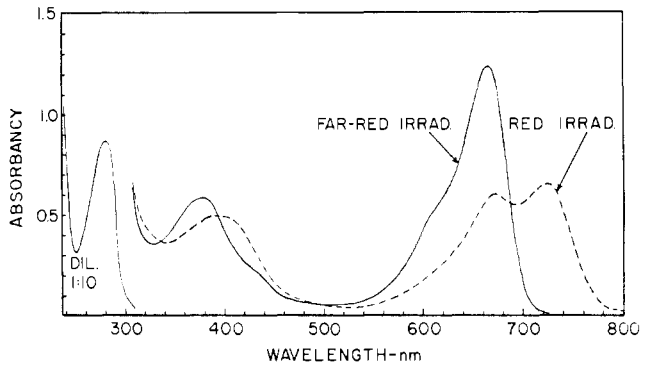

FIG. 1.—Absorption spectra of a solution of oat phytochrome, following irradiation with red and far-red light. The spectrum at wavelengths shorter than 300 mμ (nm) was measured on a solution diluted 10-fold.

seedlings and was dissolved in 0.1 M potassium phosphate buffer, *p*H 7.8, which contained 0.05 M 2-mercaptoethanol and 0.001 M EDTA. Most of the solutions used in these studies had a protein content of about 20 mg/ml of which 10% or less was phytochrome protein. The phytochrome solution used for the spectra in Figure 1 was taken through an additional stage of purification which consisted of absorption onto and gradient elution from a brushite column.

Effects of urea were determined by adding a 10 M solution, adjusted to *p*H 7.8 with potassium phosphate buffer, to an equal volume of the phytochrome solution. *p*-Mercuribenzoate and *N*-ethyl maleimide were generally made up to 5×10^{-2} M and were diluted 10-fold by addition to the phytochrome solution. Pronase (California Corp. for Biochemical Research) and trypsin (Worthington Biochemical Corp.) were made up to 1.0 mg/ml and were diluted 10-fold in the phytochrome sample. The experiments with pronase and trypsin and the experiment on dark conversion were carried out at room temperature. In all other experiments the phytochrome solution was kept near 0°.

Allophycocyanin and phycocyanin were purified from *Smithora naidum* according to the method of Haxo *et al.* (1955).

With the exception of Figures 1 and 4, spectrophotometric measurements were made with a single-beam recording spectrophotometer similar to one previously described (Norris and Butler, 1961). (The spectra in Figs. 1 and 4 were measured with a Cary recording spectrophotometer.) The sample was contained in a vertical cylindrical cell which could be surrounded by ice. The vertical optical path permitted the addition of reagents and dilution of the sample without changing the number of pigment molecules in the measuring beam.

Actinic sources of red and far-red light were available for irradiating the sample in the spectrophotometer. These sources consisted of a tungsten lamp with either a 650-mμ interference filter or a far-red cut-on filter which transmitted wavelengths longer than 730 mμ. The sample was irradiated with these sources for about 1 minute, which was more than sufficient to drive the $P_R \rightleftarrows P_{FR}$ photoconversion to completion in either direction. The sequence of the spectral measurements is indicated in the figures if it is pertinent to the design of the experiment.

RESULTS

Figure 1 shows the absorption spectra of a solution of phytochrome following irradiation with the red and far-red actinic sources. The absolute absorption

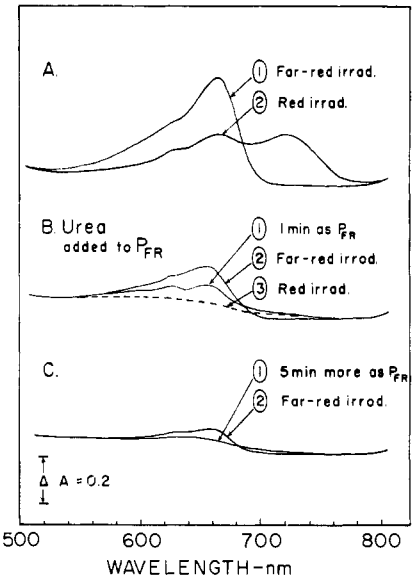

FIG. 2.—Effect of 5 M urea. (A) Absorption spectra of original solution; (B) 1 minute after adding urea to P_{FR}; (C) phytochrome kept in urea 5 minutes more as P_{FR}.

spectra indicate that the absorption maxima of the two forms are at 665 and 725 mμ (nm) rather than at the previously reported values of 660 and 730 mμ (Hendricks *et al.*, 1962) which were obtained from difference spectra. The far-red actinic light converts practically all of the phytochrome to P_R, but the red actinic light does not appear to convert all of the P_R to P_{FR}. It will be shown that a certain amount of P_R remains at the photostationary state in red light due to the absorption of P_{FR} in the red region. Many of the absorption spectra (*vide infra*) also have shoulders at about 630 mμ which vary in magnitude among different preparations. These absorption bands probably are due to inactive protochlorophyll which is present in etiolated tissue.

*P and P**.—The ratio of the maximal reversible absorbancy change in the far-red region to that in the red region, $\Delta A_{FR}/\Delta A_R$, in the phytochrome solution purified from oats (Fig. 1) is 1.0. In earlier work, with phytochrome purified from dark-grown barley seedlings, we noted that this ratio was 1.0 *in vivo* and at early stages of purification but tended to decrease at higher stages of purification to about 0.7–0.5. In some cases in which the phytochrome appeared to be mildly denatured, as indicated by a decrease of solubility, the ratio for both the soluble and insoluble phytochrome was less than 0.2 (Hendricks *et al.*, 1962). Difference spectra of the preparations with a low ratio showed that the difference band in the far-red region shifted to shorter wavelength as the ratio decreased. The variation of the $\Delta A_{FR}/\Delta A_R$ ratio at various stages of purification of barley phytochrome suggested that an alteration of the phytochrome protein resulted in an altered phytochrome, P*, which was still photoreversible. The absorption spectrum of P_R* was similar to P_R but P_{FR}* had a shorter wavelength-absorption maximum and a lower extinction coefficient than P_{FR}. Phytochrome may be denatured to various degrees. P* is not meant as definite molecular species with a fixed $\Delta A_{FR}/\Delta A_R$ ratio, but rather the sum of molecules in various degrees of denaturation. As denaturation proceeds, the absorbancy of the far-red absorption band decreases, finally going to zero, in which case the red-absorbing form cannot be regenerated by far-red light.

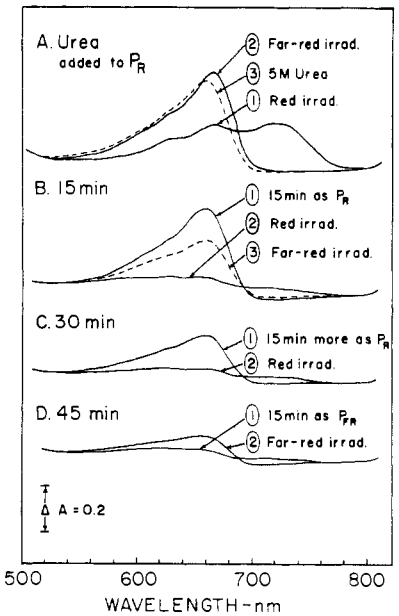

FIG. 3.—Effect of 5 M urea. (A) Curves 1 and 2, original solution; curve 3, 1 minute after adding urea to P_R; (B) phytochrome kept in dark 15 minutes more as P_R; (C) phytochrome of (3-B) kept in dark 15 minutes more as P_R; (D) phytochrome kept in dark an additional 15 minutes as P_{FR}.

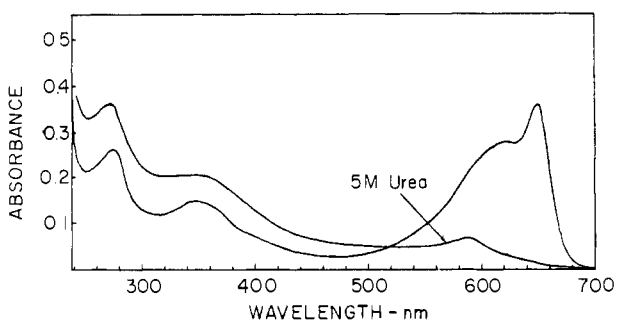

FIG. 4.—Absorption spectra of allophycocyanin before and after addition of urea.

Transformation of P to P by Urea.*—The native phytochrome can be converted to the P* system by denaturing the protein with urea. Figure 2 shows the effect of 5 M urea on a phytochrome solution that had a $\Delta A_{FR}/\Delta A_R$ ratio between 0.9 and 1.0. The addition of urea to P_{FR} caused an immediate (within 1 minute) decrease in the P_{FR}-absorption band (Fig. 2B, curve 1). The P_R, which was present at the photostationary state in red light before the addition of urea, remained. The phytochrome was still photoreversible. Irradiating the sample with far-red and red light produced absorbancy changes with a $\Delta A_{FR}/\Delta A_R$ ratio between 0.1 and 0.2, which is typical of conversions between P_R* and P_{FR}*. If the sample remained as P_{FR}* in the presence of urea for 5 minutes (Fig. 2,C), the absorption bands and the reversible absorbancy changes decreased further.

Addition of urea to P_R does not cause the rapid deterioration of the absorption bands that occurred in Figure 2 where the urea was added to P_{FR}. The addition of urea to P_R (Fig. 3) caused an immediate shift of the absorption maximum from 665 mμ to 660 mμ with only a slight loss of absorption. The absorption spectrum did not undergo further change during the next 15 minutes (Fig. 3 B, curve 1). In other experiments the 660-mμ band was stable in urea for over an hour.

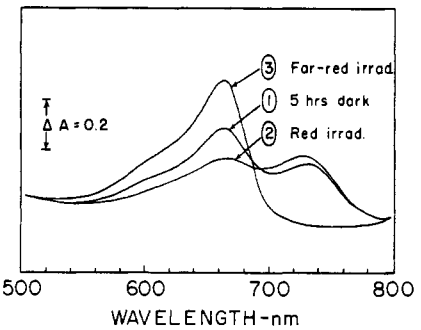

FIG. 5.—Absorption spectra of barley phytochrome. Curve 1, absorption spectrum after standing at room temperature in the dark for 5 hours after an irradiation with red light. Curve 2, absorption spectrum after subsequent irradiation with red light. Curve 3, absorption spectrum after irradiation with far-red light.

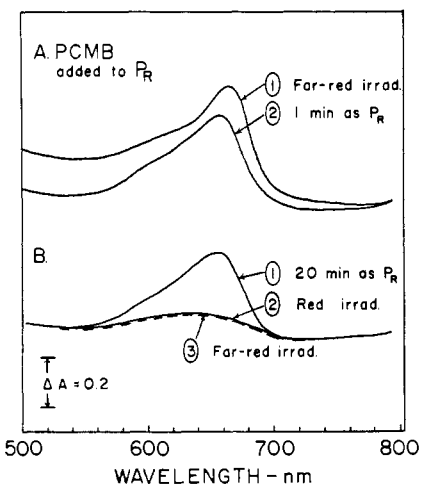

FIG. 6.—Effect of 5×10^{-3} M *p*-mercuribenzoate (PCMB). (A) *p*-Mercuribenzoate added to P_R; (B) phytochrome kept in dark for 20 minutes as P_R. The phytochrome solution did not contain mercaptoethanol.

Irradiating the sample with red light, however, gave a typical P_R*-to-P_{FR}* conversion. The urea denatured P_R to P_R*, as indicated by the 665- to 660-mμ shift of the absorption maximum and the transformation to the low-extinction P_{FR}* on irradiation with red light, but P_R* was stable in urea. Irradiation of P_{FR}* with far-red light did not produce as much P_R* as was originally present, but the P_R* that was formed was still stable in 5 M urea. (Compare curve 3, part B, with curve 1, part C, of Fig. 3, measured 15 minutes later.) The absorption bands were less, however, (Fig. 3D) after the solution stood for 15 minutes as P_{FR}*.

The action of 5 M urea on P_R was reversed by removing the urea. Urea was added to a solution of phytochrome ($\Delta A_{FR}/\Delta A_R$ = 0.9) in the P_R form. The shift of the absorption maximum from 665 to 660 mμ indicated that P_R was denatured to P_R.* After 10 minutes in the P_R* form the urea was rapidly removed by passing the solution through Sephadex G-25, all operations being carried out in darkness or with a dim green safe-light. The absorption spectra of the collected fractions showed that P_R was quantitatively regained from the column. The phytochrome was in the 665-mμ-absorbing P_R form which, on irradiation with red light, converted to P_{FR} with a $\Delta A_{FR}/\Delta A_R$ ratio of 0.9. The results from a similar experiment in which urea was added to and removed from P_{FR} showed that approximately 30% of the phytochrome,

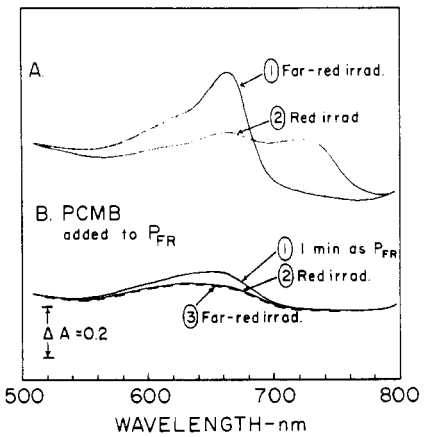

FIG. 7.—Effect of 5×10^{-3} M *p*-mercuribenzoate (PCMB). (A) Original solution; (B) *p*-Mercuribenzoate added to P_{FR}. The phytochrome solution did not contain mercaptoethanol.

with a $\Delta A_{FR}/\Delta A_R$ ratio close to unity, was regained from the column.

Denaturation of Allophycocyanin by Urea.—The action of urea on the absorption spectrum of allophycocyanin was examined because of the similarity between the absorption spectra of allophycocyanin and P_R. Ó hEocha (1963) and Jones and Fujimori (1961) showed that the absorption bands of C-phycocyanin, allophycocyanin, and phycoerythrin were decreased by denaturation. Our results with allophycocyanin, which are similar to those reported by Ó hEocha, are shown in Figure 4. Addition of urea to a concentration of 5 M completely abolished the main 650-mμ absorption band within a few minutes. C-Phycocyanin behaved in a similar manner. We were not able to restore the chromophore absorption by rapidly diluting the urea-treated sample 10-fold or by removing the urea by gel filtration. It is somewhat surprising that the absorption spectra of these chromophores, which are noted for their stability (the chromophoric groups are split from the protein by hydrolysis in 12 N HCl), are readily destroyed by urea, presumably through denaturation of the protein.

Dark Transformation of P_{FR} to P_R*.*—Phytochrome solutions were examined for dark conversions. Bonner (1962), working with solutions of phytochrome purified from dark-grown pea seedlings, reported that the far-red-absorbing form reverted to the red-absorbing form in the dark. A similar transformation was shown with barley preparations which appeared to be partially denatured (Hendricks *et al.*, 1962). The highly purified solutions of phytochrome from oats, such as that of Figure 1, exhibited no transformation in the dark when freshly prepared. However, after aging or after mild denaturation, the oat phytochrome often showed some degree of dark conversion. The ratio of the reversible absorbancy changes, $\Delta A_{FR}/\Delta A_R$, for the phytochrome which transforms in the dark indicated that the transformation was from P_{FR}* to P_R*.

The dark conversion *in vitro*, obtained at room temperature with a phytochrome sample purified from maize, is shown in Figure 5. Curve 1 is the absorption spectrum of the solution 5 hours after an irradiation with red light. The dark period was extended to 5 hours to ensure that the dark reaction had gone to completion. Most of the transformation occurred in the first hour. Irradiating the sample with red light after the dark period (curve 2, Fig. 5) restored the absorption spectrum to that of the initial red-irradiated sample and showed that the phytochrome which trans-

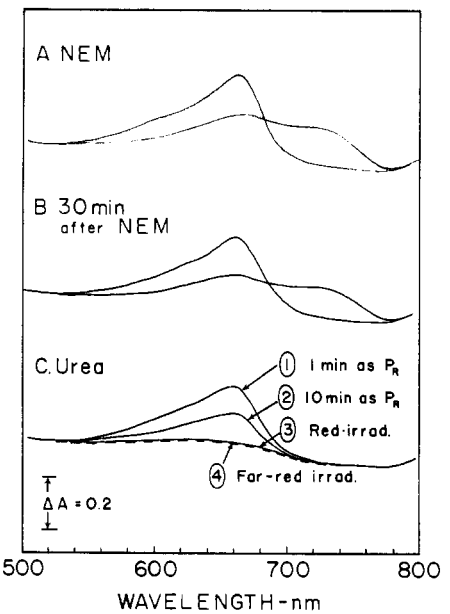

FIG. 8.—Effect of 5×10^{-3} M *N*-ethylmaleimide (NEM). (A) Original solution; (B) 30 minutes after addition of *N*-ethylmaleimide; phytochrome kept 20 minutes as P_R and 10 minutes as P_{FR}; (C) curve 1, urea added to P_R (5 M); curve 2, kept 10 minutes in dark as P_R*; curve 3, irradiated with red light; and curve 4, irradiated with far-red light. The phytochrome solution did not contain mercaptoethanol.

formed in the dark had a $\Delta A_{FR}/\Delta A_R$ ratio of about 0.2. Apparently the solution, which had a $\Delta A_{FR}/\Delta A_R$ ratio of 0.8–0.9 for the photoreversible absorbancy changes, was a mixture of P and P*. There could also have been some denaturation of P to P* during the prolonged dark period at room temperature. P_{FR}* reverted to P_R* during the dark period, but P_{FR} remained.

Effect of Sulfhydryl-reacting Reagents.—The influence of phytochrome SH groups on the absorption bands was examined by adding *p*-mercuribenzoate, a reagent which reacts with SH groups, to phytochrome solutions which were free of mercaptoethanol. The addition of *p*-mercuribenzoate to a concentration of 5×10^{-3} M (Fig. 6) had little effect on the red-absorption band other than to shift the absorption maximum to a slightly shorter wavelength. This change is similar to the addition of urea to P_R and may represent a denaturation of P_R to P_R*. The nonspecific change in the baseline slope of the spectra in Figure 6A is probably caused by a change in the scattering characteristics of the particular protein solution. A similar change of slope is also often observed on the addition of urea to P_R (though not in Fig. 3). After the initial spectral shift, the red-absorption band was not altered by standing for 20 minutes in 5×10^{-3} M *p*-mercuribenzoate (curve 1, Fig. 6 B). On irradiation with red light, however, the red-absorption band was lost without the concomitant formation of any absorption band in the far-red. An immediate irradiation with far-red light did not re-form any of the red-absorption band. The *p*-mercuribenzoate attacked the P_{FR} chromophore as soon as it was formed. Thus the integrity of a specific SH group (or groups) is required for the characteristic absorption band of the P_{FR} chromophore. In higher concentrations *p*-mercuribenzoate can attack the chromophore of P_R or P_R*, although relatively slowly. In one experiment in which *p*-mercuribenzoate was added to P_R to a concentration of 10^{-2} M, the red-absorption band gradually decreased to zero over a period of 30 minutes.

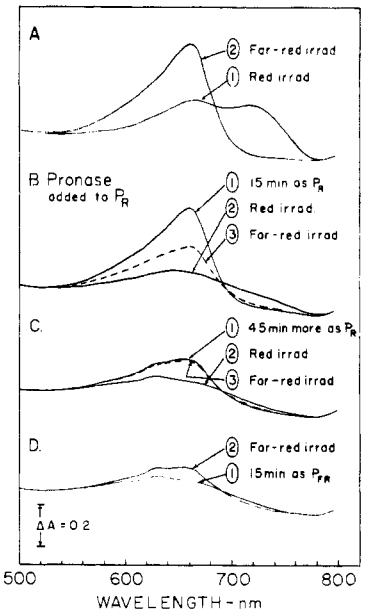

FIG. 9.—Effect of 0.1 mg/ml pronase at room temperature. (A) Original solution; (B) 15 minutes after adding pronase to P_R; (C) phytochrome of (3-B) kept 45 minutes as P_R*; (D) phytochrome kept additional 15 minutes as P_{FR}.

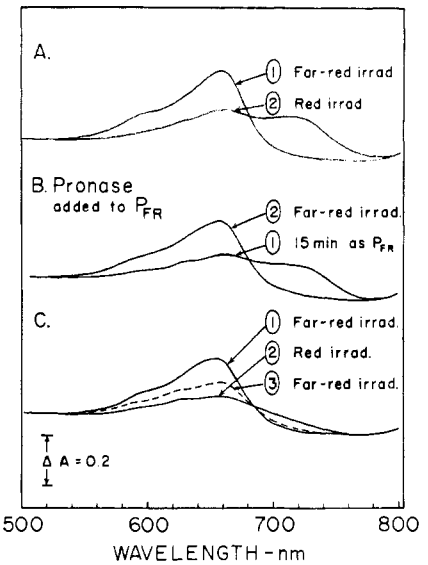

FIG. 10.—Effect of 0.1 mg/ml pronase at room temperature. (A) Original solution; (B) 15 minutes after pronase was added to P_{FR}; (C) immediately following (B).

When *p*-mercuribenzoate was added to solution which had been irradiated with red light (Fig. 7), the absorption band of P_{FR} was immediately lost. However, the P_R which was present at the photostationary state in curve 2 of Figure 7A remained. Irradiating the sample with red light (Fig. 7B) converted the remaining P_R* and completed the destruction of the photoreversible pigment. The spectra indicate that 10–20% of the phytochrome at the photostationary state in red light is P_R.

The amount of *p*-mercuribenzoate required to effect these changes varied to some extent with different phytochrome solutions, in part because of varying amounts of nonphytochrome protein which also binds the *p*-mercuribenzoate. In most of the solutions used in these studies, the phytochrome protein was in the order of 10% of the total protein. The susceptibility of the phytochrome protein to the denaturation of P to P* also appears to play a role. We have had a phytochrome solution in which 10^{-2} M *p*-mercuribenzoate had no effect on the absorption bands of either P_R or P_{FR}. With this solution the *p*-mercuribenzoate was not able to denature the phytochrome to the P* system.

Another reagent which reacts with SH groups, *N*-ethyl maleimide, had little effect on phytochrome when present at a concentration of 5×10^{-3} M. The photoreversible absorbancy changes decreased only about 25% after standing in 5×10^{-3} M *N*-ethyl maleimide for 30 minutes (Fig. 8B), during which time the solution was irradiated with both red and far-red light. After addition of urea, however, the *N*-ethyl maleimide acted like *p*-mercuribenzoate. In Figure 8C the absorption band of P_R*, in the presence of 5×10^{-3} M *N*-ethyl maleimide and 5 M urea, decreased about 50% in 10 minutes, which was similar to the action of 10^{-2} M *p*-mercuribenzoate on P_R. On irradiating the solution with red light, all absorption was lost immediately and no red-absorption band could be re-formed by a subsequent irradiation with far-red light. In another experiment, addition of *N*-ethyl maleimide (5×10^{-3} M) to P_{FR}*, after the addition of urea (5 M), resulted in the immediate loss of the P_{FR}* absorption. Thus, the SH group or groups required for the absorption bands of phytochrome are susceptible to attack when phytochrome is in the partially denatured P_{FR}* form.

Urea attacks the chromophore of P_{FR}* more rapidly in the absence of mercaptoethanol, although not so rapidly as *N*-ethyl maleimide plus urea. We noted previously with partially denatured phytochrome solutions from barley that P_{FR}* was much more stable than P_R* and that mercaptoethanol exerted a protective effect on P_{FR}*. Solutions of native phytochrome are stable in the absence of mercaptoethanol. These observations also suggest that specific SH groups are much more susceptible in the P_{FR}* form than in the P_R*, P_R, or P_{FR} forms.

Effects of Proteolytic Enzymes.—The effects of pronase and trypsin on phytochrome were studied at room temperature at a concentration of 0.1 mg/ml. This concentration gave a measurable effect in a reasonably short period of time. Fifteen minutes after adding pronase to P_R, the red-absorption band showed little change except for a slight shift to shorter wavelength. Irradiation with red light, however, produced the low-extinction P_{FR}* (curve 2, Fig. 9 B). Thus, as in the case of urea, pronase altered the protein of P_R to give P_R*, which immediately transformed to P_{FR}* on irradiation. Also, the chromophore was relatively stable in the P_R* form but deteriorated with time in the P_{FR}* form.

When pronase was added to P_{FR} the expected conversion to P_{FR}* was not observed. Figure 10, part B, curve 1, shows that the absorption spectrum of the red-irradiated solution changed very little in 15 minutes after the addition of pronase to P_{FR}. Irradiation of the solution with far-red light after 15 minutes produced the typical P_{FR}-to-P_R conversion. An immediate reirradiation with red light (Fig. 10C), however, produced the low-extinction P_{FR}*. Thus, the formation of P_{FR}* required not only the proteolytic enzyme, but also the photoconversion of P_R or P_R*.

The action of trypsin was essentially the same as that of pronase. The similarity between the effects of the proteolytic enzymes and of urea on the chromophore will be examined in the discussion.

Denaturation by Acid and Base.—The chromophores of P_R and P_{FR} are stable in the *p*H range between 5 and 9. The protein in the solution generally precipitates

below *p*H 6, but the phytochrome is fully photoreversible. At *p*H values below 5 and above 9 both forms begin to lose absorbancy so that the $\Delta A_{FR}/\Delta A_R$ ratio remains about the same. In one experiment 80% of the photoreversible absorbancy changes were lost at *p*H 4.4 but, on bringing the *p*H back to 7.1, the reversible absorbancy changes were restored to 85% of their original value. If the acid treatment is more drastic, the loss of absorbancy is irreversible. In another experiment the photoreversible absorbancy changes were irreversibly lost at *p*H 10.8.

Precipitation of phytochrome by 5% trichloroacetic acid gives a slightly blue precipitate which has a broad flat absorption band through the 580- to 680-mμ region similar to that shown by the denatured solutions. The absorption spectrum of this precipitate is not affected by light.

Discussion

The purpose of the denaturation studies was to examine some molecular properties of phytochrome. The results with urea show that the chromophores of P_R and P_{FR} have different sensitivities to denaturation. P_R is denatured to P_R*, but no further loss of chromophore occurs so long as the phytochrome remains as P_R*. If P_R* is converted to P_{FR}* by light, or if urea is added to P_{FR} to form P_{FR}*, the chromophore deteriorates with time. The deterioration can be prevented by returning P_{FR}* to P_R*. The different susceptibilities of P_R and P_{FR} to denaturation suggest differences of protein conformation.

The effect of proteolytic enzymes on the chromophore of P_R is similar to the effect of urea on P_R. The absorption maximum shifts to a slightly lower wavelength, indicating a change of P_R to P_R*, but no further change of the chromophore is observed as long as it remains P_R*. On irradiation of the chromophore with red light, P_{FR}* is formed. P_{FR}* deteriorates with time unless it is returned to the stable P_R*. The greater resistance of P_R* to the effects of the proteolytic enzymes also suggests a difference of protein conformation. The proteolytic enzymes and urea appear to have similar effects on the absorption spectra of chromophores. The chromophore change probably reflects a general loosening of the protein structure.

The effect of the proteolytic enzymes on P_{FR}, however, is somewhat different from that of urea. The addition of urea to P_{FR} rapidly transformed it to P_{FR}*, whereas the action of pronase had very little effect on the chromophore of P_{FR}. Pronase had acted on the protein of P_{FR}, however, because the red-far-red irradiation sequence immediately produced the low-extinction P_{FR}*. Apparently the peptide bonds broken by pronase had no direct influence on the chromophore of P_{FR}, but the protein conformational changes involved in the photoconversion were sufficient to loosen the protein structure in the region of the chromophore after the bonds had been broken.

Other cases are known where the state of a prosthetic group affects the conformation of the attached protein. Fridovich (1962) presented evidence that the protein conformation of ferricytochrome c was different from that of ferrocytochrome c. Nozaki *et al.* (1958) have also reported that ferrocytochrome c was more resistant to proteolytic digestion than was ferricytochrome c.

The effect of *p*-mercuribenzoate indicates that a specific SH group (or groups) is intimately involved with the chromophore in the P_{FR} form. The observation that urea attacks the chromophore of P_{FR}* more rapidly in the absence of mercaptoethanol also suggests the involvement of SH groups. Urea probably causes an unfolding of the protein. The experiments with *N*-ethyl maleimide showed that this SH reagent could not readily attack the specific SH groups in the native P_{FR} but could if P_{FR} were partially denatured to P_{FR}* by urea, presumably because denaturation opened the protein. The opening of the protein by urea is accompanied by a decrease in the far-red absorption band. As denaturation proceeds the absorption band of P_{FR}* progressively decreases, presumably as the protein progressively opens or unfolds. When the absorbancy of P_{FR}* goes to zero the ability to regenerate P_R* is lost.

The molecular properties of phytochrome, which we observed in the denaturation studies, may relate to the physiological action of phytochrome. It has been postulated on physiological grounds that P_{FR} is the active form of an enzyme (Borthwick and Hendricks, 1960), while P_R is inactive. The apparent change of protein conformation which accompanies the interconversions between P_R and P_{FR} is attractive for such a hypothesis. Furthermore, our attention is drawn to the specific SH group or groups which are involved with the chromophore of P_{FR}. The SH groups in some way determine the absorbancy of the chromophore and the chromophore somehow controls the accessibility of the SH groups. The SH groups are much more susceptible to attack in the presumably active P_{FR} form than in the inactive P_R form. Perhaps the substrate for phytochrome must complex with these SH groups, and the act of enzyme activation involves making the SH groups available. If a substrate-SH complex did occur we would expect a change in the absorption spectrum because of the sensitivity of the chromophore to the SH groups. This could afford an experimental means of looking for the substrate. The absorption band of the complex might vanish as it does when the SH groups complex with *p*-mercuribenzoate. This would have the physiological implication that phytochrome would not be sensitive to light while acting on the substrate.

The phytochrome system has some similarities to rhodopsin. Light-induced changes of rhodopsin are not normally reversible, but at temperatures between $-196°$ and $-140°$ the pigment is reversibly photochromic (Yoshizawa and Wald, 1963): rhodopsin (λ_{max} = 498 mμ) $\rightleftarrows$ pre-lumirhodopsin (λ_{max} = 518 mμ). Light of 440 mμ favors the forward reaction while light of 600 mμ drives the reaction to the left. These changes apparently involve the isomerization of 11-*cis*-retenene to the all-*trans* form with little change of the protein. Transformations of phytochrome do not occur at these temperatures, perhaps because a protein conformational change which is obligatory for the transformation is prevented by the rigidity of the medium. At higher temperatures, pre-lumirhodopsin goes over to lumirhodopsin and further to metarhodopsin, presumably as the protein opens. In the transformation of rhodopsin to metarhodopsin, two additional SH groups become exposed (Wald and Brown, 1953). Wald *et al.* (1963) have suggested that rhodopsin may be a proenzyme which is transformed by light to the active enzyme, metarhodopsin.

Changes of protein conformation resulting from changes of a prosthetic group appear to be the rule rather than the exception. Phytochrome, once it is available in sufficient purity, should be an ideal protein for such studies because of its property of photoreversibility.

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Immunologically Active Fragments of Rabbit Gamma Globulin*

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Fragment III of papain-digested rabbit gamma globulin was digested with crystalline pepsin within a dialysis bag. A variety of degradation products resulted, some of which were too large to pass through the dialysis membrane. These retained the capacity to precipitate with antiserum against rabbit gamma globulin and demonstrated that a separation of antigenic determinants on the parent molecule had been effected by digestion. Other fragments were small enough to pass through the dialysis bag, did not precipitate with antibody, but were able to inhibit precipitation of antibody with fragment III. Fractionation by starch-gel electrophoresis and chromatography on Sephadex gels yielded a variety of fractions, some of which were able to combine with specific antibody, elicit reversed passive cutaneous anaphylaxis, and react with rheumatoid sera. One of the active fractions was comprised of peptides small enough to enter Sephadex G-50, indicating a molecular weight range of 2,000–10,000.

Rabbit γ-globulin possesses a number of properties of interest to immunologists in addition to its behavior as specific antibody. Degradation of the γ-globulin molecule by papain (Porter, 1959) yields a fragment (III) which, while devoid of antibody activity, carries the γ_2-globulin-specific determinants of the parent molecule (Porter, 1959), the ability to fix complement (Ishizaka *et al.*, 1962), the capacity to sensitize tissue for reversed passive cutaneous anaphylaxis (Ovary and Karush, 1961), reactivity with sera from cases of rheumatoid arthritis (Goodman, 1961), and the capacity to be recognized as γ-globulin by homologous cells (Brambell *et al.*, 1960).

Pepsin also splits γ-globulin into smaller units (Nisonoff *et al.*, 1960), but apparently further degrades the portion of the molecule corresponding to fragment III. This suggested the possibility that smaller pieces of fragment III bearing the above activities might be obtained by "controlled" treatment with pepsin. Accordingly, it was decided to digest fragment III within a dialysis sac under mild pressure, in order to protect peptides which were small enough to pass through the membrane from further degradation. An earlier report described preliminary results employing this procedure (Goodman, 1963).

MATERIALS AND METHODS

Rabbit γ-Globulin.—Rabbits were bled by cardiac puncture and the sera were pooled. γ-Globulin was prepared from the pooled sera by precipitation with sodium sulfate (Kekwick, 1940).

Antisera.—Goat antisera to rabbit γ-globulin and fragment III of papain-digested rabbit γ-globulin were kindly supplied by Dr. Melvin Cohn. The immunochemical characterization of these antisera has been described (Goodman and Gross, 1963). Another goat antiserum against rabbit γ-globulin provided by Dr. Leon S. Kind was used in passive cutaneous anaphylaxis experiments. From precipitin tests this antiserum was found to contain approximately 180 μg antibody N/ml.

Enzymatic Digestions.—Rabbit γ-globulin was digested by twice-crystallized mercuripapain (Worthington Biochemical Co.) and chromatographed on the cation-exchange resin carboxymethyl-cellulose (Carl Schleicher and Schull Co.), following the procedures described by Porter (1959). Three fractions were obtained, designated fragments I, II, and III in order of elution from the column. These were dialyzed exhaustively against water and lyophilized.

Fragment III of papain-digested γ-globulin was dissolved in water and the *p*H was adjusted to 4.0. The protein concentration was about 5%. Twice-crystallized pepsin (Mann Chemical Co.), dissolved in water at *p*H 4.0, was added at a concentration of 3% that of fragment III. The mixture was placed in a dialysis sac (Fisher Scientific Co.) which had been washed with running tap water for at least 12 hours. The dialysis bag was exposed to about 2 psi air pressure and placed in a vessel containing water at *p*H 4.0 at room temperature. The dialysate, about 50 ml, was changed twice daily for a period of 4 days. During this pressure dialysis the volume within the bag decreased and a crystalline precipitate formed. At the end of the fourth day both the dialysate and the material remaining within the bag were adjusted to *p*H 7.5 and the dialysate was lyophilized.

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