pyridine ring. Perhaps once NAD or NADH has done its job and is oxidized or reduced, its properties are changed sufficiently to reduce its affinity for the binding site on the enzyme. This could contribute to the efficiency of the catalyzed reaction.

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Chemical Evidence for Conformational Differences between the Red- and Far-Red-Absorbing Forms of Oat Phytochrome†

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ABSTRACT: The observation that glutaraldehyde inhibits the photoreversibility of the red-absorbing form of phytochrome (Pr) more than that of the far-red-absorbing form (Pfr) suggested that Pr and Pfr might have different reactive sites available to the aldehyde. An approach to evaluating this suggestion was made by performing amino acid analyses on highly purified phytochrome which had been reacted with glutaraldehyde as Pr, as Pfr, and while cycling between Pr and Pfr under constant red light (here called Pcy). Results of the analyses indicated that of the 27 lysine residues (per 60,000 molecular weight) in the control phytochrome, 13 in Pr and Pcy had reacted with glutaraldehyde compared to only 11 in Pfr under the conditions employed. These results were repeated several times with a standard deviation of less than 3%. The data suggest that there are two more aldehyde-accessible lysines in Pr and Pcy than in Pfr and are consistent with the hypothesis that the Pr → Pfr transformation involves a protein conformation change along with a change in the chromophore group. Similar experiments, with trinitrobenzenesulfonic acid (TNBS) as the reagent, were performed with results numerically different from the glutaraldehyde experiments but still consistent with the conformational change hypothesis. Peptide map analyses of TNBS-reacted and trypsin-digested Pr and Pfr were also consistent with the same hypothesis.

Phytochrome is a blue-green biliprotein widely distributed throughout the plant kingdom. The action spectra of several light-induced morphological changes in plants suggest that they are controlled by phytochrome and so this protein is thought to play a key role in plant development.

Phytochrome has two spectrally differing forms; one, Pr[1], has an absorption maximum at about 667 nm, and the other, Pfr, has a maximum at about 725 nm. These forms are interconverted, through short-lived intermediates, by irradiation with light of the appropriate wavelength.

$$\mathrm{Pr} \underset{\text{far-red light } (ca.\ 725\ \text{nm})}{\overset{\text{red light } (ca.\ 665\ \text{nm})}{\rightleftharpoons}} \mathrm{Pfr}$$

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[1] The abbreviations used are: Pr, the red-absorbing form of phytochrome; Pfr, the far-red-absorbing form of phytochrome; TNBS, 2,4,6-trinitrobenzenesulfonic acid; HA, hydroxylapatite; TNP, trinitrophenyl.

The spectral data described in Roux and Hillman (1969) suggested that Pr and Pfr had different sites available to reaction with glutaraldehyde. The differential reactivity of Pr and Pfr has been inferred also from their spectral response to reaction with urea, *p*-mercuribenzoate, trypsin (Butler *et al.*, 1964b), ammonium sulfate (Briggs *et al.*, 1968), and a low molecular weight substance in peas (Furuya and Hillman, 1966; Fox and Hillman, 1970). This question of the possible differential reactivity of Pr and Pfr is examined more directly here by using the technique of amino acid analysis to estimate the number of chemically modified (and thus sterically accessible) lysines in each form after treatment with a lysine reagent. Oat phytochrome contains approximately 27 lysines per 60,000 molecular weight (Mumford and Jenner, 1966; Roux, 1971).

Two different lysine reagents were used: glutaraldehyde (*cf.* Bowes and Cater, 1968; Jansen and Olson, 1969; Richards and Knowles, 1968) and 2,4,6-trinitrobenzenesulfonic acid (TNBS; *cf.* Ando *et al.*, 1965; Freedman and Radda, 1968; Habeeb, 1966; Satake *et al.*, 1960). Both react with the ϵ-amino group of lysine to give products which are stable under conditions of acid hydrolysis. Amino acid analysis of the hydrolysate of a treated protein shows a reduced number of free (unmodified) lysines and the loss is presumably proportional to the number of lysines in the protein which had reacted. As a way of complementing the amino acid analysis data, peptide maps of trypsin digests of TNBS-reacted Pr and Pfr were prepared. Similar maps of glutaraldehyde-reacted phytochrome could not yield reproducible results because of the polymerization and inter-, intramolecular bonds (Habeeb and Hiramoto, 1968) formed with proteins by this reagent.

By providing more direct data on the question of the differential reactivity of Pr and Pfr, these experiments are relevant also to the question of whether the spectral changes in the Pr $\rightleftarrows$ Pfr phototransformation are accompanied by a protein conformation shift.

Materials and Methods

Phytochrome. Six different preparations of phytochrome were used. Two were used in the glutaraldehyde experiments, both supplied by Dr. F. Mumford of DuPont and prepared from etiolated oat tissue by the method of Mumford and Jenner (1966). They were stored frozen in a potassium phosphate buffer, pH 7.8, containing 0.5 M sucrose and 0.7% β-mercaptoethanol, and were approximately 90% pure (for both, the A_{280}/A_{667} of Pr, an index of purity or PI, was about 1.2). The thawed samples were dialyzed against 1 l. of 0.05 M pyrophosphate, 0.1% mercaptoethanol, pH 8.6 for 40 min prior to use. The short dialysis time used was not sufficient to remove all the sucrose. All 4 preparations of phytochrome used for the TNBS experiments were prepared from etiolated oat tissue by modifications of a method described in Gardner *et al.* (1971). The procedure (Roux, 1971) utilizes 4 column chromatographic steps: Brushite (calicum phosphate gel), DEAE-cellulose, hydroxylapatite (HA), and Bio-Gel P-150. HA can be substituted for DEAE in the above procedure (giving a total of two separate HA runs in the protocol). Phytochrome prepared by the two HA columns elutes differently from the final Bio-Gel P-150 column, *i.e.*, closer to the void volume and thus with a higher apparent molecular weight than phytochrome purified by a DEAE–HA column series. Phytochrome prepared by either procedure was stored at 4° in 0.1 M sodium pyrophosphate-HCl buffer, pH 8.5, used within 24 hr after elution from the Bio-Gel column. The PI's of the phytochrome preparations used for the TNBS experiments ranged from 1.4 to 1.1 (70–95% pure), as indicated in Table IV. All of these preparations had been exposed to sucrose prior to chromatography on Bio-Gel P-150, but the sucrose was presumed to have been removed by the gel filtration. None of the phytochrome preparations used for these experiments was analyzed for molecular weight; but all were certainly less than 150,000 (eluted after the void volume of Bio-Gel P-150 column), and most were probably 60,000 (Roux, 1971). Recent important data bearing on the possible significance of different molecular weight species of phytochrome are given in Gardner *et al.* (1971).

Conversion of Phytochrome to Pr, Pfr, and Pcy (Cycling) Forms. The Pr form of phytochrome was prepared by irradiating the sample with 5 min of far-red (725 nm) light; Pfr by irradiating the sample with 5 min of red (660 nm) light. Pr and Pfr were removed to darkness for 1 min prior to the addition of reagent, which was introduced under dim green light. Under constant red light, phytochrome cycles between Pr and Pfr, thus Pcy was formed by keeping the protein under constant red light for 4 min prior to the addition of the reagent, during the addition, and during the course of the incubation period. The red light source was a 500-W incandescent spotlight approximately 25 cm above the sample and separated from it by 5 cm of water, a 650-nm interference filter, and an accessory Corning CS 2-62 red filter. The far-red source consisted of four 150-W incandescent spotlights over 10 cm of water and 0.3-cm thickness of Rohm and Haas V-58015 "black" Plexiglas.

Reaction of Phytochrome with Glutaraldehyde. Two sets of experiments were performed according to the following protocol.

Experimental Set 1. The dialyzed phytochrome preparation was divided among 6 hydrolysis tubes (0.325 mg/0.5 ml per tube). Two were converted to Pr, two to Pfr, and two unirradiated samples were used as controls. To each of the light-treated tubes, 0.02 ml of 5% glutaraldehyde (Union Carbide; charcoal purified by the method of Anderson (1967) just prior to use) buffered with 0.05 M sodium pyrophosphate at pH 8.2 was added to give a final aldehyde concentration of 0.19%. The samples were incubated in the dark for 45 min, after which the reaction was stopped by adding 0.1 ml of 12 N HCl. After 10 more min in the dark, 0.5 ml of 12 N HCl was added to the 4 tubes and also to the 2 untreated control tubes. Except where noted, all the procedures to this point were under a green safelight at 4°. The hydrolysis tubes were then all sealed under vacuum (<30 μ) in the light and hydrolyzed for 22 hr at 110°.

Experimental Set 2. The protocol for set 2 was essentially the same as for set 1, except that the original sample, different from that in set 1, was divided among 27 tubes, 15 tubes with 0.210 mg (in 0.5 ml/tube) and 12 tubes with 0.105 mg (in 0.25 ml/tube). The distribution of the sample in the group of 15 tubes was: 4 Pr in 0.13% glutaraldehyde; 4 Pfr in 0.13% glutaraldehyde; 4 Pcy in 0.13% glutaraldehyde; 3 untreated controls. One each of the samples of reacted Pr, Pfr, and Pcy was not hydrolyzed but was measured for photoreversibility with a 2-filter difference spectrophotometer as described in Hillman (1965).

The distribution of the sample in the group of 12 tubes was: 3 Pr in 0.07% glutaraldehyde; 3 Pfr in 0.07% glutaraldehyde; 3 Pcy in 0.035% glutaraldehyde; 1 Pr in 0.035% glutaraldehyde; 1 Pfr in 0.035% glutaraldehyde; 1 Pcy in 0.035% glutaraldehyde. The last three tubes were measured for photore-

TABLE I: Amino Acid Composition of Pr and Pfr Forms of Phytochrome after Reaction with Glutaraldehyde (Experimental Series 1).[a]

Amino Acid	Number of Residues		
	Control	Pfr + Glutaraldehyde	Pr + Glutaraldehyde
Lysine	28.3	15.8	13.8
Histidine	13.5	12.9	13.2
Ammonia			
Arginine	18.8	18.5	17.8
Aspartic acid	44.6	46.0	44.3
Threonine	19.5	17.6	18.2
Serine	34.2	28.9*	29.8
Glutamic acid	48.4	46.1	45.8
Proline	26.1	24.5	23.6
Glycine	34.2	32.9*	31.7
Alanine	45.8	47.4	47.1
Half cystine			
Valine	29.8	29.2	28.9
Methionine	9.7	8.5	8.5
Isoleucine	20.7	20.1	20.4
Leucine	47.1	47.1	47.1
Tyrosine	8.2	6.6	6.0
Phenylalanine	19.8	18.8	19.4

[a] These numbers are the average of two analyses and represent the number of residues per 60,000 molecular weight. The two analyses agreed with one another within 3% in all cases except the two marked with asterisks. In both these cases the two analyses differed from one another by 7%. The ratio of milligrams of protein to per cent glutaraldehyde in the reaction mixture was 1.5 for all the reacted samples analyzed.

versibility after the reaction; the other nine were run on the short column (only) of the amino acid analyzer.

Reaction of Phytochrome with TNBS. The TNBS used in all the experiments was purchased from Pierce Chemicals. Because TNBS is photochemically decomposed to relatively unreactive picric acid by light <450 nm (Satake and Okuyama, 1959), the dry reagent was stored in the dark and 2–5% solutions were made up fresh in the dark prior to each experiment. All the reactions were stopped by the addition of an equal volume of 12 N HCl, which both stabilized lysine–trinitrophenyl (TNP) bonds and converted excess TNBS to picric acid (Palmer and Peters, 1969). Subsequent exposure of the reacted sample to light, while probably decomposing TNP-lysine to picramide (Satake and Okuyama, 1959), had no effect on the amino acid analysis results, since neither TNP-lysine nor picramide chromatograph under the lysine peak on amino acid analysis chromatograms.

Five sets of analyses were run (experimental sets 3–7) on TNBS-reacted phytochrome. The phytochrome and TNBS concentrations and the reaction times for each of the experimental sets are given in the legend for Table IV. After the reaction of each sample was stopped by the addition of HCl, the tube was evacuated and sealed, and the contents hydrolyzed at 110° for 22 hr. In experimental set 6, the reacted protein was precipitated in 10% trichloroacetic acid and washed free of buffer and excess picric acid with ether prior to hydrolysis and analysis.

Amino Acid Analyses of Reacted Phytochrome. In general, the preparation of all the samples, including the acid hydrolysis, was done according to the method of Moore and Stein (1963). All the hydrolyses were carried out at 110° for 22 hr a time chosen to maximize the free lysine count in phytochrome (Mumford and Jenner, 1966). The analyzers used were modifications of the model of Spackman *et al.* (1958), automated as described by Alonzo and Hirs (1968).

In the analyses of glutaraldehyde-reacted phytochrome, the charred residue present in the tubes after hydrolysis (probably carbonaceous deposits from hydrolyzed sucrose) was filtered out prior to placing the sample in the analyzer. The analyzer used for both sets of these analyses had a 15-cm short column with Beckman PA resin 35 and a 50-cm long column with Technicon Chromobeads resin type 104.

All the analyses of TNBS-reacted phytochrome were on the short column only (15 cm, Bio-Rad Aminex AS resin) of the analyzer.

It should be noted here how the estimate of the number of residues in Tables I, II, and IV was arrived at. As described in detail by Moore and Stein (1963), the only information directly calculable from amino acid chromatograms is the number of micromoles of each residue. This fixes the ratio of any one amino acid to another, but does not say what is the absolute number of free residues of any one amino acid. This number can vary widely depending on the constant one chooses by which to multiply the micromole values. To estimate the number of residues for the controls of Tables I and II that constant factor was chosen which, when multiplied by the micromole values of the amino acids, gave values which, converted to molecular weights and added together, would yield 58,000, the assumed molecular weight of phytochrome minus tryptophan. The constant factor chosen to convert micromoles to number of residues in the analyses of glutaraldehyde-reacted phytochrome was, in the case of Table I, such as to yield the same number of leucines as in the control, and in the case of Table II, such that the sum of the deviations from the control number of prolines, alanines, valines, isoleucines, and leucines would be as close to zero as possible.

The estimate of residues in Table IV could not be done as above since only short column residues were chromatographed. Instead, for the control analyses in Table IV that constant factor was chosen which would convert the micromole value of lysine to 27 residues. Then, for the analyses of reacted samples, that factor was chosen which, when multiplied by the micromole values for histidine and arginine, gave values for these residues which most closely approximated those of the untreated control. The constant was not weighted in favor of histidine or arginine, so that the per cent deviation of these from the control was equal. Thus, for example, using a higher constant in calculating the Pr + TNBS analysis from experimental set 4 would bring the histidine value (12.7) more in line with that of the control (13.0), but in so doing would also make the Pr + TNBS arginine value (18.1) even higher than the control (17.6) than it already was; and so the compromise. Whatever constant factor was decided on in this way for a given analysis was used to calculate the number of free lysine, histidine, and arginine residues for that analysis.

Peptide Mapping of TNBS-Reacted Pr and Pfr. The starting material was 3 mg of phytochrome in 5.0 ml of 0.1 M potassium phosphate, pH 7.8 (purity index = 1.1). Half of the sample was treated with 4 min of red light (= Pfr); half treated with 4 min of far-red light (= Pr). To Pr and Pfr was added: (a) 0.05 ml of 20% TNBS and 0.05 ml of 1.0 M K_2HPO_4. This reaction was allowed to proceed in the dark for 3 hr; (b) 0.5

TABLE II: Amino Acid Composition of Pr, Pfr, and Pcy Forms of Phytochrome after Reaction With Glutaraldehyde (Experimental Series 2).[a]

	Number of Residues				
Amino Acid	Control	Pfr + Glutaraldehyde	Pr + Glutaraldehyde	Pcy + Glutaraldehyde [A]	Pcy + Glutaraldehyde [B]
Lysine	27.0 ± 0.4	16.3 ± 0.5	14.1 ± 0.3	14.1 ± 0.0	19.4 ± 0.1
Histidine	14.4 ± 0.5	14.1 ± 0.0	14.1 ± 0.3	14.1 ± 0.2	14.0 ± 0.2
Ammonia					
Arginine	18.8 ± 0.5	19.1 ± 0.3	18.8 ± 0.2	18.8 ± 0.3	18.8 ± 0.2

[a] These numbers are the average of three analyses (± the standard deviation, expressed in residues) and represent the number of residues per 60,000 molecular weight. The ratio of milligrams of protein to per cent glutaraldehyde in the reaction mixture was 1.5 for all the reacted samples analyzed except for Pcy + glutaraldehyde B, in which the ratio was 3.0.

ml of concentrated HCl to stop the reaction; (c) 1.0 ml of 20% trichloroacetic acid to precipitate out protein. The precipitate was centrifuged down, washed several times in water, and then frozen.

Reduction and Alkylation. To the trichloroacetic acid precipitates of reacted Pr and Pfr was added: (a) 5 ml of 6 M guanidine-HCl, pH 7.6, plus 0.143 M β-mercaptoethanol; this was incubated for 3 hr at 45°; (b) enough iodoacetimide (~470 mg) to make solution 0.5 M; this was incubated for 15 min in dim light, titrating with NaOH to keep the pH between 6.5 and 9.0, then was kept for 10 min at *ca.* pH 8.3 with stirring in dim light; (c) enough mercaptoethanol to make the solution 1.0 M.

Trypsin Digestion. The carboxymethylated samples were dialyzed against 8 M urea in 0.1 M NH_4HCO_3 for 2 hr, then against 0.1 M NH_4HCO_3 exhaustively for 2 days, changing the buffer about every 6 hr. After the dialyzed samples were lyophilized to dryness, 1 ml of 0.2 N NH_4HCO_3 plus 38 μg of L-1-tosylamido-2-phenylethyl chloromethyl ketone (Schoellmann and Shaw, 1963) trypsin was added to each of them. The samples were then incubated for 3 hr at 37° on a rotary shaker, then frozen, and lyophilized.

Chromatography and Electrophoresis. Each of the lyophilized samples was dissolved in 0.15 ml of pyridine acetate buffer, pH 6.5. Core (undigested) protein was centrifuged down, then 0.12 ml of supernatant from each sample was removed and applied in 0.005-ml aliquots to separate sheets of Whatman No. 3MM paper (18.5 × 22.25 in.). The chromatographic separation was carried out (descending, 20 hr) with butanol–acetic acid–water (4:1:5 by volume). Electrophoretic separation was done at pH 6.5, in toluene solvent, for 45 min at 2500 V. After drying, the final map was developed with a ninhydrin–collidine stain (60 ml of absolute ethanol–20 ml of glacial acetic acid–8 ml of collidine–0.1 g of ninhydrin).

Results

I. Glutaraldehyde Experiments. Tables I and II summarize the amino acid analyses of experimental sets 1 and 2. Both tables show a 14% higher count of free lysines in the analyses of Pfr-reacted phytochrome over those of Pr-reacted phytochrome. Table II shows that reacted Pfr also has a 14% higher free lysine count than Pcy. Though significant decreases of free tyrosine, serine, threonine, glycine, and methionine in reacted phytochrome are evident, differences between reacted Pr, Pfr, and Pcy in the count of all residues other than lysine are within the standard deviation and so are not statistically significant. Just as for experimental set 1, the reacted protein from experimental set 2 was analyzed for all amino acid residues except cystine and tryptophan; but since the results were essentially the same for both sets, analyses results for only the basic amino acids are given from set 2 to avoid unnecessary repetition.

Table III summarizes the effects of two different concentrations of glutaraldehyde on the photoreversibility of phytochrome. It illustrates that the magnitude of the differential effect of glutaraldehyde on the spectra of Pr and Pfr *in vitro* is partially dependent on glutaraldehyde concentration. This is discussed in more detail in Roux (1971).

II. TNBS Experiments. Table IV records free lysine, histidine, and arginine residues in the analyses of TNBS-reacted Pr and Pfr. The table shows a consistent difference of one more free lysine remaining in Pr than in Pfr after reaction with TNBS, while showing no consistent difference between reacted Pr and Pfr in the numbers of free histidine and arginine. Note the contrast between these results and the glutaraldehyde results given in Tables I and II. Table IV also indicates that the number of free histidine and arginine residues in reacted phytochrome was roughly equal to that in the control, whereas lysine losses in reacted phytochrome ranged from

TABLE III: The Effects of Two Different Concentrations of Glutaraldehyde on the Photoreversibility of Pfr, Pr, and Pcy.[a]

	Final Δ (ΔA)[b] Treated/ Final Δ (ΔA) Control	
Sample	Set 1	Set 2
Pfr + G	0.54	0.73
Pr + G	0.13	0.63
Pcy + G	0.44	0.47

[a] The ratio of milligrams of phytochrome to per cent glutaraldehyde in the reaction: for set 1: 1.5 (0.2 mg/0.13% glutaraldehyde); for set 2: 3.0 (0.1 mg/0.033% glutaraldehyde). [b] Δ (ΔA) = photoreversibility = total change in absorbance at 660 nm + the total change in absorbance at 720 nm in the Pr → Pfr or Pfr → Pr phototransformation.

TABLE IV: Estimation of Lysine, Histidine, and Arginine Residues in TNBS-Reacted Pr and Pfr by Amino Acid Analysis and Average of the Results from Experimental Sets 3–7.[a]

		Amino Acid Composition of Pr and Pfr Forms of Phytochrome after Reaction with TNBS					
	Sample	No. 3	No. 4	No. 5	No. 6	No. 7	Av No. of Residues
Lys	CONTROL	27.0	27.0	27.0	27.0	27.0	
	Pr + TNBS	21.2 ± 0.1	20.7 ± 0.4	18.4	18.6	24.5 ± 0.3	20.7
	Pfr + TNBS	20.2 ± 0.2	19.9 ± 0.4	17.3	17.2	23.5 ± 0.2	19.6
His	CONTROL	13.0 ± 0.3	13.0 ± 0.3	12.2	12.1	12.8 ± 0.3	
	Pr + TNBS	13.1 ± 0.1	12.7 ± 0.1	12.2	12.2	13.0 ± 0.2	12.7
	Pfr + TNBS	12.7 ± 0.1	13.1 ± 0.1	12.0	12.2	12.8 ± 0.2	12.6
Arg	CONTROL	17.6 ± 0.0	17.6 ± 0.0	20.4	20.6	18.4 ± 0.3	
	Pr + TNBS	17.5 ± 0.1	18.1 ± 0.1	20.5	20.4	18.0 ± 0.1	19.0
	Pfr + TNBS	18.1 ± 0.4	17.6 ± 0.1	20.7	20.5	18.3 ± 0.2	19.1

[a] The sets were counted equally in figuring the average, even though sets 5 and 6 represent only one analysis and all the others represent two. The phytochrome and TNBS concentrations, expressed as the ratio, OD_{280} phytochrome (cm path length)/%TNBS, the purity indexes,[b] and the reaction times for each of the experimental sets were:

Set No.	Ratio: OD_{280} phytochrome/% TNBS	PI	Reaction Time (hr)
3	1.33 (= 0.16/0.12)	1.4	3
4	0.67 (= 0.16/0.24)	1.4	3
5	1.67 (= 0.20/0.12)	1.3	2
6	1.56 (= 0.72/0.45)	1.2	2.5
7	3.60 (= 0.43/0.12)	1.1	2.5

[b] Purity index = A_{280}/A_{667} of Pr.

10 to 40%, depending on the ratio of TNBS to phytochrome and the reaction time of the experiment.

Figure 1a,b compares the peptide maps of reacted Pr and Pfr. One spot, noted by the arrows, is strongly stained on the Pr map but barely visible on the Pfr map. Otherwise the two maps are identical.

Discussion

Earlier data (Roux, 1971) indicated that, over a narrow range of low glutaraldehyde concentrations, changing the concentration of glutaraldehyde altered the photoreversibility of Pr far more than that of Pfr. The data in Table III were consistent with that earlier observation (note that the glutaraldehyde concentration per milligram of protein in set 1 is twice that in set 2) and dictated the choice of glutaraldehyde concentration for the reactions which would be assayed by amino acid analysis for loss of lysine. That is, if lysines were involved in the observed unequal alteration of Pr and Pfr photoreversibility by glutaraldehyde, one might hope to detect the most unequal loss of Pr and Pfr lysines to glutaraldehyde at the same concentration of glutaraldehyde which gave the most unequal alteration of Pr and Pfr photoreversibility.

But when the data from the amino acid analyses were tabulated (Tables I and II), notable noncorrelations with Table III became evident. Amino acid analyses indicated that at one glutaraldehyde concentration Pr and Pcy lost the same number of lysines, but spectral data showed that at the same concentration Pr lost far more photoreversibility than Pcy. At two different glutaraldehyde concentrations the photoreversibility of Pcy was approximately the same though it lost 5 fewer lysines to glutaraldehyde at the lower concentration. Of course, these data do not rule out the possible necessity of precise shifts in lysine side chain positions for a spectrally "normal" Pr to Pfr phototransformation. But they suggest caution before drawing any conclusions from the one obvious correlation of analysis and spectral data: Pr loses 2 more lysines to glutaraldehyde *and* more photoreversibility than Pfr. Because glutaraldehyde lacks specificity as a protein reagent (Vallee and Riordan, 1969) any one of several amino acids might be potential sites where glutaraldehyde could react to alter the spectral properties of phytochrome, and the experiments here do not aid in selecting which one.

Pcy, which is pretreated with red light just as is Pfr but kept under constant irradiation during the reaction with glutaraldehyde, resembles Pr in its greater loss of lysines. Since both Pr and Pfr forms are available to glutaraldehyde in the Pcy state, one would predict that the loss of lysine in Pcy would be at least as great as that in the form with the greater loss. The fact that Pcy does not lose more than Pr is consistent with the idea that the intermediate forms of phytochrome (Linschitz *et al.*, 1966) also present in Pcy, expose no lysine sites reactive to glutaraldehyde other than those already present in either Pr or Pfr.

The results of Table I might indicate that glutaraldehyde is reacting with other amino acids besides lysine. While this cannot be absolutely ruled out, other plausible explanations are available. Serine, threonine, and tyrosine are all particularly labile to destruction or conversion (Downs and Pigman, 1969; Moore and Stein, 1963) during acid hydrolysis. The presence of glutaraldehyde in the hydrolysis tubes might be expected to affect these residues more than the other more stable amino acids Tyrosine losses in glutaraldehyde-reacted proteins have been noted before (Bowes and Cater, 1968). Intramolecular cross-linking probable under the conditions

of these experiments (Habeeb and Hiramoto, 1968), might slow the acid hydrolysis of certain segments of glutaraldehyde-reacted phytochrome relative to the untreated control, decreasing the number of free copies of the amino acids in these segments. Lysine, however, definitely reacts with glutaraldehyde (*cf.* Anderson, 1967; Bowes and Cater, 1968; Habeeb and Hiramoto, 1968; Richards and Knowles, 1968), and its significant loss noted in Tables I and II can be attributed almost exclusively to this reaction.

The data of Tables I and II suggest strongly that Pr and Pcy have two more lysines available to react with glutaraldehyde than does Pfr under the experimental conditions chosen. Although this difference of two repeated itself with remarkable consistency through 8 analyses, the data have no predictive values about the relative quantities of lysines one may find on the outer surfaces of Pr and Pfr when three-dimensional maps of them become available. Rather, the conclusion inferred should be qualitative: some of the lysines in Pr are in a different microenvironment than those same lysines in Pfr, making those Pr lysines relatively more accessible to glutaraldehyde. This, in turn, implies Pr and Pfr differ not only in their spectral properties, but in their conformational shape as well.

The amino acid analyses of TNBS-reacted phytochrome (Table IV) suggest the same general conclusion as the analyses of glutaraldehyde-reacted phytochrome: Pr and Pfr have different conformations because under identical *in vitro* conditions they have different numbers of lysines available to reaction with the same reagent. In the case of the glutaraldehyde experiments there were two more reactive lysines in Pr than in Pfr; in the case of these TNBS experiments there appears to be one more reactive lysine in Pfr than in Pr. This contrast in the results deserves an explanation, though it would have been rather more surprising if two reagents as different as TNBS and glutaraldehyde reacted with and only with the same lysines. Probable factors in the contrasting results were the differences in the reaction mixtures: (1) sucrose and β-mercaptoethanol were both present in the glutaraldehyde phytochrome reactions and not in the TNBS–phytochrome reactions. It is not difficult to imagine how both these reagents could introduce differences in lysine accessibility (*cf.* Clement-Metral and Yon, 1968). (2) The phytochrome preparations themselves, while similar in purity index, were prepared by different techniques, and showed differences in amino acid analysis (Roux, 1971).

Though Table IV gives ample testimony to the consistency of the results, it should be pointed out that no one of the vertical columns there could stand alone as convincing evidence of the one lysine difference between the analyses of reacted Pr and Pfr, since the expected standard deviation of $\pm 3\%$ allows some probability of reducing that difference of one to near zero. So, given the precision of amino acid analysis, such a result could only become credible upon numerous repetitions. Table IV highlights the consistency of the results by comparing the average number of free lysines, histidines, and arginines found over the five experiments in reacted Pr with those in reacted Pfr. The average lysine difference of 1.1 looms large when compared with the average histidine and arginine difference of 0.1.

The peptide maps of reacted Pr and reacted Pfr are consistent with the amino acid analysis results. Since reacted Pr had one more free lysine than did reacted Pfr (Table IV), one would expect more trypsin cleavage and consequently more peptide map spots from reacted Pr than from reacted Pfr. The arrow in Figure 1a points to a deeply stained spot on the map of reacted Pr which appears as only a trace in the corresponding position on the map of reacted Pfr. This trace probably reflects the 20% "contamination" of Pr which is always present in Pfr (Butler *et al.*, 1964a). Otherwise, the two maps are identical. Granted the one lysine difference demonstrated in Table IV is real, then these results offer hope of identifying which one it is by correlating information on the sequence in the "extra" peptide on the map of reacted Pr with sequence data on the intact phytochrome molecule.

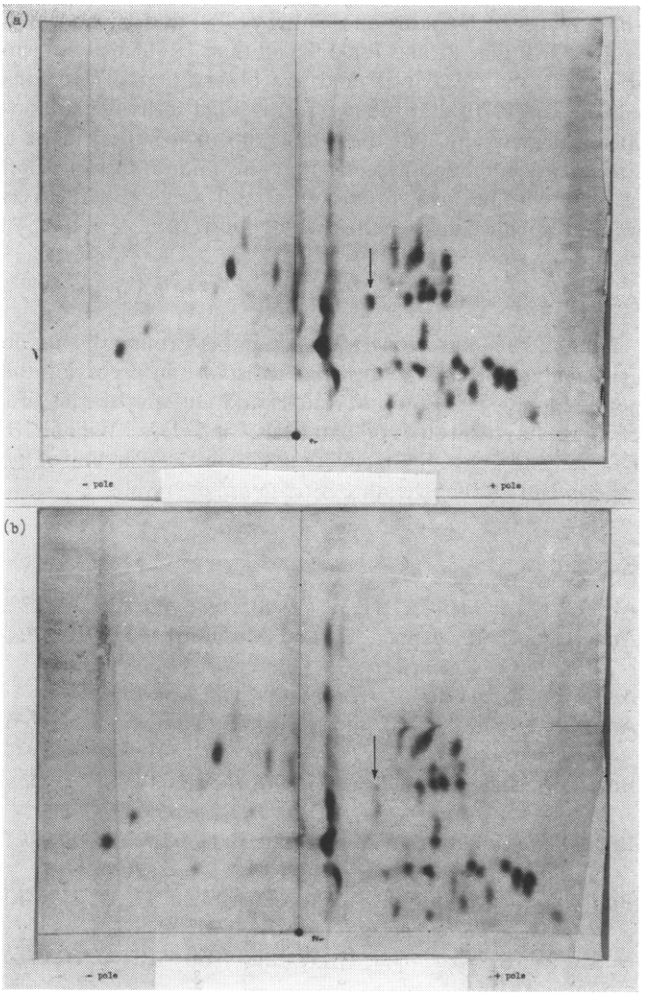

FIGURE 1: Peptide maps of TNBS-reacted (a) Pr and (b) Pfr forms of phytochrome after trypsin digestion.

The aim of the TNBS experiments was to provide data which would allow by comparison, a better evaluation of the results from the glutaraldehyde experiments. The data support the same general conclusion as the glutaraldehyde experiments: Pr and Pfr showed a different reactivity to the same reagent under the same conditions, and a shape difference between Pr and Pfr was again invoked as the probable explanation for this. The results of the amino acid analyses were also different enough with the two reagents (Pr showed greater lysine reactivity to glutaraldehyde; Pfr showed greater lysine reactivity to TNBS) to demonstrate clearly that these sorts of data only permit generalized qualitative judgments: Pr and Pfr have different reactivities—not generalized quantitative ones, since the extent of the differential reactivity is not fixed but will vary depending on the concentrations and kinds of reagents used.

The results obtained with both reagents are consistent with

the findings of Hopkins and Butler (1970) and of Anderson *et al.* (1970) that Pr and Pfr differ in their circular dichroism spectra in the ultraviolet, and the observation of Hopkins and Butler (1970) that the two forms differ in their immunochemical reactivity. All these data, taken together, make a strong case for hypothesizing that the phototransformation of phytochrome involves structural shifts in the protein as well as resonance shifts in the chromophore(s).

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