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Studies on Phytochrome

SOME PROPERTIES OF ELECTROPHORETICALLY PURE PHYTOCHROME

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1. Phytochrome was purified from etiolated oat (*Avena sativa*) seedlings either by gel-filtration chromatography and ion-exchange chromatography or by gel-filtration chromatography and calcium phosphate chromatography. Differences were observed in the spectral properties of phytochrome isolated by the two methods. 2. Electrophoresis of pure phytochrome at pH values between 9.0 and 6.0 showed the tendency of phytochrome to form different molecular species. Studies in the ultracentrifuge did not show a corresponding change in the sedimentation coefficient with the change in pH. 3. Tryptic digestion of electrophoretically pure phytochrome gave 17 peptides and a photoactive core. The amino acid composition of the core is reported and compared with the analysis of whole phytochrome. 4. Some properties of phytochrome isolated from *Pisum sativum* are compared with those of phytochrome from *A. sativa*. 5. The properties of phytochrome purified by other workers are compared with our findings.

In the preceding paper (Walker & Bailey, 1970) we reported some properties of two photoactive proteins purified from etiolated oat seedlings by a combination of gel-filtration chromatography, ion-exchange chromatography and polyacrylamide-gel electrophoresis. Since it appeared that the extraction procedure was having a denaturing effect on the properties of phytochrome it was decided to purify phytochrome by two separate methods to investigate the effect of the purifying procedure on the spectral characteristics of phytochrome. Our findings in this respect and on other properties of phytochrome are reported.

MATERIALS AND METHODS

Etiolated oat (*Avena sativa* var. Condor, grown in Scotland) seedlings were grown and harvested as previously described (Walker & Bailey, 1968). Etiolated pea (*Pisum sativum* var. Meteor) seedlings were grown for 14 days under the same growth conditions as the oat seedlings.

Extraction of oat phytochrome. The extraction procedure used in these experiments was similar to that described previously (Walker & Bailey, 1970) with the following exceptions. The seedlings (9–10kg) were blended as before in 1.5kg lots with 1.5 litres of 0.1M-potassium phosphate buffer, pH7.8, containing 0.05M-2-mercaptoethanol. Unless otherwise stated all buffers were at pH7.8, and contained 2-mercaptoethanol. The crude extract (13–14 litres) was clarified by filtration and centrifugation and concentrated to 1.6 litres with dry Sephadex G-50 beads. The concentrated extract was divided into two and each half purified separately by a different procedure.

(I) One half was chromatographed on two Sephadex G-100 columns (60cm × 10cm) equilibrated with 0.01M-potassium phosphate buffer. The columns were eluted with the equilibrating buffer and the photoactive fractions from Sephadex G-100 chromatography were then treated as described in the preceding paper (Walker & Bailey, 1970) except that Sephadex G-200 chromatography was carried out on a column 100cm × 3.5cm instead of 125cm × 2.5cm.

(II) The other half of the extract was chromatographed on two Sephadex G-100 columns (60cm × 10cm) equilibrated with 0.005M-potassium phosphate buffer; elution was again carried out with the equilibrating buffer and the photoactive fractions bulked and loaded on to a column (40cm × 7cm) of partially altered brushite equilibrated with 0.005M-potassium phosphate buffer, pH7.8. The column was eluted with a gradient of 150ml of 0.005M-potassium phosphate buffer and 1.5 litres of 0.4M-potassium phosphate buffer, both at pH7.8, the eluate was collected in 30ml fractions and the photoactive fractions were located and combined. The photoactive solution was equilibrated with 0.005M-potassium phosphate buffer by passing it down a Sephadex G-50 column

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(45cm × 7cm) equilibrated with this buffer. After irradiation with far-red light for 20min the photoactive protein mixture was adsorbed on brushite equilibrated with 0.005M-potassium phosphate buffer by stirring an arbitrarily chosen amount of brushite into the active solution. The slurry was stirred on a magnetic stirrer for 30min and then filtered on a sintered glass funnel. The proteins were desorbed from the brushite with 0.25M-potassium phosphate buffer, pH7.8, and the active solution was chromatographed on a column of Sephadex G-200 identical with that being used in the DEAE-cellulose half of the experiment. The column was eluted with 0.25M-potassium phosphate buffer by use of a peristaltic pump to obtain a constant flow rate of 15ml/h and the eluate was collected in 5ml fractions, which were monitored for E_{280} and photoreversibility. After combination of those fractions with highest activity the active solution was re-equilibrated with 0.005M-potassium phosphate buffer on a Sephadex G-50 column and reconcentrated by adsorption on and desorption from brushite as described above. For preparative polyacrylamide-gel electrophoresis the photoactive solutions were treated as described by Walker & Bailey (1970). Scheme 1 presents a flow diagram of the two comparative extraction procedures.

Extraction of pea phytochrome. The 14-day-old etiolated pea seedlings were harvested under dark-green light (Ilford Spectrum Green Safelight, transmission band 510–530nm) to minimize the effect of the 'killer' factor present in etiolated pea seedlings (Furuya & Hillman, 1966). Electrophoretically pure phytochrome was obtained from extracts of etiolated pea seedlings by using the same sequence of Sephadex G-100 chromatography, DEAE-cellulose chromatography and Sephadex G-200 chromatography that has been described for the purification of phytochrome from etiolated oat seedlings.

Calcium phosphate. Partially altered brushite was prepared by the method described by Siegelman, Wieczorek & Turner (1965). The characteristics of brushite prepared by this method changed on storage; consequently the brushite was prepared freshly 10 days before an extraction.

Analytical polyacrylamide-gel electrophoresis. Analytical gel electrophoresis was carried out on columns of gel polymerized in constant-bore glass tubing (5cm × 0.6cm). The buffers used were 0.05M-tricine [*N*-tris(hydroxymethyl)methyl glycine]–KOH at pH9.0, 8.6 and 8.2 and 0.05M-potassium phosphate buffer starting at pH6.0 and changing by increments of 0.2 to pH8.0. The gel composition used was 7.5% (w/v) acrylamide with 1.66% cross-linking.

After equilibration of the samples to the electrophoresis buffers by dialysis, sucrose was added to the samples and portions containing approx. 100μg of protein were overlaid on the gel surface. Electrophoresis was carried out for 60min at 150V at 5mA/tube. The proteins were fixed and located in the gel columns with 0.1% Amido Black in aq. 7% (v/v) acetic acid.

Performic acid oxidations. Performic acid was prepared by allowing 1ml of 30% hydrogen peroxide to react with 19ml of 98% (v/v) formic acid at room temperature for 2h. The reaction mixture was then cooled to −11°C. Protein samples were dissolved in 1–2ml of anhydrous methanol–98% formic acid (1:6, v/v) and treated with a 12-fold excess of performic acid mixture for 3h at −11°C. At the end of the reaction time the reaction was stopped by the addition of a large excess of glass-distilled water and the sample was freeze-dried. The whole of the freeze-dried sample was hydrolysed for amino acid analysis.

Amino acid analysis. Amino acid analysis was carried out on weighed amounts of freeze-dried electrophoretically pure phytochrome. The same hydrolysis technique was used as described in the preceding paper (Walker & Bailey, 1970).

Tryptic digestion. Solutions of electrophoretically homogeneous phytochrome were equilibrated with 0.1M-ammonium acetate buffer, pH8.0, by dialysis. The solutions for digestion normally contained 5–10mg of protein/ml. Approximate concentrations of protein were determined from the E_{280}/E_{260} ratio (Layne, 1957). Initially tryptic digestion was carried out in a Radiometer pH-stat with Worthington trypsin (three times crystallized) in the ratio 1:100 phytochrome (w/w). 0.01M-Ammonia was used in the pH-stat burette to keep the pH of the digestion at 8.0. Digestion was carried out at 37±0.1°C for 1h or 3h. The peptides were recovered by exhaustive dialysis against glass-distilled water and the diffusate was freeze-dried. Tryptic digestion was also carried out at 4°C for 24h. The photoactive solution was irradiated with far-red light for 30min before being placed in a small glass-stoppered conical flask that had been covered with aluminium foil. Trypsin was added in the same ratio as used above and the mixture stirred gently during the digestion period in the cold-room. The peptides of digestion were recovered as before. The photoactive core was recovered by chromatography on Sephadex G-200 or Sephadex G-75 columns (30cm × 2.5cm), both equilibrated to 0.1M-ammonium acetate buffer, pH8.0.

Peptide 'maps'. Peptide 'maps' were prepared by electrophoresis followed by paper chromatography at right angles to the direction of electrophoresis. Electrophoresis was carried out in a commercially available apparatus of the type described by Gross (1961) for 25min at 70–80mA and 5kV. The buffer used was 2M-formic acid–acetic acid, pH2.0. The freeze-dried peptide mixture was redissolved in 1–2ml of the electrophoresis buffer and a suitable amount loaded on to a 15cm-wide strip of Whatman 3MM chromatography paper 10cm from the anode end.

After electrophoresis the strip was dried for 2h at 80°C. A piece of Whatman 3MM chromatography paper (57.5cm × 47cm) was sewn along one edge of the electrophoresis strip and the peptides were chromatographed for 23h in butan-1-ol–acetic acid–water (3:1:1, by vol.). The chromatogram was oven-dried and the peptides were located by spraying with a commercial ninhydrin spray and developing the spots in the oven at 100°C.

Sedimentation studies. Sedimentation studies on the purified protein were carried out in the Spinco model E analytical ultracentrifuge. The protein samples were equilibrated to the required pH by dialysis against a 4000-fold excess of buffer. 0.05M-Tricine–NaOH buffer was used at pH values between 9.0 and 7.5; 0.05M-potassium phosphate buffer was also used between pH7.0 and 8.0. Centrifugation was also carried out at pH8.4 in 0.05M-tris-acetate buffer.

Tricine. This was prepared as described by Good (1962).

RESULTS

Oat phytochrome. We have previously shown (Walker & Bailey, 1970) that phytochrome migrated as a single peak in both ion-exchange chromatography and gel-filtration chromatography. Active fractions from the initial Sephadex G-100 chromatography when chromatographed on partially altered brushite also migrated as a single photoactive component (Fig. 1). Subsequent Sephadex G-200 chromatography of the active fractions from the brushite columns gave elution patterns similar to those from Sephadex G-200 chromatography of the phytochrome solutions from DEAE-cellulose chromatography. The elution diagrams of Sephadex G-200 chromatography for both halves of the experiment are presented in Figs. 2(*a*) and 2(*b*). The eluates from both Sephadex G-200 columns were concentrated and the spectra of the concentrates determined. Figs. 3(*a*) and 3(*b*) show the spectra of the photoactive solutions after Sephadex G-200 chromatography. The effect of DEAE-cellulose treatment on the spectral characteristics of phytochrome was very apparent and its effect on the recovery of active phytochrome as determined by $\Delta(\Delta E)$/cm can be seen from the results presented in Table 1.

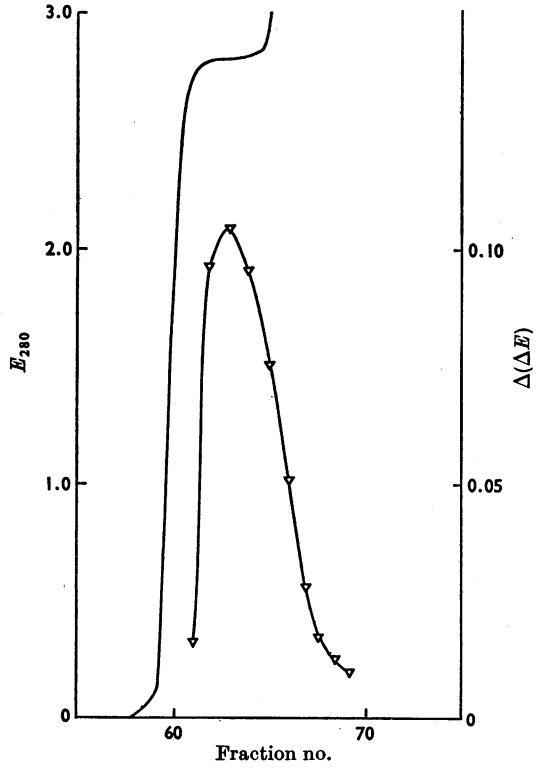


Fig. 1. Chromatography on partially altered brushite of active fractions from Sephadex G-100 chromatography. ——, E_{280} read in 10mm cuvettes. ▽——▽, Photoreversibility read in 40mm cuvettes. The results are expressed in the diagrams as $\Delta(\Delta E)$/cm.

In contrast with our previous experience (Walker & Bailey, 1970) subsequent preparative polyacrylamide-gel electrophoresis at pH 8.4 showed that the preparation was at this stage electrophoretically homogeneous (Fig. 4). The same result was obtained for phytochrome solutions purified by either method and for mixtures of these phytochrome solutions. Analytical electrophoresis between pH 9.0 and 8.0 showed that phytochrome migrated as a single band with increasing mobility at the more alkaline values. Below pH 8.0 however, the mobility of the front band remained constant and an increasing number of diffuse bands appeared so that at pH 6.0 the total number of bands staining was five.

Sedimentation studies on the purified protein, in contrast with the electrophoretic studies, did not indicate the formation of polymeric forms. The sedimentation coefficient was determined at pH 9.0, 8.5, 8.0 and 7.5 in tricine–sodium hydroxide buffer and also in potassium phosphate buffer at pH 7.5 and 7.0 so that the possibility that the polymeric forms observed in electrophoresis were the consequence of using phosphate buffer could be eliminated. The corrected sedimentation coefficients (Table 2) show a small increase in the sedimentation coefficient with increase of pH, the opposite effect to that indicated by electrophoresis studies. Phytochrome prepared by ion-exchange chromatography and gel-filtration chromatography did not sediment at a rate different from that prepared by brushite chromatography and gel-filtration chromatography, nor was there any indication of two differently sedimenting proteins in the same preparation.

Tryptic digestions were carried out on phytochrome purified by chromatography on brushite and gel-filtration chromatography. Preliminary tests on the dialysis residue of 37°C tryptic digest showed that a considerable portion of the phytochrome molecule (the tryptic core) had remained undigested, but the residual part was not photoactive. When phytochrome was tryptically digested at 4°C for 24h the solution did remain photoactive although the photoactivity was considerably diminished. Peptide 'maps' prepared from the diffusible peptides of 4°C tryptic digests showed the same patterns as those recovered from 37°C tryptic digests (Fig. 5). Chromatography of the 4°C tryptic digest on columns of either Sephadex G-200 or G-75 showed that the photoactive component migrated as a single peak and retained its photoactivity. Subsequent digestion of the photoactive core with chymotrypsin did not yield further

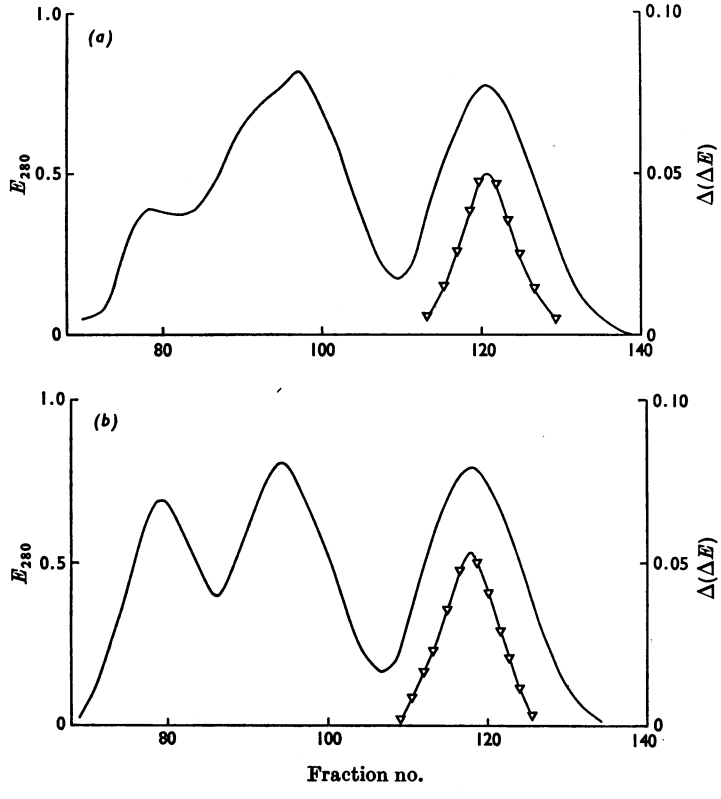


Fig. 2. Elution diagrams of Sephadex G-200 chromatography of (*a*) concentrated active solution from DEAE-cellulose chromatography and (*b*) concentrated active solution from partially-altered-brushite chromatography. E_{280} (——) was determined in 5mm cuvettes and photoreversibility (▽—▽) in 10mm cuvettes.

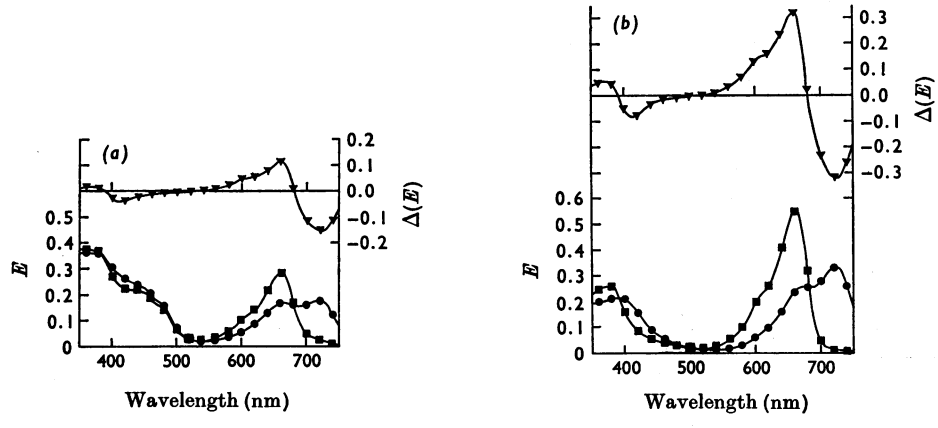


Fig. 3. P_r (■), P_{fr} (●) and difference spectra (▼) of phytochrome solutions after (*a*) DEAE-cellulose chromatography and Sephadex G-200 chromatography, (*b*) calcium phosphate chromatography and Sephadex G-200 chromatography. The difference spectra were obtained with the 645nm irradiated sample in the reference beam.

Table 1. *Recoveries of photoactive material from DEAE-cellulose and brushite purification procedures*

The results for two similar experiments are presented. In each column (*a*) are the values for the DEAE-cellulose half of the experiment; (*b*) are the values for the calcium phosphate half of the experiment. $\Delta(\Delta E) = [(E_{660}-E_{725})_{730\ \text{irrad.}}-(E_{660}-E_{725})_{645\ \text{irrad.}}]/\text{cm}^{-1}$. Photoreversible units are $\Delta(\Delta E)/\text{cm}^{-1} \times$ vol. (ml) and therefore have the dimensions $\Delta(\Delta E)\cdot\text{cm}^2$.

Purification step	Vol. (ml)		$\Delta(\Delta E)$		Photo-reversible units		Recovery (% of total)		Recovery (% of previous step	
	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)
Concentrated or crude extract	600	600	0.04	0.04	24	24	100	100	—	—
	550	550	0.037	0.037	20.4	20.4	100	100	—	—
After Sephadex G-100 chromatography	1160	1180	0.014	0.017	16.2	20.1	67.7	83.9	67.7	83.9
	1200	1200	0.012	0.013	14.4	15.3	70.8	75.2	70.8	75.2
After DEAE-cellulose or $Ca_3(PO_4)_2$ chromatography	22	25	0.24	0.28	5.28	7.05	32.5	35.0	22.0	29.4
	25	30	0.41	0.37	10.1	11.7	49.8	57.5	70.3	76.4
After Sephadex G-200 chromatography	8	8	0.18	0.32	1.43	2.58	5.97	10.8	27.1	37.5
	8	8	0.21	0.32	1.67	2.54	8.21	12.5	16.5	21.7

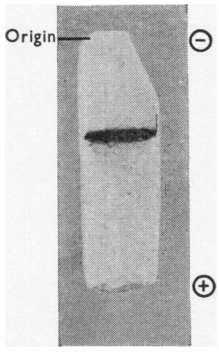


Fig. 4. Segment cut from preparative polyacrylamide-gel column and stained with Amido Black. The sample loaded was concentrated active solution after partially altered brushite and Sephadex G-200 chromatography.

peptides but the core lost its photoactivity. All photoactivity was lost from native phytochrome after digestion with pronase and initial studies with this enzyme were not continued.

Amino acid analysis of the photoactive peptide core was carried out on samples that had been recovered from Sephadex G-200 chromatography and then dialysed exhaustively against glass-distilled water before being freeze-dried. Amino acid analyses of phytochrome and the tryptic core were carried out under the same conditions. The results of the two sets of analyses were compared after correcting each set with a recovery constant calculated from the combined percentage recoveries of aspartic acid and glutamic acid. The oxidized samples showed abnormally high values for aspartic acid and glutamic acid, possibly due to oxidation products of the chromophore; the results for the oxidized samples were therefore corrected to the mean value obtained for leucine in the remaining four analyses of each set. The method suggested by Thornber & Olson (1968) was then used to determine the minimum number of amino acid residues present in the phytochrome molecule and its tryptic core. The results for the analyses are presented in Tables 3 and 4. The minimum molecular weights based on these analyses are 26 392 for whole phytochrome and 14 900 for the tryptic core. Molecular weights calculated from the corrected sedimentation coefficients for native phytochrome by using the formulae of Atassi & Gandhi (1965) or Halsall (1967) varied between 79 000 and 127 000 suggesting that phytochrome might be a trimer or tetramer of the minimum unit as calculated from the amino acid analysis.

Pea phytochrome. Electrophoretically pure pea phytochrome solutions were prepared as described above and showed similar spectral properties to oat phytochrome purified by the same procedure. The peak extinctions of the P_r and P_{fr} forms of pea phytochrome are compared with those of oat phytochrome in Table 5. Although the wavelengths of peak extinction of the P_{fr} forms are very similar, that of the P_r form of pea phytochrome is 4–6 nm nearer the red end of the spectrum than that of oat phytochrome. This has a corresponding effect on the difference spectrum in this region. In addition, the hypsochromic shift in the P_r form that occurs during the final purification step is less in pea phytochrome than in oat phytochrome.

Amino acid analysis was carried out on purified pea phytochrome by using the same procedures as in the analysis of oat phytochrome. The same method of processing the results was also used, in

Table 2. *Corrected sedimentation coefficients of electrophoretically pure phytochrome determined at various* pH *values*

The sedimentation runs were carried out at temperatures between 3°C and 4°C. The rotor speed in each of these runs was 59780 rev./min.

	Phosphate buffer		Tricine buffer			
	pH7.0	pH7.5	pH7.5	pH8.0	pH8.5	pH9.0
Phytochrome purified by brushite chromatography	4.93	—	5.71	6.06	6.16	6.15
Phytochrome purified by DEAE-cellulose chromatography	—	5.23	—	—	6.44	—

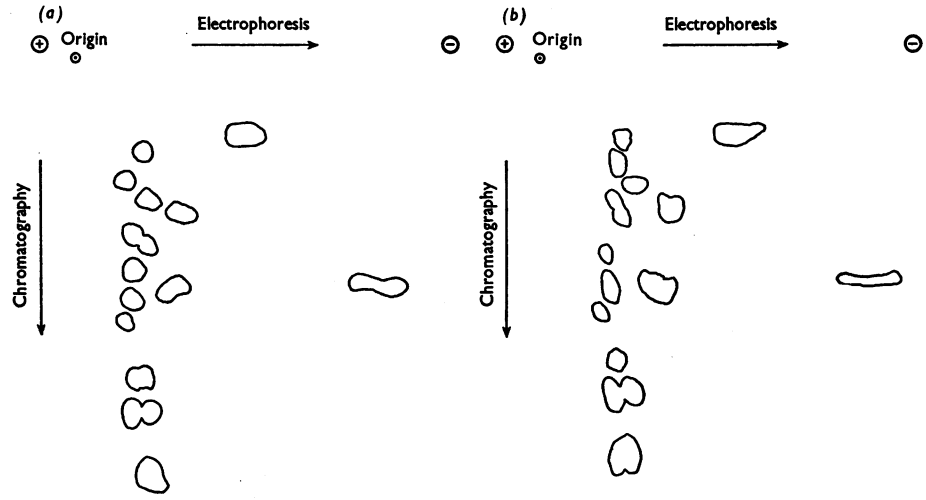


Fig. 5. Tracings taken from peptide 'maps' produced from the tryptic digests of electrophoretically pure phytochrome at (*a*) 37°C and (*b*) 4°C. Electrophoresis was carried out at 5kV at 70mA for 25min in 2M-formic acid–acetic acid, pH2.0. Chromatography was then carried out for 23h in butan-1-ol–acetic acid–water (3:1:1, by vol.).

that the results were first corrected by using a factor based on the percentage recoveries of aspartic acid and glutamic acid. The least number of residues was calculated as for the oat phytochrome analyses and these results are presented in Table 6. Smaller quantities of pea phytochrome were available from any one extraction so that it was possible to carry out hydrolyses at only three time-intervals in addition to the 24h hydrolysis of an oxidized sample.

Sedimentation-coefficient runs were carried out on pea phytochrome concentrated after Sephadex G-200 chromatography. A tracing from a run at 8°C carried out in 0.1M-tris-acetate buffer, pH8.4, is shown in Fig. 6. As in some preparations of oat phytochrome (Walker & Bailey, 1970) two components again sedimented although the preparation had run as a single component in Sephadex G-200 chromatography. The sedimentation coefficients calculated from this run were $s_{20,w}$ 6.24 and 11.0S. Molecular weights calculated from these coefficients varied from between 112790 and 152850 for the slower sedimenting component and between 264710 and 358730 for the faster sedimenting component.

DISCUSSION

In the preceding paper (Walker & Bailey, 1970) we indicated that purification of phytochrome by the use of DEAE-cellulose might be responsible for a denaturing effect, as judged by its spectral properties. The results we present in this paper confirm this observation; the results do not, however, explain the differences we previously observed both in sedimentation coefficients and electrophoretic mobility for the two photoactive proteins that we were able to separate. The method of isolation affected the spectral properties of phyto-

Table 3. *Amino acid composition of oat phytochrome*

The mean values for threonine and serine were obtained from the zero-time extrapolation of the plot of recoveries against reaction time. The mean value for valine was the mean of the last two analyses (48 h and 72 h). The value for methionine was calculated from the recovery of methionine sulphone in the oxidized sample. The value of cysteic acid obtained in the oxidized sample was taken as the basis for number of residues of cysteine used in the molecular-weight calculations.

	Amount (μmol of amino acid)						Proportions corrected to 4 methionine residues	Nearest integer
Amino acid	12 h	24 h	48 h	72 h	Oxidized 24 h	Mean		
Cysteic acid	—	0.0053	0.0190	0.0194	0.0301	0.0301	3.80	4
Asp	0.1700	0.1810	0.1822	0.1782	0.2156	0.1803	22.74	23
Thr	0.0679	0.0317	0.0183	0.0147	0.0819	0.1200	15.13	15
Ser	0.0543	0.0127	0.0040	0.0050	0.1095	0.1115	14.06	14
Glu	0.1765	0.1793	0.1743	0.1840	0.2039	0.1785	22.51	22–23
Pro	0.1086	0.1080	0.0729	0.0775	—	0.0918	11.58	11–12
Gly	0.1697	0.1651	0.1569	0.1627	0.2407	0.1638	20.64	21
Ala	0.1833	0.1884	0.1833	0.1975	0.2324	0.1883	23.74	24
Val	0.1425	0.1493	0.1561	0.1646	0.1604	0.1604	18.97	19
Met	0.0305	0.0381	0.0269	0.00	0.0318	0.0318	4.00	4
Ile	0.0916	0.1142	0.0998	0.1201	0.1003	0.1084	13.67	14
Leu	0.1663	0.1701	0.1672	0.1782	0.1705	0.1705	21.50	21–22
Tyr	0.0509	0.0635	0.0479	0.00	0.00	0.0541	6.82	7
Phe	0.0849	0.0952	0.0856	0.0953	0.0886	0.0903	11.39	11
Lys	0.1222	0.1345	0.1299	0.1239	—	0.1278	16.15	16
His	0.0428	0.0431	0.0420	0.0426	—	0.0426	5.37	5
Arg	0.0825	0.0863	0.0721	0.0658	—	0.0789	9.95	10

Table 4. *Amino acid composition of the tryptic core of phytochrome*

The mean value for threonine was obtained from the zero-time extrapolation of the plot of recoveries against time. The mean values for valine and alanine were the mean of the 48 h and 72 h analyses. The value for methionine was calculated from the recovery of methionine sulphone in the oxidized sample. The value of cysteic acid obtained in the oxidized sample was taken as the basis for the number of cysteine residues used in the molecular-weight calculations.

	Amount (μmol of amino acid)						Proportions corrected to two methionine residues	Nearest integer
Amino acid	12 h	24 h	48 h	72 h	Oxidized 24 h	Mean		
Cysteic acid	—	—	—	—	0.2185	0.2185	3.64	4
Asp	1.0128	1.0104	1.0018	1.0023	1.4815	1.0068	16.75	17
Thr	0.5560	0.5097	0.4617	0.4209	0.6904	0.5885	9.79	10
Ser	0.6026	0.6126	0.7089	0.4756	0.6208	0.5999	9.98	10
Glu	0.9004	0.9025	0.9065	0.9099	1.8750	0.9048	15.05	15
Pro	0.4275	0.4341	0.4647	0.4770	0.3010	0.4508	7.50	7–8
Gly	0.6891	0.7461	0.7840	0.7959	0.8696	0.7900	13.14	13
Ala	0.8727	0.9264	1.0446	1.0155	0.7834	1.0301	17.13	17
Cys	0.0215	0.0293	—	—	—	—	—	—
Val	0.5226	0.5938	0.5785	0.6522	0.5446	0.6154	10.23	10
Met	0.108	0.1117	0.1052	0.119	0.1203	0.1203	2.00	2
Ile	0.3040	0.3541	0.345	0.3751	0.3803	0.3446	5.73	6
Leu	0.6111	0.6235	0.6163	0.6616	0.6281	0.6281	10.45	10–11
Tyr	0.1887	0.1837	0.1688	0.1974	0.0	0.1847	3.07	3
Phe	0.2727	0.2664	0.2542	0.2938	0.2709	0.7218	4.52	4–5
Lys	0.3931	0.5343	0.4041	0.4755	—	0.4518	7.51	7–8
His	0.1403	0.1301	0.1314	0.1493	—	0.1378	2.29	2
Arg	0.2051	0.1601	0.1501	0.2525	—	0.1940	3.22	3

Table 5. *Comparison of the wavelengths of peak absorption of oat phytochrome and pea phytochrome*

		Wavelength (nm)		
		P_r form	P_{fr} form	Difference peak
Oat phytochrome	Before Sephadex G-200 chromatography	662	666–668, 722	660
	After Sephadex G-200 chromatography	658	667–668, 721	658
Pea phytochrome	Before Sephadex G-200 chromatography	666	669–670, 722	664
	After Sephadex G-200 chromatography	664	667–668, 722	662

Table 6. *Amino acid composition of pea phytochrome*

The mean values for threonine and serine were obtained from the zero-time extrapolation of the plot of the recoveries against reaction time. The value of cysteic acid obtained in the oxidized sample was taken as the basis for the number of residues of cysteine used in the molecular-weight calculations.

	Amount (μmol of amino acid)					Proportions corrected to lowest common factor	Nearest integer
Amino acid	12h	24h	60h	Oxidized 24h	Mean		
Cysteic acid	0.0096	0.0063	0.0064	0.0146	0.0146	2.17	2
Asp	0.08671	0.0875	0.08711	0.1055	0.08711	12.97	13
Thr	0.0406	0.01875	0.0014	0.0218	0.0625	9.31	9
Ser	0.0289	0.0094	0.0063	0.00728	0.0485	7.22	7
Glu	0.09633	0.09688	0.10201	0.09097	0.09841	14.65	15
Pro	0.05571	0.06563	0.06050	0.06224	0.06061	9.02	9
Gly	0.09635	0.08125	0.09517	0.07641	0.09092	13.54	14
Ala	0.10598	0.09375	0.11232	0.08733	0.10402	15.49	15
Val	0.07708	0.0750	0.07931	0.04730	0.07713	11.48	11
Met	0.01087	0.00912	0.01891	—	0.01273	1.90	2
Ile	0.05203	0.050	0.03486	0.0473	0.04563	6.79	7
Leu	0.08864	0.09063	0.08272	0.08733	0.08733	13.00	13
Tyr	0.00588	0.00625	0.00	0.00325	0.00607	0.90	1
Phe	0.0308	0.0375	0.03396	0.02183	0.03409	5.08	5
Lys	0.06744	0.05313	0.04692	—	0.05583	8.31	8
His	0.01734	0.02188	0.02169	—	0.0203	3.02	3
Arg	0.0289	0.02758	0.02621	—	0.02755	4.41	4

chrome but did not apparently alter the electrophoretic mobility or the sedimentation coefficient.

Studies on the electrophoretic mobility of the protein indicated that with decreasing pH an increasing number of polymers appeared. This behaviour was not repeated in the ultracentrifuge and may have been an artifact produced in electrophoresis. The values that we obtained for the sedimentation coefficient of phytochrome were significantly different from those reported by other workers. The value we observed most frequently was about 6S. We obtained a similar value for both pea phytochrome and oat phytochrome, although the former also gave a value of 11.02S. Attempts to reproduce the values of approximately 2S that we had previously noted (Walker & Bailey, 1970) were not successful. Correll, Steer, Edwards, Suriano & Shropshire (1966) recorded a value of 9.5S for phytochrome extracted from rye seedlings and a subunit obtained from this preparation by treatment with urea or sodium dodecyl sulphate had a value of 1.9S. Siegelman & Firer (1964), in contrast with both of these results, reported a value of 4.5S which may not have been a very accurate value for phytochrome because of the presence of other proteins in the analysed solution.

Significant differences exist in the amino acid composition of phytochrome presented by Mumford & Jenner (1966) and those found by us. To compare all of these results we have corrected each analysis to the number of residues obtained by us for aspartic acid in the analysis of oat phytochrome; these comparisons appear in Table 7. Disregarding the values obtained by us for threonine and serine in the 'green' and the 'blue' proteins since these are based only on 24h analyses and not zero-time concentration plots, the most noticeable features are the much lower values obtained by Mumford & Jenner (1966) for threonine, glycine, alanine, valine, methionine, isoleucine and lysine in com-

parison with the values obtained in all our analyses. The most striking differences between our full analyses for oat and pea phytochrome were the higher value obtained for proline and the much lower value obtained for tyrosine in the analysis of pea phytochrome. A low value for tyrosine was also observed in the previous analyses of oat phytochrome reported by us (Walker & Bailey, 1970) when only trace amounts of tyrosine were observed in the chromatograms.

Tryptic digestion of oat phytochrome at 4°C gave 17 peptides and a photoactive core. This was in direct contrast with the observations made by Correll *et al.* (1966) who had reported 36 trypsin-sensitive bonds and 34–41 tryptic peptides. Our results agree with the observation by Butler, Siegelman & Miller (1964) that tryptic digestion of phytochrome left it denatured but still photoactive. A noteworthy feature of the amino acid analysis of the tryptic core was that it showed that the cysteine residues were not removed from the active part of the protein, in agreement with the observations made by Butler *et al.* (1964), who demonstrated the effect on the photoreversibility and absorption properties of native phytochrome of thiol-reacting agents.

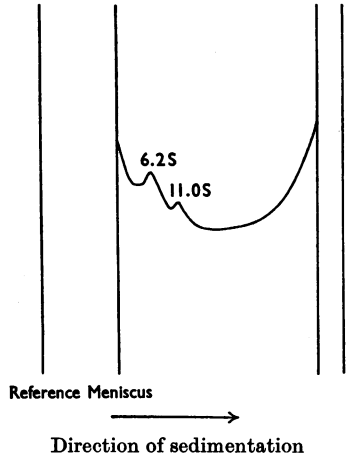


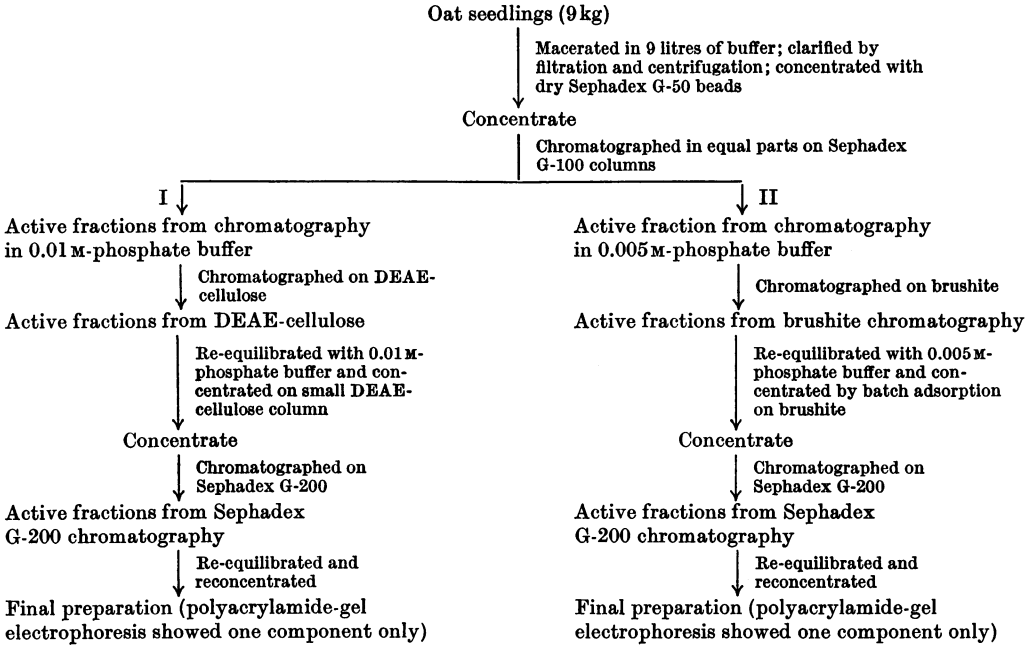
Fig. 6. Diagram of a photograph taken during a sedimentation run in the ultracentrifuge on pea phytochrome after 32 min at 50740 rev./min at 8°C.

The spectral properties of phytochrome in our preparations were not significantly different from those reported by other workers, although we did note small differences between pea phytochrome and oat phytochrome extracted by the same method. We have not, however, been able to support the general observations of others with regard to the persistence of the photoreversibility function in purified or partially purified solutions of phytochrome. Because of the continual loss of photoactivity of our preparations throughout the extraction procedures even under the most favourable conditions, we have made no attempt to define the molecular extinction coefficient of phytochrome as this would obviously bear little relationship to the true value. In our laboratory, active phytochrome was only ever successfully extracted under green light, white light rapidly destroying the photoreversible function. In contrast with others (Siegelman & Firer, 1964;

Table 7. *Comparison of the amino acid compositions of phytochrome from various sources*

All the analyses were corrected to the value of 22.74 that was obtained for aspartic acid in the oat phytochrome analysis presented in Table 2. The number of residues thus obtained was rounded up or down to the nearest whole number.

Amino acid	Pea phytochrome	Oat phytochrome	Green protein	Blue protein	Mumford & Jenner (1966) analysis
Cysteic acid	4	4	—	—	4
Asp	23	23	23	23	23
Thr	16	15	6	6	9
Ser	13	14	3	4	15
Glu	25	23	19	24	21
Pro	16	12	13	14	14
Gly	24	21	21	27	14
Ala	27	24	23	26	19
Val	20	19	20	20	14
Met	3	4	3	2	1
Ile	12	14	13	15	10
Leu	23	22	22	22	21
Tyr	2	7	—	—	7
Phe	9	11	10	9	9
Lys	15	16	16	18	12
His	5	5	6	4	7
Arg	7	10	10	8	11
Mol.wt.	25604	26392	22722	23350	22808



Scheme 1. Abbreviated scheme for the purification of phytochrome by using either brushite and gel-filtration chromatography or DEAE-cellulose and gel-filtration chromatography.

Mumford & Jenner, 1966; Briggs, Zollinger & Platz, 1968) we found that ammonium sulphate precipitation also destroyed the photoreversible function.

The denaturing effect on phytochrome of DEAE-cellulose chromatography must also call into question the conclusions of Taylor & Bonner (1967) who have assumed that the spectra they obtained for phytochrome from *Mesotinium* and *Sphaerocarpus* after purification on DEAE-cellulose represented that for the native phytochrome. In view of our results this assumption was probably incorrect.

The results presented by us in this and the preceding paper (Walker & Bailey, 1970) are significantly different in many respects from those obtained by others working with similar material. Further, the results we obtained with oats grown in different parts of the country were different although the same variety was purchased in each case. These results together with others we have already discussed lead us to conclude that although the primary photoreversible function of phytochrome is the same in all the material studied, the physical and chemical properties of the protein isolated from the various plants may well be different.

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