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ADSORPTION OF PHYTOCHROME ON SEVERAL SUBSTITUTED SEPHAROSE GELS

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Abstract—Phytochrome from *Avena sativa* shows strong adsorption with hydrophobic ligands such as octyl and phenyl Sepharose The same behaviour was observed for undegraded (MW 400 000) and degraded (MW 60 000) phytochromes in the P_r or P_{fr} form as well The pigment is photoreversible after adsorption on those gels Chromatography with amino acid ligands of degraded phytochrome was also tested The chromoprotein showed the same strong adsorption on tryptophyl Sepharose A more specific adsorption could be achieved on histidyl Sepharose but with loss of 70% of photoreversibility This can be interpreted by the accessibility and perturbation of the chromophoric site by the histidyl ligands

INTRODUCTION

Phytochrome, a pigment which regulates vegetative photomorphogenesis, is a chromoprotein containing an open tetraphyrrole group covalently bound via a cysteinyl residue to an apoprotein [1] The nature of its interactions with presumed receptors of the plant cell is ill-defined Several studies indicate the presence of hydrophobic sites which could play a role in these interactions Thus phytochrome was purified by chromatography on adsorbents showing major hydrophobic interactions Cibacron blue F3GA alkyl and ω-amino octyl agarose [2, 3] These hydrophobic properties were confirmed by two different techniques, aqueous two-phase partition and hydrophobic probe labelling [4, 5] Many results indicate that aromatic or heterocyclic amino acid residues of phytochrome as well as the chromophore are likely candidates for these interactions Spectroscopic studies [6, 7] and specific chemical modifications suggest the exposure of Tyr, Trp and His residues [8] However, other studies suggest that the chromophore is on the surface of phytochrome [9–11] Our present knowledge of the phytochrome structure allows not only the applicability of hydrophobic interactions, but also charge-transfer (CT) interactions as well as hydrogen bonding for its absorption properties Proteins with exposed aromatic residues interact by CT and hydrophobic forces with amino acid ligands attached to the insoluble polysaccharide matrix [12] In this report we show the feasibility of amino acids and analogues coupled to Sepharose as a model to study the interactions of phytochrome In this preliminary work, undegraded phytochrome as well as the chromopeptide fragment of MW 60 000 was used Thus, our results can be considered as models of inter- and intramolecular interactions

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RESULTS

The elution behaviour of the partially purified phytochrome preparation on Sepharose 4B gels coupled with different ligands such as octyl, phenyl, resorcyl, tyrosyl, tryptophyl, glycyl, histidyl and zinc histidyl was as follows

Octyl Sepharose 4B

Both degraded and undegraded phytochromes were retained on octyl Sepharose 4B but not on the unsubstituted Sepharose 4B The inclusion of 33% ethylene glycol or 1% Triton in the eluant buffer did not affect desorption of phytochrome from octyl Sepharose

Phenyl Sepharose 4B

This gel, which has a hydrophobicity between those of the straight chain alkyls *n*-butyl and *n*-pentyl, is recommended by the commercial producers for use with proteins which adsorb too strongly to octyl Sepharose However, the phenyl gel may show some specific CT interaction of the π–π-type with phenyl and tyrosyl side groups of proteins We found that desorption was incomplete, but was somewhat enhanced when 50% ethylene glycol was used instead of 33% Quantitative desorption was obtained with 3% Triton The presence of 50% ethylene glycol resulted in an immediate loss of phytochrome activity of *ca* 10%, but had a great effect on stabilization during 24 hr, Triton at 3% did not modify its activity No difference in behaviour was observed between the degraded and undegraded forms of phytochrome

We then investigated desorption by phenylalanine (a presumably weak π–π electron interaction displacer), which would compete by structural similarity to the ligands for the interaction sites with the proteins However, a saturated solution of phenylalanine (0 12 M) did not displace the phytochrome Phytochrome im-

Table 1 Adsorption behaviour and recovery of degraded phytochrome with different gels

Gel	% Recovery of active phytochrome	Relative elution volume (V_e/V_t)
Octyl Sepharose	70 (on the gel)	∞, cannot be desorbed by ethylene glycol or Triton
Phenyl Sepharose	70 (in solution)	∞, can be totally desorbed with Triton
Tryptophyl Sepharose	70 (on the gel)	∞, immobilized on the gel in the active form
Tyrosyl Sepharose	80 (in solution)	2–3
Resorcyl Sepharose	20 (in solution)	∞, but can be partially desorbed by competitive
	70 (on the gel)	elution with tyrosine
Glycyl Sepharose	70 (in solution)	0 96
Histidyl Sepharose	30 (in solution)	1 07
Zinc histidyl Sepharose	30 (in solution)	∞, can in part be desorbed by 1 M NaCl

mobilized on the gel was found to be photoreversible (Table 1) The adsorption maxima and isosbestic points were the same for adsorbed and for free phytochrome Photoreversible activity decreased slightly more in the case of the immobilized phytochrome during successive cycles of photoreversion (16 cycles)

Resorcyl Sepharose

This ligand has a phenyl ring with a hydroxyl in the *meta* position to the other bridge Degraded phytochrome was partially desorbed by a saturated solution of tyrosine

Tyrosyl Sepharose

This matrix did not give irreversible fixation with degraded phytochrome, but did retard elution The retardation is a measure of the strength of the interaction

Tryptophyl Sepharose

Degraded and undegraded phytochromes were so strongly adsorbed on the gel that it could not be eluted even by a saturated solution of tryptophan, 50% ethylene glycol or 3% Triton However, 70% of the initial activity was retained on the gel

Glycyl Sepharose

It is known that even simple amino acids such as glycine may be involved in weak CT interactions with appropriate partners [13] We expected that a more suitable adsorbent might be prepared by the use of glycine In order to study the strength of adsorption as a function of ligand concentration, we prepared gels with different degrees of substitution Surprisingly, all of the glycine-substituted gels gave almost identical chromatograms with degraded phytochrome This is in disagreement with the results obtained with model substances (unpublished work) The experiments of Table 1 were repeated with the gel having the highest concentration of glycine (coupling time 16 hr) with degraded phytochrome Figs 1a, 1b and 1c show the elution patterns obtained with partially purified degraded phytochrome in the presence and absence of sodium chloride (1 or 4 M) A small protein fraction eluted at $V_e/V_t = 0\,4$ whereas the major protein peak at $V_e/V_t = 0\,96$ contained all the phytochrome activity Some nucleoprotein contaminants were retarded slightly more in the presence of sodium chloride However, the protein maximum and activity distribution did not coincide The relative elution volumes were the same for the undegraded or degraded form of phytochrome whether in the P_r or P_{fr} form initially

Histidyl Sepharose

Histidine with its imidazole ring may be a better ligand than glycine for interacting more specifically with the apoprotein and chromophore Figs 2a and 2c show a fraction eluted, non-retarded at $V_e/V_t = 0\,46$, and a protein peak containing the activity eluted at $V_e/V_t = 1\,07$, which contains nucleoproteins as judged from the spectrum Elution in the presence of 1 M sodium chloride (Fig 2b) showed a disappearance of the nonretarded form corresponding to a possible dissociation of the aggregated forms of phytochrome 70% of the activity was lost in solution, as compared with only a 30% loss in the case of glycyl Sepharose The possibility that some phytochrome remained on the gel should be considered

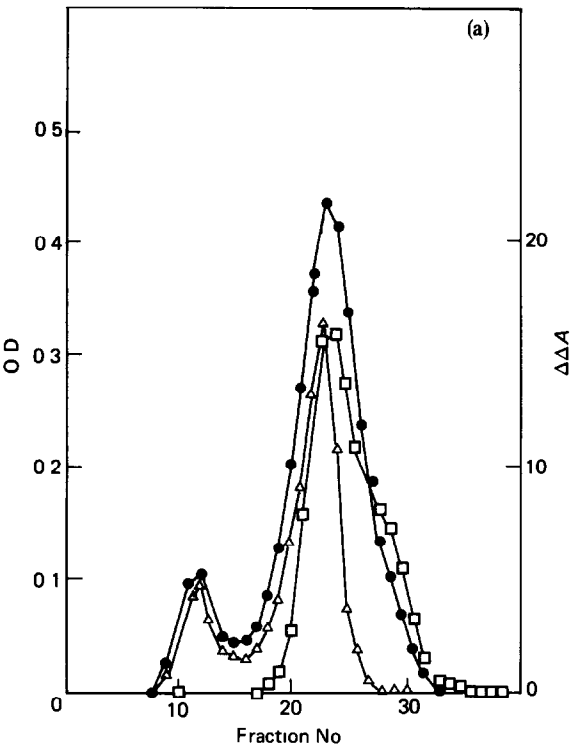
Zinc histidyl Sepharose 4B

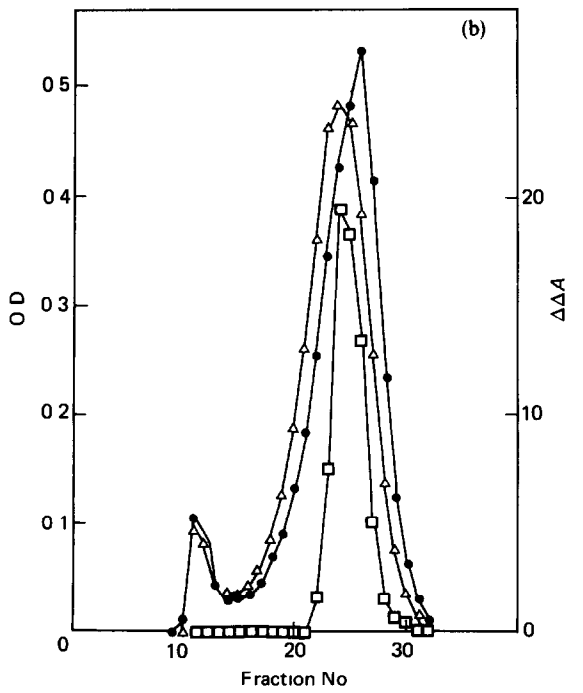
According to the work of Vijayalakshmi [14], CT interactions occurring in histidine-coupled gels are enhanced by the adsorption of Zn^{2+} or Cu^{2+} onto these same gels On the other hand, Lisansky and Galston [15] have shown inactivation of phytochrome by Zn^{2+} in solution We tried to study phytochrome behaviour on histidyl Sepharose gel, first without and then with Zn^{2+} immobilized on the same gel Under our elution conditions, degraded phytochrome was adsorbed to histidyl zinc Sepharose and was partially desorbed by 1 M sodium chloride (Figs 3a and 3b) with an activity loss of 70%, i e the same as in the case of histidyl Sepharose gel However, a higher proportion of ninhydrin-reactive compounds were retained on the zinc-loaded gel

DISCUSSION

The strong adsorption of undegraded and degraded phytochromes to octyl Sepharose indicates the presence of a hydrophobic site Phytochrome thus behaves similarly with these ligands to hydrophobic proteins such as membrane proteins, or tetrapyrrolic proteins such as cytochrome oxidase or haemoglobins This is in agreement with the hydrophobicity found by phytochrome partition in two phases [4], hydrophobic labelling [5] and ω-amino octyl agarose chromatography [3] However, we

did not find any significant difference between the P_r and P_{fr} forms of degraded and undegraded phytochromes for the hydrophobic adsorbents octyl and phenyl Sepharose The hypothesis of irreversible adsorption by multiple fixations of the ligand may be the reason why no difference has been found with octyl and phenyl Sepharose This is in disagreement with ω-amino octyl agarose results, but for this gel the hydrophobic interactions are probably weakened by the presence of the amino group





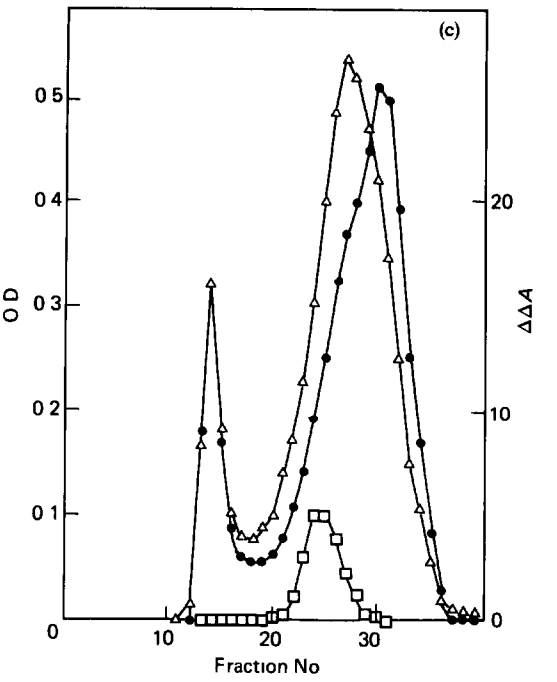


Fig 1 Elution pattern of phytochrome extract (MW = 60 000) on a glycyl Sepharose 4B column (1 x 35 cm) at 4° Sample volume 1 ml, fraction volume 1 08 ml Ethylene morpholine acetate buffer 0 1 M at pH 7 was used with addition of different sodium chloride concentrations (a) Without sodium chloride, (b) with 1 M sodium chloride, (c) with 4 M sodium chloride □, Phytochrome activity, ●, absorbance at 260 nm, △, absorbance at 280 nm

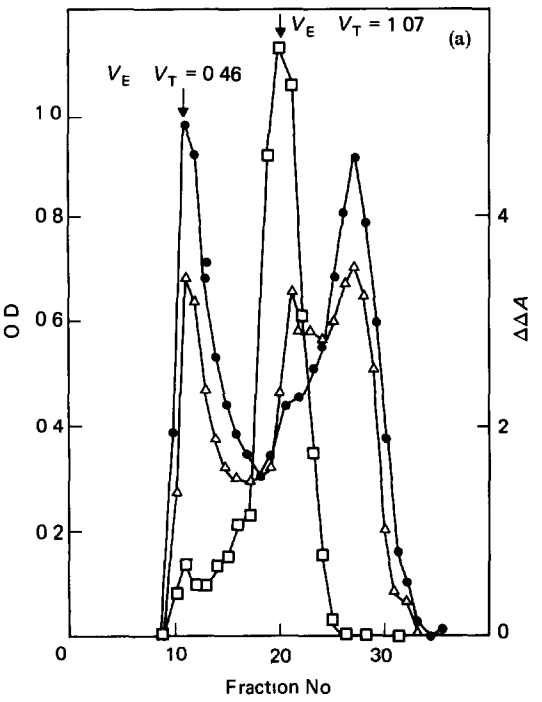
From the similarity in properties of the two preparations, we inferred that the major site responsible for adsorption is located around the chromophoric sites and not in the sections which were removed by proteolysis Therefore, we considered it worthwhile to discuss the interactions of other ligands with degraded phytochrome only The adsorption onto phenyl Sepharose is probably not attributable solely to hydrophobic interactions, but could also be due to 'aromatic stacking' In fact, the adsorption onto the resorcyl, tyrosyl or tryptophyl Sepharose gels, where 'aromatic adsorption' is likely to be stronger, favours the latter interpretation On the other hand, the possibility of direct electron transfer with the chromophore is indicated by the bleaching of phytochrome after hydrophobic labelling with 8-anilinonaphthalene-I-sulfonate [5] Moreover, the chromopeptides from phycocyanin which have a chromophore analogous to phytochrome show interactions with the same gels as those used with phytochrome (in preparation)

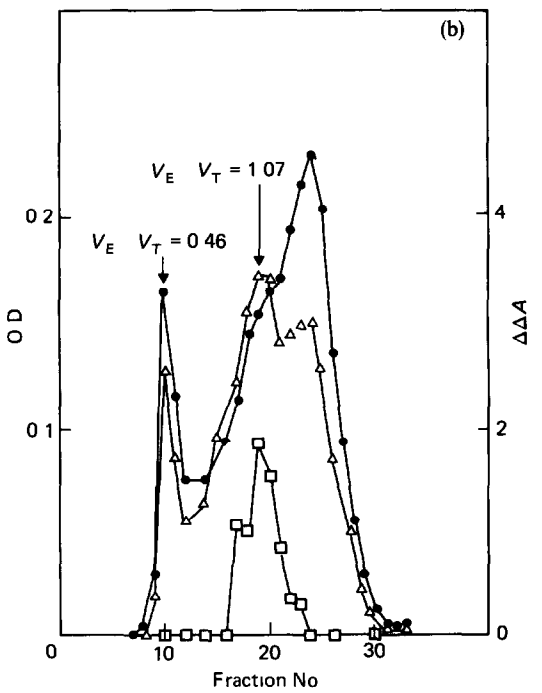
Other interactions of *n*-type donors are possible in the case of phytochrome In the case of glycine and histidine coupled to Sepharose 4B, such interactions with the free electron pairs on the ligands can contribute to the adsorption The effects in the case of coupled histidine are particularly interesting The involvement of electron coupling seems probable to us in view of the adsorption

differences obtained with and without zinc bound to the histidine ligands Since in the latter case (zinc histidyl) the phytochrome can to some extent be desorbed by raising the ionic strength from 0 1 to above 1 0, charge–charge attraction is likely to be of considerable importance

Although the same 70 % loss of activity was obtained on histidyl Sepharose, two different molecular mechanisms may be involved for each gel We could mimic the loss of phytochrome activity on histidyl Sepharose by the bleaching of phycocyanin chromopeptides on this gel, on zinc iminodiacetate Sepharose or with zinc in solution (unpublished results) Thus, the loss of phytochrome activity on zinc histidyl Sepharose can be explained by the action of zinc alone in a similar way that is reported for phytochrome in the presence of soluble zinc [15]

However, a more specific interaction of histidyl with the chromophoric site cannot be excluded for the histidyl Sepharose gel The chromopeptides (obtained by digestion with pepsin) of phycocyanin or phycoerythrin can be separated on histidyl Sepharose without bleaching by differential retention of the chromophore containing peptides (unpublished results) In the same way, synthetic peptides containing histidyl or tyrosyl residues are delayed on the basis of CT interaction [14] On the contrary, octyl Sepharose or DEAE-Sepharose give poor separation with the same chromopeptides We think that histidyl Sepharose may form CT interactions with the chromophore, the residues of histidyl and to a lesser extent tyrosyl of biliproteins The loss of phytochrome activity in the case of histidyl Sepharose might be interpreted by the global perturbation of the intramolecular interactions of the chromophoric site on adsorption of phytochrome on the histidyl ligand The histidyl ligand might produce secondary effects in the chromophoric site even in regions different from the site





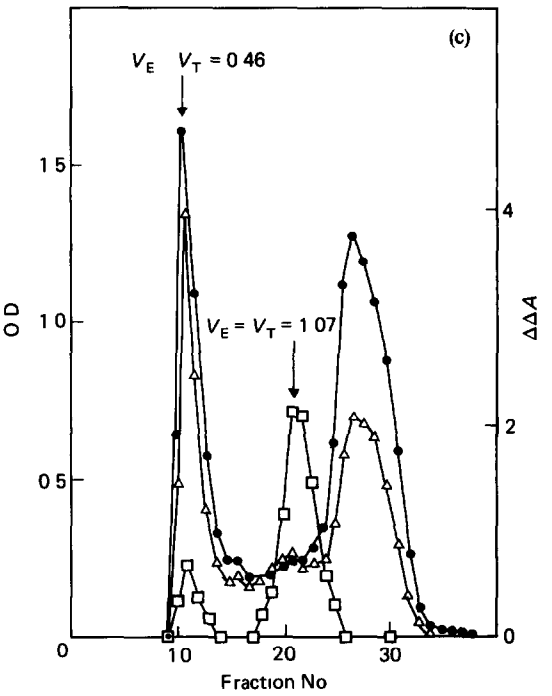


Fig 2 Elution pattern of phytochrome extract (MW = 60 000) on a histidyl Sepharose 4B column (1 × 31 cm) at 4° Sample volume 1 ml, fraction volume 1 01 ml □, Phytochrome activity, ●, absorbance at 260 nm, △, absorbance at 280 nm (a) Elution of the P_r form with 0 1 M ethylene morpholine acetate buffer at pH 7 without sodium chloride, (b) elution of the P_r form with 0 1 M ethylene morpholine acetate buffer at pH 7, 1 0 M sodium chloride, (c) elution of the P_{fr} form with 0 1 M ethylene morpholine acetate buffer at pH 7 without sodium chloride

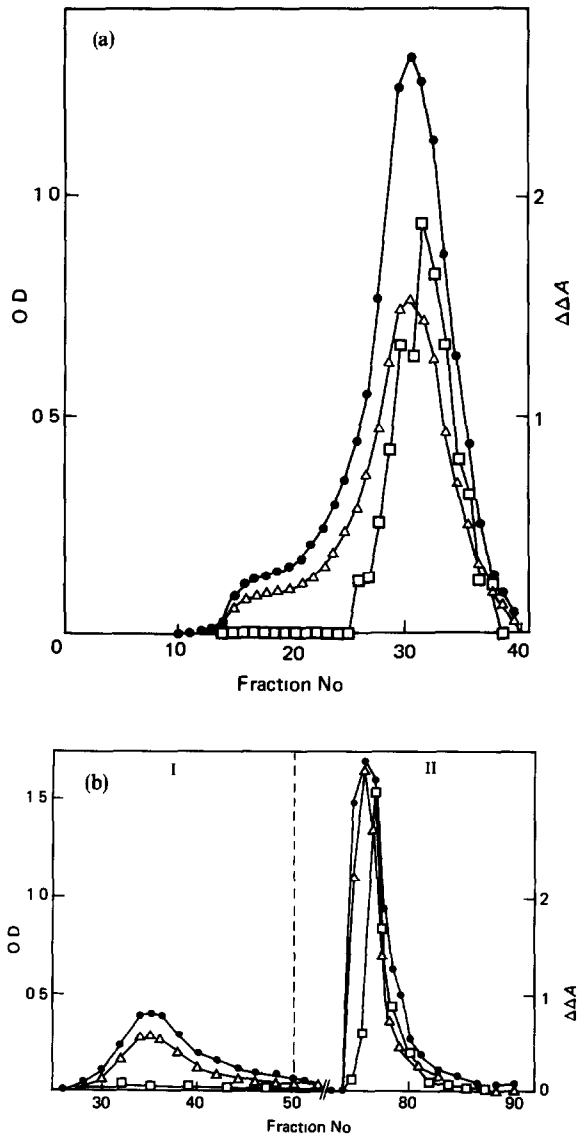


Fig 3 Elution pattern of phytochrome extract (MW = 60 000) on a zinc histidyl Sepharose 4B column (1 × 38 5 cm) at 4° □, Phytochrome activity, ●, absorbance at 260 nm, △, absorbance at 280 nm (a) Ethylene morpholine acetate buffer, 0 1 M, pH 7, 1 M sodium chloride Sample 1 ml extract, fraction volume 1 01 ml (b) Buffer I ethylene morpholine acetate, 0 1 M, pH 7 Buffer II desorption buffer ethylene morpholine acetate, 0 1 M, pH 7, with 1 M sodium chloride Sample 3 ml extract, fraction volume 1 01 ml

of fixation of the ligand This is not contradictory to the results showing the accessibility of a histidyl and two tyrosyls at the level of the phytochrome chromophore [8] Although in that case only the modification of tyrosyl but not that of the histidyl caused the loss of activity, histidyl Sepharose might interact with a site different from the histidyl modified by these authors

In this study of different adsorbents, undegraded and degraded phytochromes showed the existence of multiple interaction sites as ionic, hydrophobic and charge-transfer This may account for phytochrome interaction with presumed receptors of the plant cell as well as for many *in vitro* artefacts Our results should be clarified in relation to the specificity of interactions of histidyl with chromophore or the protein part of phytochrome, and to the general interactions of bilin chromophores with the amino acids coupled On the other hand, our work with histidyl, tyrosyl and tryptophyl Sepharose should be repeated with pure, undegraded phytochrome in order to detect modifications of accessibility between the P_r and P_{fr} forms of phytochrome

EXPERIMENTAL

Preparation of phytochrome The phytochrome was extracted from 4-day-old etiolated *Avena sativa* cv Noire de Mayencourt plants according to the scheme shown in Fig 4 All the extractions and chromatography were done at 4° under dim green light [16] Characterization of fractions of undegraded and degraded phytochromes was done by gel filtration on Sephacryl S300 or Sephadex G200 for each extraction The gels were equilibrated with 10 mM Tris (pH 7 8), 100 mM KCl, 1 mM EDTA, 1 mM NaN_3 and calibrated with dextran blue, ferritin, catalase, aldolase, bovine serum albumin, and cytochrome *c* Chromatography on coupled gels was completed within 4 hr of the end of $(NH_4)_2SO_4$ fractionation, while the gel filtration made in parallel was finished in 9 hr The MW was determined from an independent expt by electrophoresis on 7 5% acrylamide SDS gels [17] and after the following additional step of purification Degraded and undegraded phytochromes from $(NH_4)_2SO_4$ fractionation were eluted on Sephacryl S300 The pool of activated fractions was adsorbed on a DEAE-Sephacel column and eluted by a 0 1–0 3 M KCl gradient The phytochrome was concd by 50% $(NH_4)_2SO_4$ precipitation and dialysed against the electrophoresis buffer After electrophoresis the gels were stained by Coomassie blue

Preparation of different adsorbents Sepharose 4B, octyl Sepharose, phenyl Sepharose, DEAE-Sephacel, Sephadex G200 and Sephacryl S300 were all commercial products from Pharmacia Hydroxylapatite-Ultrogel was obtained from IBF (France) Tryptophyl, resorcyl, tyrosyl, histidyl and glycyl ligands were coupled to epoxy-activated Sepharose 4B according to ref [12]

Chromatography Preliminary expts were carried out on small columns (total vol 2 ml) at a flow rate of 10 ml/hr per cm^2 All the elutions were then repeated with standard Pharmacia columns, diameter 1 6 cm, with a bed height of 30 cm at a flow rate of 20 ml/hr per cm^2 The results were expressed in relative elution vol, i e the ratio of the elution vol (V_e) of the substance eluted to the total vol (V_t) of the column bed For octyl and phenyl Sepharose the expts were repeated with the three following buffers, with inclusion of ethylene glycol and Triton X-100 for desorption 160 mM KPi, pH 6 8, 10 mM Tris (pH 7 8)–100 mM KCl–1 mM EDTA–1 mM NaN_3, 100 mM ethylene morpholine acetate, pH 7 For the other coupled gels, only the ethylene morpholine acetate buffer was used Modifications of the buffer composition, such as NaCl addition, are given in the figure legends

Phytochrome photoreversibility measurement The double difference spectra method of ref [18] was employed using a modified spectrophotometer constructed in the Photobiology Laboratory of Rouen (France) [19] Although the samples used were initially optically clear, the method for turbid samples was used A highly diffusing medium ($CaCO_3$ 1 g/ml) was added to the soln before the spectroreversible activity measurement [16] We have found that this technique of saturation with the solid

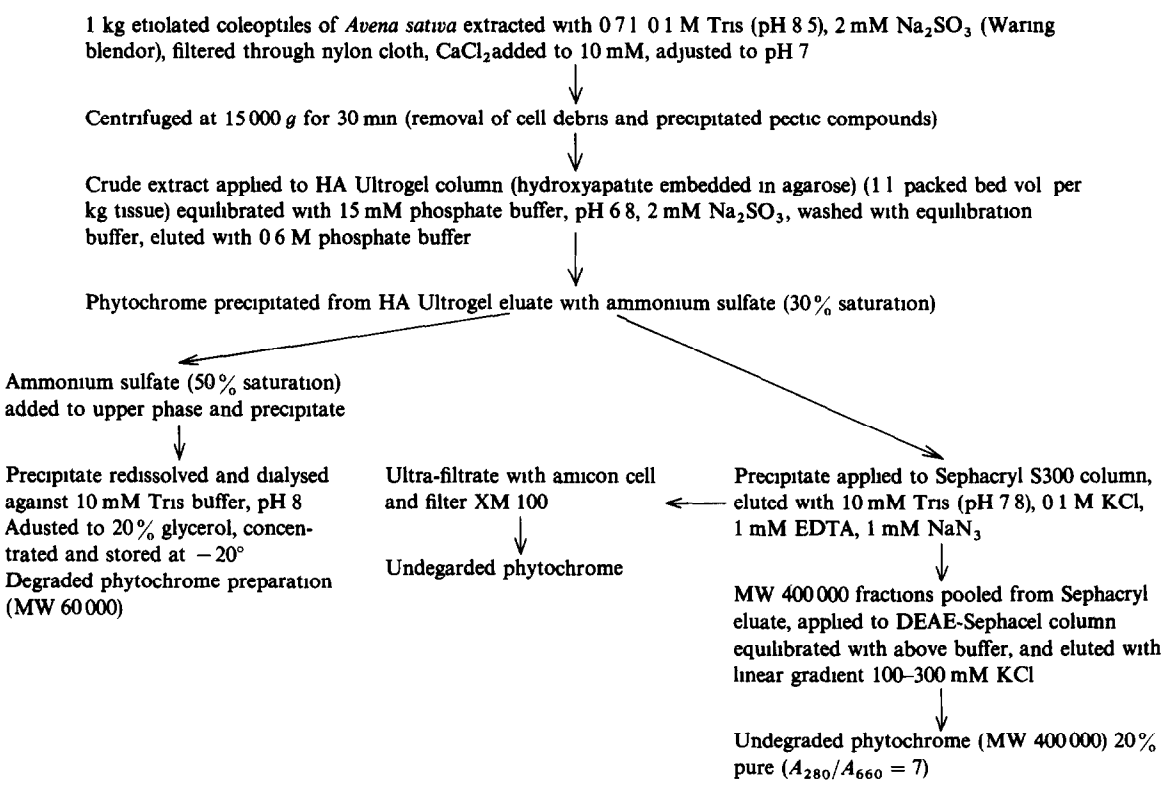


Fig 4 Procedure for partial purification of undegraded and partly degraded forms of phytochrome

phase $CaCO_3$ reduces the effects of chemicals enhancing ($CaCl_2$) [15] or lowering (ethylene glycol) the photoreversible activity in such a way that the variations of measurement for different buffers are within experimental errors To compare these measurements with those obtained with clear samples, the spectrophotometer was calibrated with aliquots using both techniques The results obtained with a scattering medium are thus converted to values corresponding to the measurement with a clear sample of equivalent concn One unit was defined as an absorbance variation of one-thousandth A at 730 nm following two consecutive irradiations at 660 and 730 nm for a clear sample and 0 5 cm light path ($\Delta\Delta A$ in the figures) The wavelength of the reference beam was 805 nm The photoreversibility of phytochrome adsorbed onto different gel matrices was measured by a similar method as follows a suspension of the Sepharose-based adsorbents containing 1 ml buffer per 2 ml gel was allowed to settle in cuvettes of 5 mm path length for 1 hr before measurements Sepharose 4B in suspension was used as a standard for the evaluation of the influence of light dispersion in the samples The precision was the same as for $CaCO_3$ measurements

Irradiation Chromatography of the P_{fr} forms was made both with irradiation of the sample before loading the column, and by chromatography under continuous illumination of the column This was made with a projector equipped with a double-line filter at 660 nm [16]

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