

The Aggregation States of Phytochrome from Etiolated Rye and Oat Seedlings

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DAVID L. CORRELL AND JOHN L. EDWARDS

Radiation Biology Laboratory, Smithsonian Institution, Rockville, Maryland 20852

ABSTRACT

Initial extracts from etiolated plants contained two aggregates of phytochrome. A major fraction was almost excluded by Sephadex G-200 and was within the fractionation range of Sepharose 4 B. A minor fraction was within the fractionation range of Sephadex G-200. Modifications of a previous isolation procedure which allowed retention of this aggregation state are reported. With respect to gel filtration, the major fraction of phytochrome from oat and rye seedlings was identical. The aggregates of rye and oat phytochrome were also separated by diethylaminoethyl cellulose chromatography.

Highly purified phytochrome, isolated from etiolated oat seedlings, has been reported to fractionate on Sephadex G-200 and Bio Gel P-100 in a manner indicative of a molecular weight of 55,000 to 62,000 (4). Partially purified phytochrome from etiolated oat seedlings was also reported to resolve into two fractions on Sephadex G-200. The larger aggregate was almost excluded and the smaller was fractionated. With time and handling the larger aggregate was lost (1). Highly purified phytochrome from etiolated rye seedlings was reported to consist primarily of a tetramer (mol wt 180,000 as determined by high speed equilibrium centrifugation) and a minor component thought to be the hexamer (3). These two components were observed to separate on Sephadex G-200, and the larger aggregate decreased with time and handling (3). The goals of the present study were to (a) determine if any differences existed between the aggregation properties of phytochrome obtained from rye and oat seedlings, (b) determine the principal species of aggregates present in initial buffer extracts, and (c) determine the conditions necessary to stabilize the phytochrome aggregates.

METHODS

Materials. Studies were made with phytochrome from both etiolated winter rye shoots (*Secale cereale* L. cv Balbo) and etiolated spring oat shoots (*Avena sativa* L. cv Clintland). Phytochrome was extracted and purified as reported previously (2) except for the following modifications. It was observed that the most critical factor in stabilizing the large phytochrome aggregates found in crude extracts was the maintenance of a constant pH of 7.8. This was difficult, if not impossible, when concentrated protein solutions were dialyzed against buffer, since most of the proteins in the solutions are polyanions (Gibbs-Donnan effect). Therefore, each dialysis step was replaced with a Sephadex G-50 column pre-equilibrated with 0.01 M potassium phosphate,

pH 7.8, containing 0.1 M 2-mercaptoethanol. A second change was in the method of disrupting the tissue. Instead of grinding with a mortar and pestle in buffer, alumina, and glass, the tissue was quick-frozen with liquid nitrogen, broken by impact with a tamping bar, and thawed to 2° in a constant temperature bath with a measured amount of tris buffer to titre the pH of the suspension to about 7.8. Phytochrome isolated by the two procedures was identical.

Assays. Optical density, 280 nm, was determined with 1-cm cuvettes in a Gilford model 2000 spectrophotometer. All densities above 3 were determined on diluted aliquots. Units of photo-reversible phytochrome were determined with a Ratiospect (Agricultural Specialties Co., Beltsville, Md.) at 730 vs. 800 nm as described previously (2).

Gel Filtration Studies. Crude extracts were concentrated for some experiments by adding dry, coarse mesh Sephadex G-50 to the extracts (10 g/100 ml of extract) (5). The slurry was then poured into a column, and the excluded fraction was eluted with buffer. By a series of three consecutive concentrations of this type a final concentration of about 30-fold was obtained quickly at 2° with little loss of phytochrome photoactivity.

Various samples were tested for their fractionation on Sephadex G-200 columns (5 cm × 45 cm) and Sepharose 4B columns (4 cm × 45 cm), (Pharmacia Fine Chemicals, Piscataway, N. J.). Columns were calibrated by measuring their total volume (V_t) by the elution position of sodium chloride and their void volume (V_0) by the elution position of blue dextran 2000. Sample volumes applied were usually 2 ml/cm² of column cross section. Volumes of elution were always measured from the middle of the sample volume applied to the volume of the center of an eluted peak. Fractionation was then expressed as $(V_s - V_0)/(V_t - V_0) \times 100$ where V_s was the volume of elution of a given fraction from a sample. Sephadex G-200 columns were equilibrated at 2° in 0.01 M potassium phosphate, pH 7.8, which also contained 0.09 M NaCl and 0.1 M 2-mercaptoethanol and were eluted with the same buffer. Sepharose 4B columns were run in the same way except that the NaCl concentration of the buffer used was 0.04 M.

Sedimentation Analysis. Phytochrome samples were layered onto the top of linear 5 to 20%, w/w, sucrose density gradients and centrifuged 8 to 16 hr with a SW-39 rotor in a Spinco model L ultracentrifuge at 39,000 rpm. They were then observed by eye for blue-green bands and eluted through a Beckman 1-cm light path quartz flow cell in a Gilford model 2000 spectrophotometer while recording volume eluted vs. OD at 660 nm or 280 nm.

RESULTS AND DISCUSSION

Quaternary Structure of Phytochrome. The results of several experiments in which crude extracts were concentrated by adding dry Sephadex G-50 and then applied to Sephadex G-200 or to Sepharose 4B columns, indicated that phytochrome from etiolated seedlings existed as two principal aggregates. These clearly separated on Sephadex G-200 (Fig. 1). The larger of these aggre-

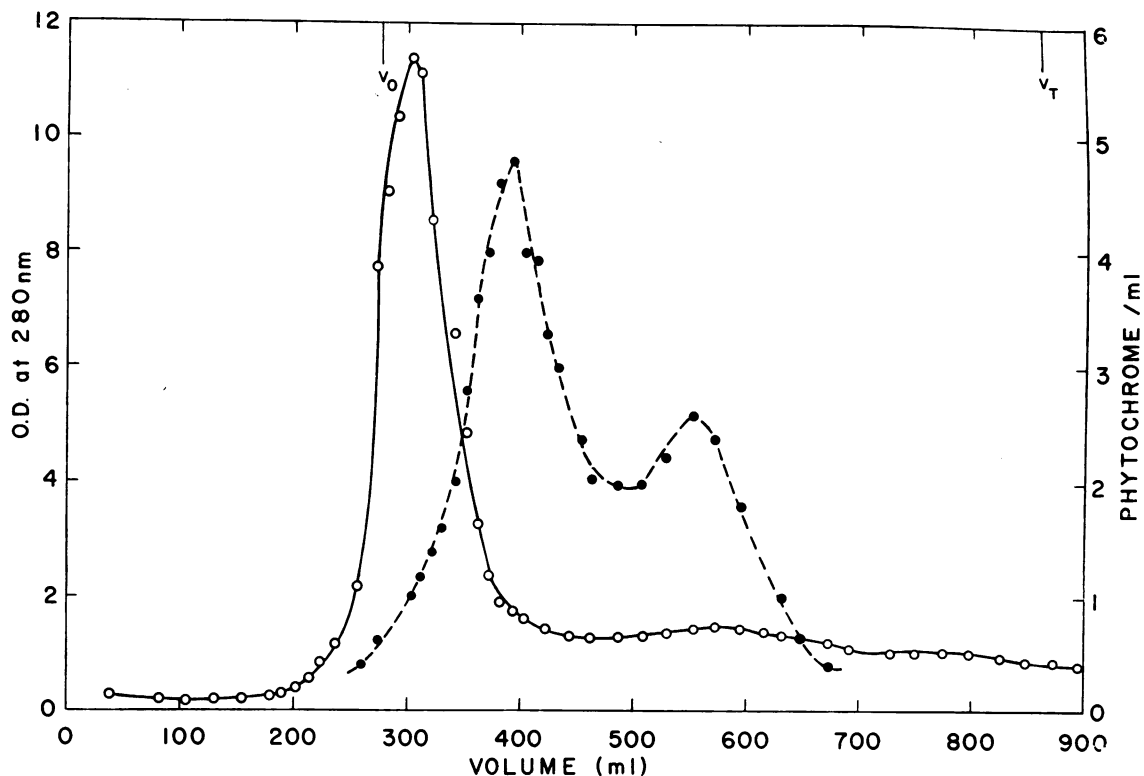


FIG. 1. Sephadex G-200 column (5×45 cm) elution pattern for crude extracts of oat phytochrome after 30-fold concentration with dry Sephadex G-50. Open circles are 280 nm OD readings and closed circles are units of photoreversible phytochrome as assayed by the change in OD at 730 nm when solutions are given saturation 730 nm and 660 nm irradiation alternately.

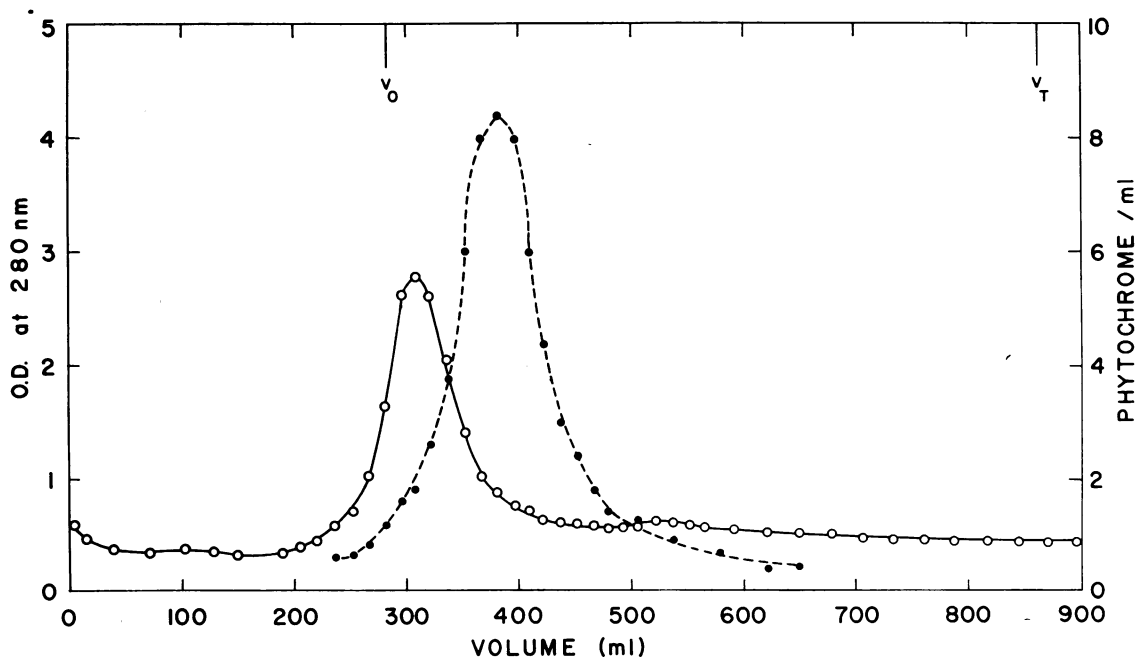


FIG. 2. Sephadex G-200 columns (5×45 cm) elution pattern for oat extracts which have been purified by ammonium sulfate fractionation (0-200 g/l) and subsequent Sephadex G-50 gel filtration. Open circles are 280 nm OD readings; closed circles are units of phytochrome.

gates (more excluded) was consistently the principal fraction (comprised about two-thirds of the total photoactivity). Fractionations (see "Methods" for formula) of the large and small aggregates were $16 \pm 1\%$ and $45 \pm 5\%$, respectively. The large aggregate had a fractionation of about $55 \pm 5\%$ on Sepharose 4B. The separation pattern on Sephadex G-200, after purifications

by ammonium sulfate fractionation and Sephadex G-50 filtration, showed a selective loss of the small aggregate (Fig. 2). The rest of the smaller aggregate could be recovered by using a higher ammonium sulfate level (adding 210 g of dry ammonium sulfate per liter instead of the usual 200 g) in the fractionation. The fractionation of the large aggregate remained the same on G-200

(16%) and on Sepharose 4B (55%) (Fig. 3). After calcium phosphate chromatography these patterns did not change. In the case of oat phytochrome the large aggregate had a fractionation on Sepharose 4B of 56% and for the large aggregate of rye phytochrome the fractionation was 57% (Fig. 4). While investigating

the effects of chromatography on DEAE-cellulose, it was discovered that the two rye phytochrome (Fig. 5) or oat phytochrome (Fig. 6) aggregates could be separated directly with the proper gradient elution. A convex gradient of phosphate buffer resolved the phytochrome into weakly bound (fraction 1) and

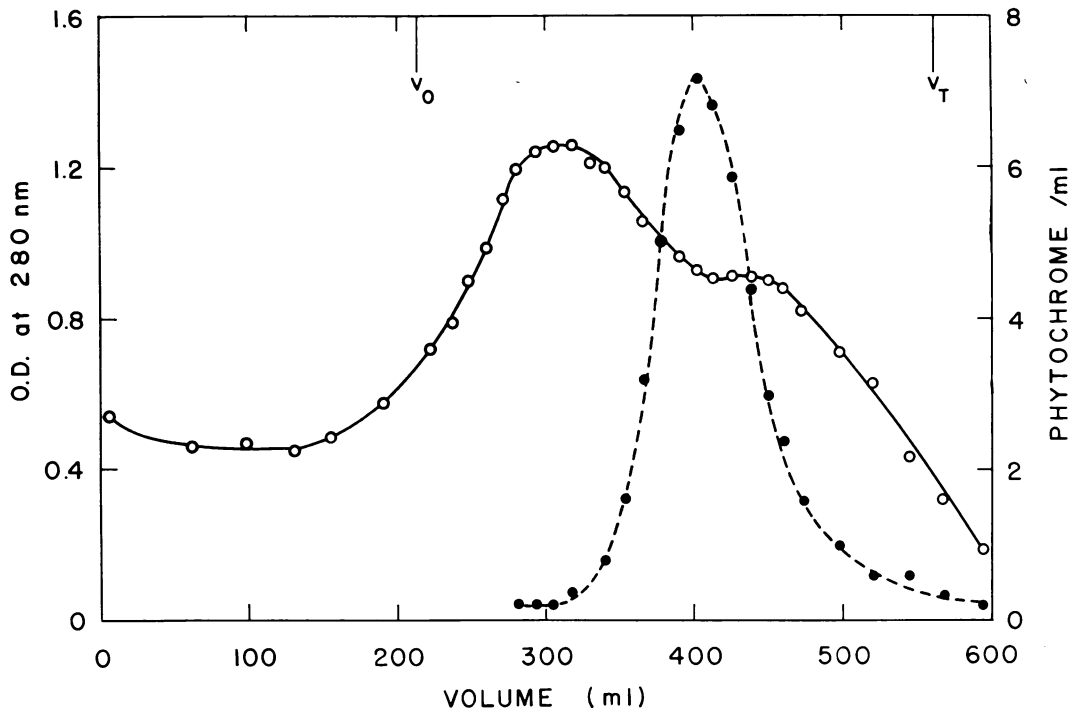


FIG. 3. Sepharose 4B (4×45 cm) column elution pattern for oat extracts prepared as for Figure 2. Open circles are 280 nm OD readings; closed circles are units of phytochrome.

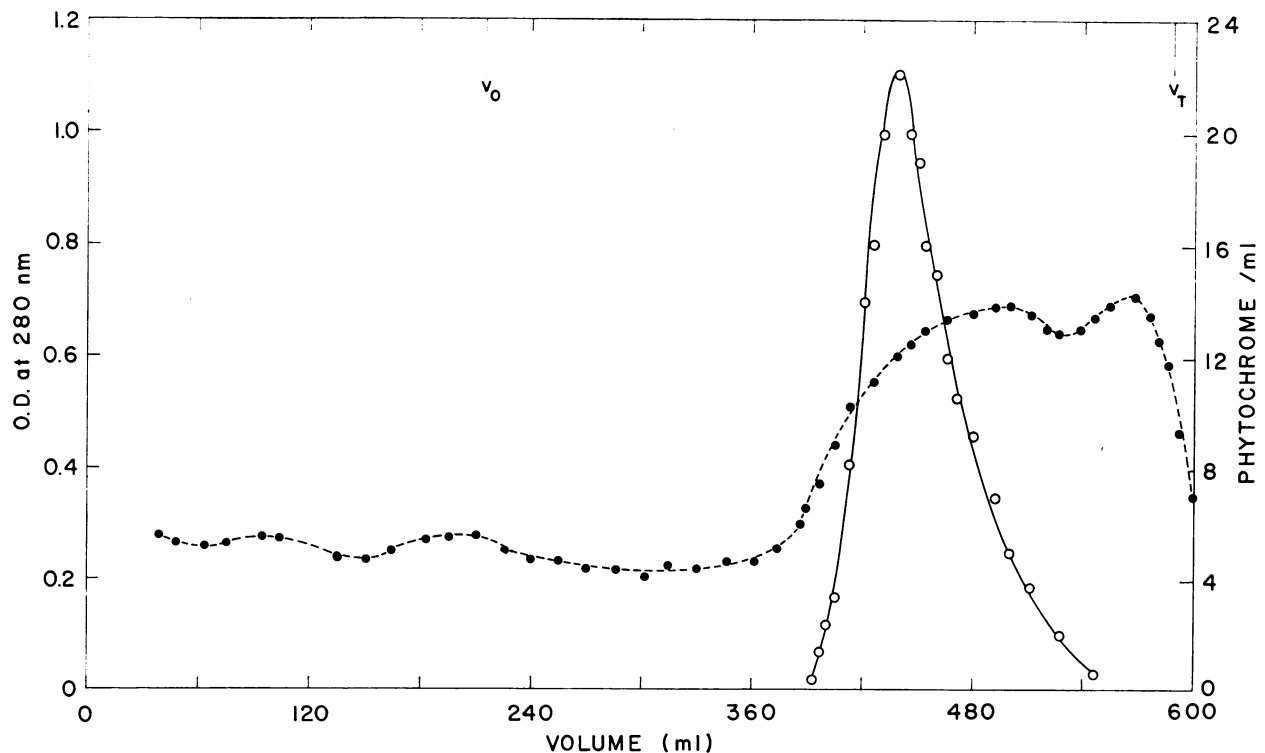


FIG. 4. Sepharose 4B (4×47 cm) column elution pattern for rye extracts which have been purified as for Figure 2 followed by calcium phosphate column chromatography. Closed circles are 280 nm OD readings; open circles are units of phytochrome.

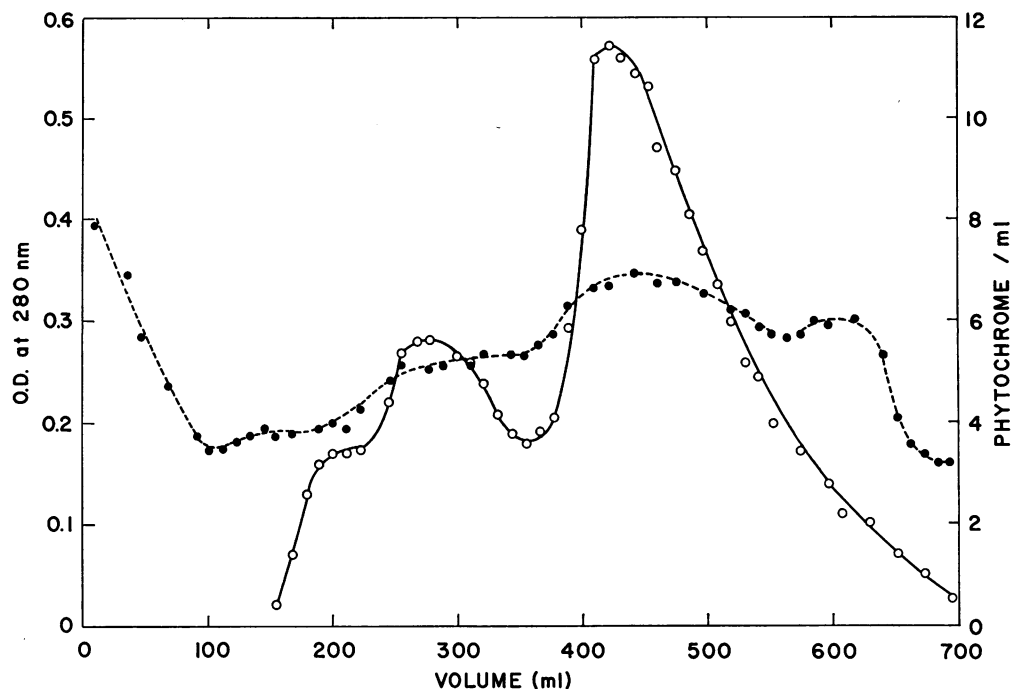


FIG. 5. DEAE-cellulose column (1.2×35 cm) elution pattern for rye phytochrome which had been purified by ammonium sulfate fractionation, Sephadex G-50 gel-filtration, calcium phosphate column chromatography, and a second ammonium sulfate fractionation. The elution gradient was generated by connecting the column to a closed, 300-ml mixing flask initially filled with 0.01 M potassium phosphate, pH 7.8, which contained 0.1 M 2-mercaptoethanol. This flask was then connected to a reservoir of 0.20 M buffer. Closed circles are 280 nm OD readings; open circles are phytochrome readings.

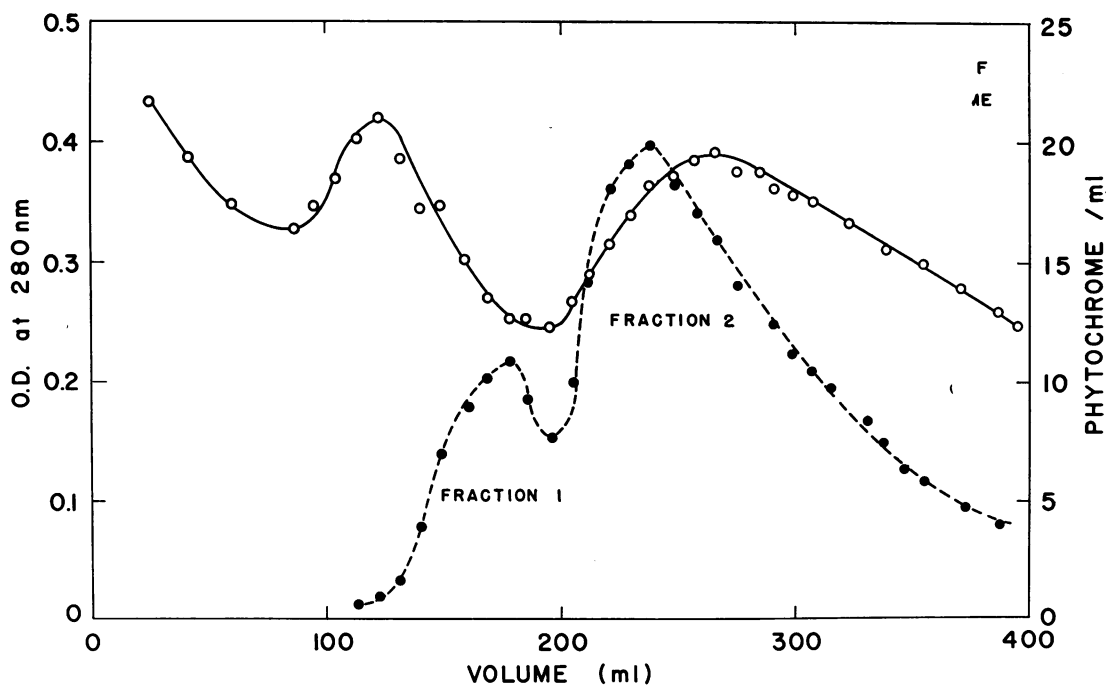


FIG. 6. DEAE-cellulose column (1.2×45 cm) elution pattern for oat phytochrome which has been purified as in the case of Figure 5. The elution gradient was generated as in the case of Figure 5 except that the reservoir contained 0.25 M buffer. Open circles are 280 nm OD readings; closed circles are phytochrome readings.

more strongly bound (fraction 2) components (Fig. 6). Each of these fractions was then passed through a Sephadex G-200 column (Fig. 7). Fraction 1 (weakly bound) was predominantly small aggregate with a fractionation of 46% while fraction 2 (strongly bound) was predominantly large aggregate with a frac-

tionation of 16% (Fig. 7). When dialysis steps were used to remove residual buffers or salts, a shift toward the small aggregate occurred. This is believed to be due to a shift to a lower pH within the dialysis tubing (Gibbs-Donnan effect). Perhaps this factor could explain the low molecular weight phytochrome which was

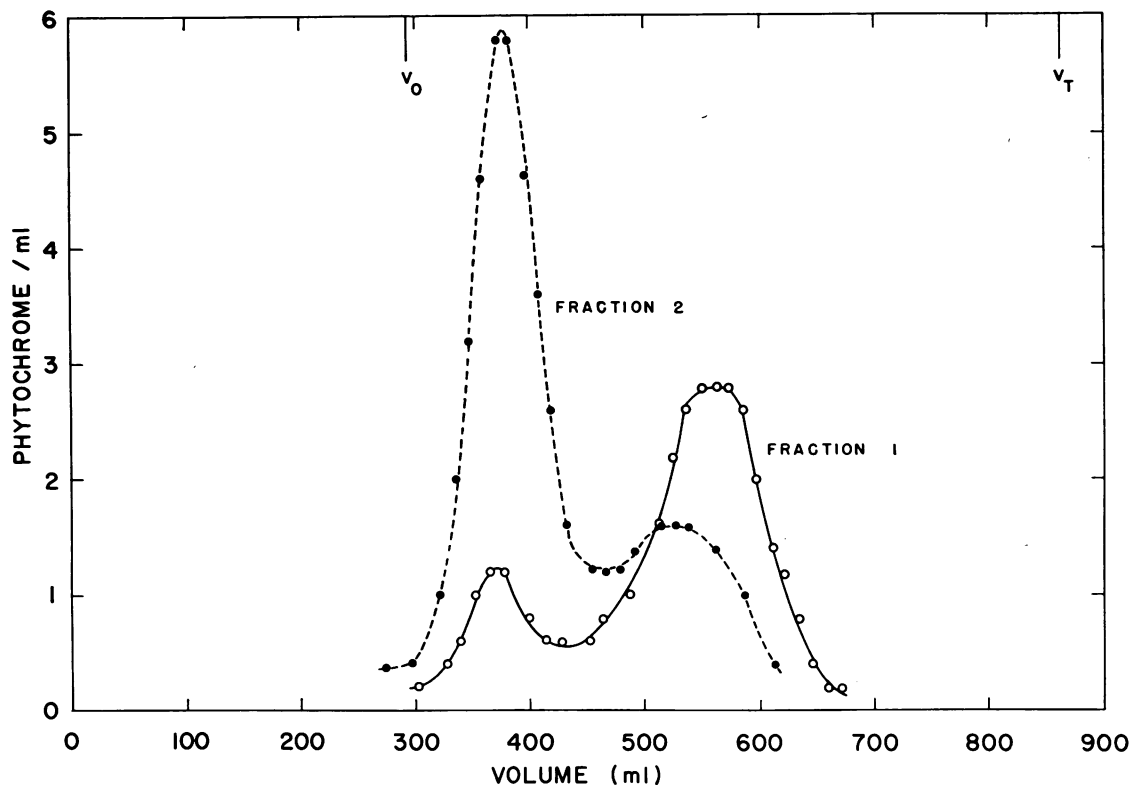


FIG. 7. Sephadex G-200 column (5 × 45 cm) elution patterns for aliquots of fraction 1 and fraction 2 from a DEAE-cellulose chromatogram (Fig. 4).

Table I. Sedimentation of Phytochrome in a Linear (5–20%, w/w) Sucrose Density Gradient at 39,000 rpm in a Spinco SW-39 Rotor

Time	Distances of Bands from Origin	
	280 nm	660 nm
<i>hr</i>	<i>mm</i>	
8	7	13
13.2	11	20
16	12	23

isolated (4). This shift was reduced by desalting with G-50 Sephadex, leaving the large aggregate as the predominant species. It was previously reported (3) as a result of high speed equilibrium centrifugation that the phytochrome protomer has a molecular weight of 42,000. The aggregates found in phytochrome isolated by this general procedure were the tetramer and the hexamer (3). Sedimentation analysis of phytochrome, which had been purified by the revised procedure without any dialysis steps, also indicated that it was composed of only two principal 280 nm-absorbing fractions, both of which contained phytochrome

photoactivity. One fraction (which comprised the bulk of the phytochrome) sedimented at almost twice the rate of the minor fraction (Table I). The presence of two aggregates which fractionate in roughly the same manner on Sephadex G-200 has been reported (1) for extracts of oats which have been purified to various degrees. They also reported a shift with time and handling toward smaller aggregates.

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