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Short Communication

Phytochrome: A Re-examination of the Quaternary Structure[1, 2]

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WILLIAM O. SMITH, JR., AND DAVID L. CORRELL
Smithsonian Radiation Biology Laboratory, Rockville, Maryland 20852

ABSTRACT

Highly purified phytochrome samples from rye (*Secale Cereale* cv. Cougar) were fractionated by ultracentrifugation in isokinetic sucrose density gradients. Three protein species were separated with estimated sedimentation coefficients of 6.5S, 8.0S, and 11.5S. The 6.5S and 8.0S forms contained photoreversible phytochrome and produced a single subunit of 120,000 molecular weight upon reduction and electrophoresis in the presence of sodium dodecyl sulfate. The 11.5S species contained no detectable phytochrome. Reduction and electrophoresis of the 11.5S species in the presence of sodium dodecyl sulfate produced a major polypeptide of 32,000 molecular weight and a minor polypeptide of 48,000 molecular weight. The square tetrameric structures, observed by electron microscopy and previously thought to be phytochrome molecules, were found to be due to the presence of this 11.5S species in phytochrome preparations.

Phytochrome was first isolated from rye seedlings by Correll *et al.* (1). Basing their conclusions on analytical ultracentrifugation studies, they proposed that the purified phytochrome consisted mostly of an aggregate with a sedimentation coefficient of 9S which dissociated to a monomer with a sedimentation coefficient of 2S. The mol wt of this species was estimated to be 42,000 (3). Electron microscopy of phytochrome preparations using negative staining methods produced fields containing square tetrameric structures in the appropriate size range to be consistent with the ultracentrifugal data.

More recently others have purified rye phytochrome (7, 8) and confirmed the sedimentation coefficient of the major species *in vitro* to be about 9S (4); however, on investigation of the monomer mol wt by SDS-polyacrylamide electrophoresis an estimate of 120,000 was obtained (7, 8). A sedimentation coefficient of 9S is consistent with a protein of approximately twice this mol wt so it was inferred that phytochrome existed as a dimer in solution instead of a tetramer.

The purpose of this communication is to demonstrate that the tetrameric structures observed in electron micrographs of phytochrome preparations are, in fact, not phytochrome but another protein present in the extract.

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MATERIALS AND METHODS

Extraction and Purification of Phytochrome. Phytochrome extractions were made from dark-grown seedlings of winter rye (*Secale cereale* cv. Cougar) which was grown and harvested as previously reported (2). The purification procedure was a modification of that reported by Correll *et al.* (1). The details will be published elsewhere (W. O. Smith, Ph.D. thesis, University of Kentucky, Lexington, 1975). In summary it consisted of extraction in tris buffer, ammonium sulfate precipitation (140–210 g/l), brushite adsorption and batch elution, a second brushite column, DEAE cellulose chromatography, gel filtration on a column of 8% agarose (Bio-Gel A-1.5m), and finally, ultracentrifugation in an isokinetic sucrose density gradient. All steps of the purification were carried out in the dark or in dim green light at 2 to 4 C. Mercaptoethanol was included in all the buffers except the final one in which 1 mM dithiothreitol was used.

Ultracentrifugation. Isokinetic sucrose density gradients were formed as described by McCarty *et al.* (6). Twelve-milliliter gradients were prepared from 0.3 to 1.4 M sucrose in 25 mM tris-HCl at pH 7.5 containing 25 mM KCl and 1 mM dithiothreitol. Half-milliliter protein samples were layered on the gradients and overlaid with 0.5 ml of buffer without the sucrose. The tubes were centrifuged in an SW 40 Beckman rotor at 40,000 rpm for 27 hr at 2 C. Gradients were fractionated by puncturing the bottom of the tubes and pumping the gradients out the top of the tubes with a dense chase solution. An Isco density gradient fractionator was used for this purpose. The gradients were continuously scanned at 280 nm and fractions of 0.3 ml collected. The fractions were assayed individually for phytochrome using the Ratiospect as previously described (2). Catalase (11.3S), aldolase (7.5S), and ovalbumin (3.5S) were used as marked proteins for estimation of sedimentation coefficients.

Sodium Dodecyl Sulfate-polyacrylamide Electrophoresis. Proteins were analyzed by the method of Weber *et al.* (9) for subunit mol wt. Samples were incubated for 5 min at 100 C in 1% SDS and 1% 2-mercaptoethanol prior to electrophoresis on 7.5% gels containing 0.1% SDS. Proteins used as mol wt markers were carbonic anhydrase (29,000), rabbit muscle aldolase (40,000), pig heart fumarase (49,000), bovine liver catalase (58,000), BSA (68,000), rabbit muscle phosphorylase-a (100,000), and β-galactosidase (130,000). The migration of the dye marker, bromophenol blue, was marked before staining the gels by insertion of a length of 26-gauge nichrome wire. Gels were stained with Coomassie brilliant blue as described by Weber *et al.* (9) and destained by diffusion.

Electron Microscopy. Samples were prepared for electron microscopy by dialysis against 0.01 M potassium phosphate buffer, pH 7.8. Proteins of the proper dilution were examined

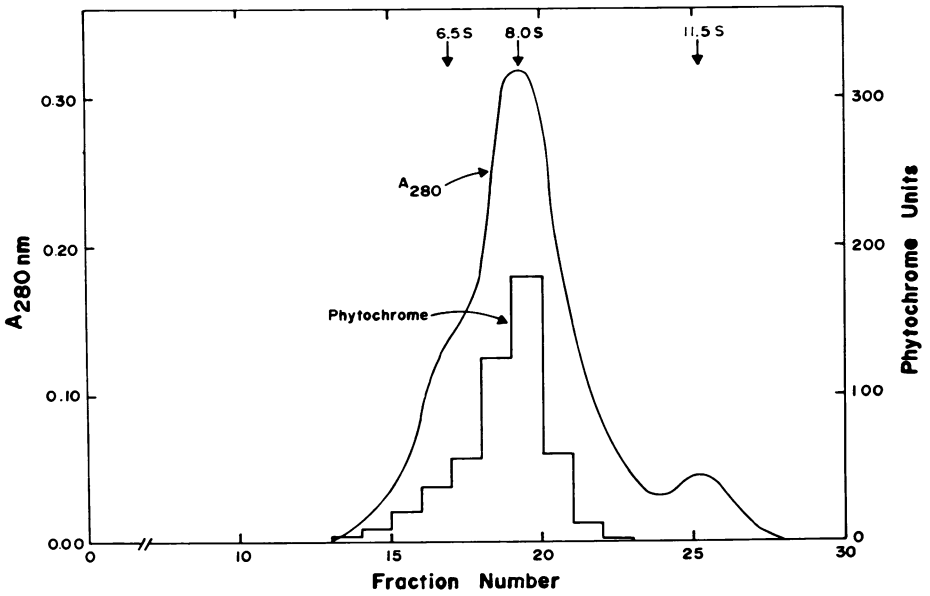

FIG. 1. Isokinetic sucrose density gradient profile of phytochrome purified through the 8% agarose step. Sedimentation coefficients are based on the migration of marker proteins in identical gradients.

by negative contrast techniques using a variety of stains. Phosphotungstic acid and ammonium molybdate were most satisfactory, with the latter being the one of choice for this work. Protein solutions after dilution were mixed 1:1 with 1% ammonium molybdate, pH 6.8, and placed on formvar and carbon-coated copper grids. Excess solution was drawn off with a piece of filter paper and the grid examined in a Phillips EM 300 electron microscope operated at 80 kv.

RESULTS AND DISCUSSION

The density gradient profile of purified phytochrome is shown in Figure 1. There was a major 280 nm-absorbing peak with an estimated sedimentation coefficient of 8.0S with a shoulder at about 6.5S. Another protein sedimented at an estimated 11.5S. The 6.5S species and the 8.0S species were found to be photoreversible phytochrome while no phytochrome spectrophotometric activity could be detected in the 11.5S peak.

SDS-polyacrylamide electrophoresis of protein samples from the three peaks in the sucrose density gradient was performed (Fig. 2). The 6.5S species and the 8S species yielded one major band of an estimated mol wt of 120,000. The 11.5S species produced a major band at about 32,000 with a minor band at about 48,000.

On investigation of phytochrome purified through the 8% agarose step by electron microscopy (Fig. 3a) previous observations (3) were confirmed. Typical fields consisted of amorphous appearing material with square tetrameric structures scattered throughout. The addition of a centrifugation procedure to the purification of phytochrome brought about a complete separation of the amorphous structures and the tetrameric structures. Examination of the three species separated on the sucrose density gradients revealed that the 6.5S and the 8.0S phytochrome species produced no interpretable features (Fig. 3b) while the 11.5S species yielded homogeneous fields of the tetrameric structures (Fig. 3c). This was observed repeatedly on different phytochrome preparations. Phosphotungstic acid, ammonium molybdate, uranyl acetate, and sodium silicotungstate were all used with the same result. The

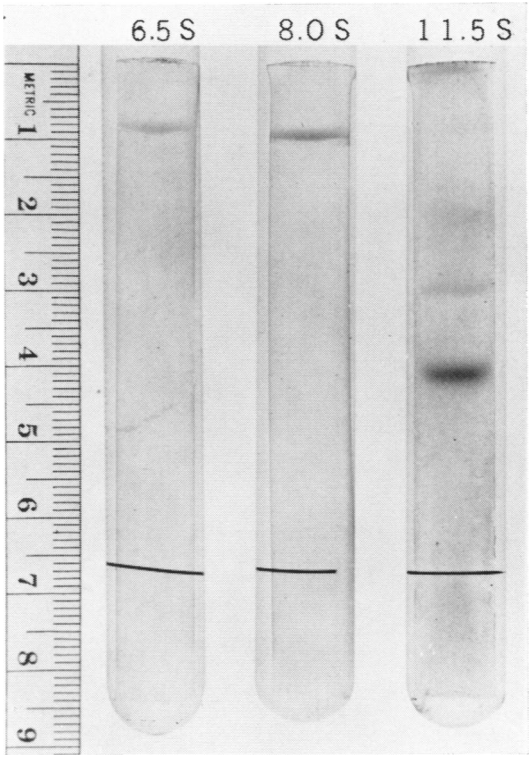

FIG. 2. SDS-polyacrylamide electrophoresis of 6.5S, 8.0S, and 11.5S species on 7.5% gels.

6.5S and 8.0S material appeared amorphous regardless of the protein concentration used or the age of the preparation. In contrast, the 11.5S material appeared quite stable and yielded the tetrameric structures in preparations that had been stored at 4 C for 1 month.

The fact that no recognizable structure could be obtained from the phytochrome species might be interpreted as supportive of phytochrome existing as a dimer *in vitro*. It has been observed (5) that many protein dimers are difficult to visualize

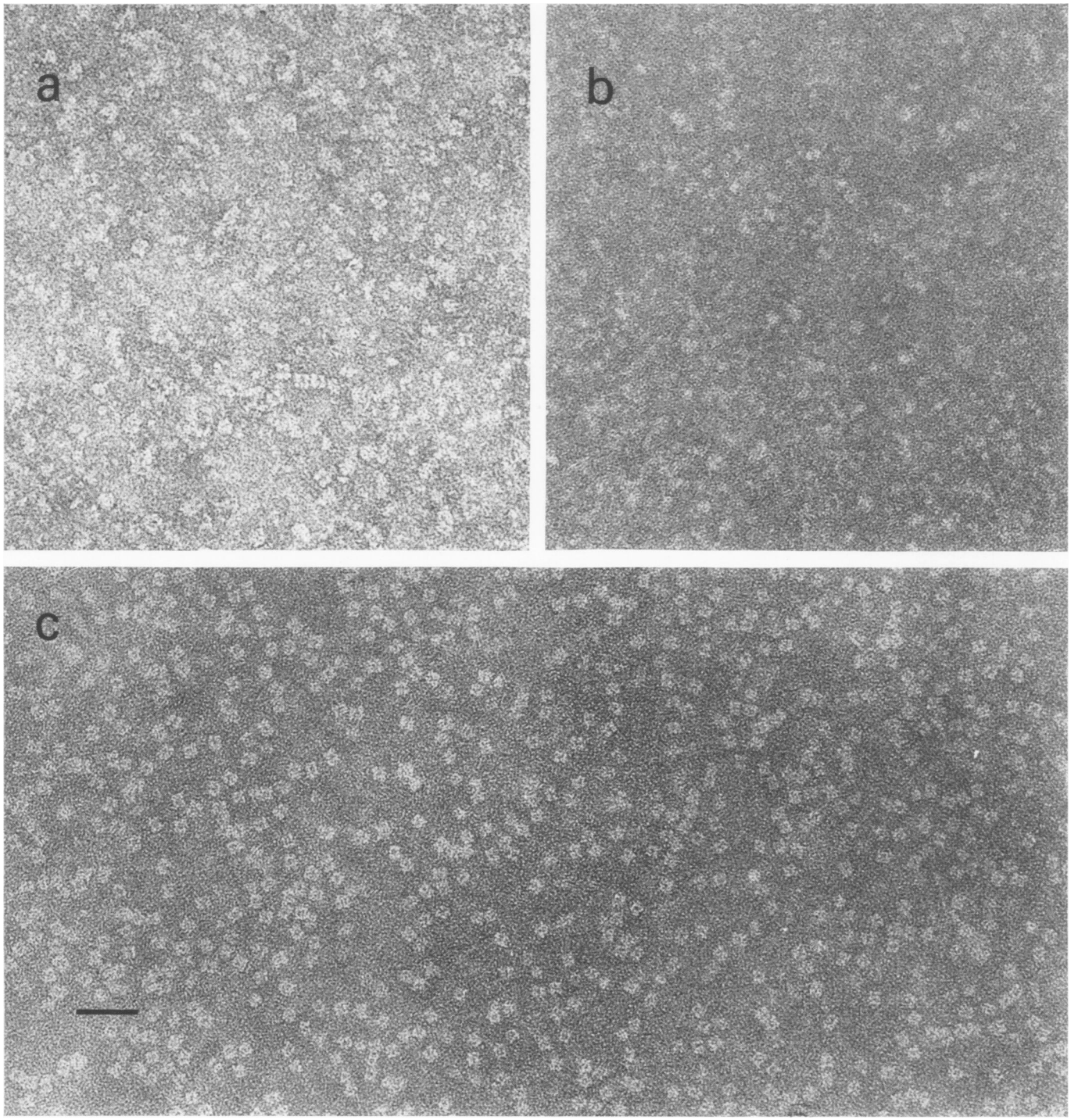

FIG. 3. Electron micrographs of rye phytochrome preparations negatively stained with 0.5% ammonium molybdate. The bar represents 50 nm. a: Phytochrome preparation before ultracentrifugation; b: 8.0S species from sucrose density gradient; c: 11.5S species from the sucrose density gradient.

by negative staining procedures. The 8.0S species observed in this work appears to be the phytochrome dimer, whereas the 6.5S species appears to be the monomer. No aggregation states of phytochrome higher than the 8.0S dimer were observed.

The actual identity of the 11.5S species is not known at this time. Its size and charge properties are similar enough to those of phytochrome to result in its presence in phytochrome preparations that have been extensively purified. In this laboratory the only successful procedure for completely separating this species from phytochrome has been ultracentrifugation.

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