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SUBUNIT STRUCTURE OF HOLOCHROME

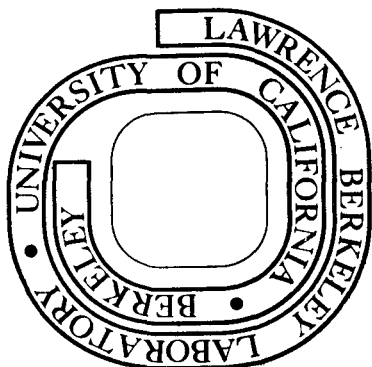
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SUBUNIT STRUCTURE OF HOLOCHROME

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**Analysis of the Subunit Structure of Protochlorophyllide  
Holochrome by SDS Polyacrylamide Gel Electrophoresis<sup>1</sup>**

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<sup>2</sup>Abbreviations:

PCH	Protochlorophyllide holochrome
CH	Chlorophyllide holochrome
RuDP-Case	Ribulose diphosphate carboxylase
SDBS	Sodium dodecyl benzene sulfate
Kd	Kilodaltons
Bis	N,N'-methylene-bis-acrylamide
TEMED	N,N,N',N'-tetramethylethylenediamine



ABSTRACT

The subunit structures of protochlorophyllide - holochrome (PCH) and chlorophyllide - holochrome (CH) were studied by SDS polyacrylamide gel electrophoresis. PCH from leaves of dark grown beans (Phaseolus - vulgaris, var. red kidney) is a polymeric pigment - protein complex of approximately 600,000 daltons. It is composed of 12-14 polypeptides of 45,000 daltons, when examined prior and immediately following photoconversion. The protochlorophyllide or chlorophyllide pigment molecules are associated with these polypeptides. Subsequent to photoconversion, the absorption maximum of newly formed chlorophyllide shifts from 678 nm to 674 nm upon standing in darkness. Following the 678 to 674 spectral shift, the chlorophyllide is associated with a polypeptide with a molecular weight of 16,000 daltons. In addition, sucrose gradient centrifugation of PCH and CH under non-denaturing conditions indicates that during the course of the dark spectroscopic shift, the 600,000 dalton chlorophyllide holochrome undergoes dissociation into a small chlorophyllide protein. The dissociation of the chlorophyllide holochrome, the change in the molecular weight of the chlorophyllide polypeptide from 45,000 to 16,000 daltons as well as the dark spectroscopic shift are temperature dependent and blocked below 0°C. It was also found that each holochrome molecule of 600,000 daltons contains at least four protochlorophyllide pigment molecules.

## INTRODUCTION

The assembly of the photosynthetically-active membrane of chloroplasts is triggered by Chl synthesis. This process takes place when dark-grown seedlings are subjected to illumination. It involves a photoreduction of PChlide to Chlide at a protein site. The photoconversion can be studied using an extracted pigment-protein complex called PChlide-holochrome (PCH). The biochemistry and physiology of PChlide holochrome have been extensively reviewed by Boardman (4), Kirk (16), and more recently by Rebeiz and Castelfranco (22). Bogorad et al. (5) have presented evidence that the 600,000 dalton PChlide-protein complex from dark grown bean leaves undergoes dissociation following photoreduction, thus producing Chlide attached to a small protein.

Henningsen et al. (13) observed the change of a 63,000 dalton molecular weight photoactive Chlide subunit to a 29,000 dalton Chlide subunit following the dark spectroscopic shift in a dark grown barley leaf homogenate.

Guignery et al. (10) demonstrated in Zea mays L. leaves that PChlide is associated with two polypeptide chains of 21,000 and 29,000 daltons. The same study also showed that Chl is associated with four polypeptide chains of 21,000, 25,000, 29,000 and 70,000 daltons.

The exact number of PChlide chromophores per protein macromolecule is still a matter of controversy. Circular dichroism measurements performed on PChlide holochrome suggest a dimer of interacting PChlide molecules as the basic unit (Schultz and Sauer (24), Mathis and Sauer (19)). This spectroscopic result is in agreement with structural information obtained by Schopfer and Siegelman (23), who found at least two molecules of PChlide per 550,000 daltons molecular weight protein. On the other hand, fluorescence spectroscopy studies at  $-196^{\circ}\text{C}$  performed by Kahn et al.

(14) indicate a basic aggregate of at least 4 chromophores per unit. Thorne (27) suggested an aggregate of 20 chromophores. His estimate was based on studies of energy transfer between PChlide and Chlide in etiolated bean leaves at  $-196^{\circ}\text{C}$ .

In this paper we examine the subunit structure of PChlide-holochrome purified from dark grown bean leaves. The fate of the pigment-protein complex after photoreduction and after the completion of the dark spectroscopic shift (25) is studied both on the macromolecular level and the polypeptide level. The results provide further evidence for the dissociation of the nascent Chlide-holochrome. The number of PChlide molecules attached to the protein complex is determined for the most purified PCH preparation.

#### MATERIALS AND METHODS

Preparation of holochrome. Bean seedlings (Phaseolus vulgaris, variety red kidney) were grown on vermiculite  $12 \pm 2$  days in the dark at  $22^{\circ}\text{C}$ . The leaves (50 to 100 g) were harvested and ground in a buffer containing 10 mM Tris-HCl, pH 8.5; 2 mM  $\text{MgSO}_4$ ; 1 mM EDTA and 25% (v/v) glycerol. In some cases, Triton X-100 (in final concentration of 0.06%, v/v) was added to the extracting solution, and is referred to as buffer + Triton. All manipulations were done in a cold room ( $4^{\circ}\text{C}$ ) under a green safe light. Two different methods were used for preparation of PCH. Method 1: The leaves were ground in buffer + Triton in a Waring blender during four 60-sec intervals, separated by 5 min. The homogenate was filtered through 6 layers of cheesecloth and centrifuged at  $78,000 \times g$  in a Beckman (Model L-2) ultracentrifuge (No. 30 rotor) at  $0^{\circ}\text{C}$  for 1 hr. The supernatant was treated with a 50% polyethylene glycol-6000 solution to a final concentration of 17% and centrifuged at  $48,000 \times g$  ( $-2^{\circ}\text{C}$ ) in a Sorvall RC-2B centrifuge for 1 hr. The precipitate was redissolved in buffer + Triton and clarified by centrifugation

at 48,000 x g for 30 min. Before redissolution, the precipitate was stored with a layer of fresh buffer + Triton for about 1 hr, to facilitate the resuspension. The clarified supernatant was adjusted to 0.2 M KCl and applied to an equilibrated hydroxylapatite column (7.5 x 20 cm). The column was washed with 2 to 3 liters of Tris buffer containing 0.2 M KCl. We found that Tris buffer containing 0.1 M potassium phosphate (pH 8.0) was sufficient to elute the PChlide-holochrome. The flow rate was maintained at 10 ml/min. All fractions with an absorption maximum at 639 nm containing PCH were combined. This partially purified pigment-protein complex was used immediately or stored at -20°C. This procedure was similar to the one reported by Schopfer and Siegelman (23). The partially purified holochrome was very stable, and maintained its photoconversion activity for at least a week at 4°C.

Method 2: This procedure was adapted from Akoyunoglou et al. (1). Etiolated leaves were homogenized in Tris buffer (same as above) and filtered through 6 layers of cheesecloth, followed by centrifugation at 48,000 x g for 30 min in a Sorvall RC-2B centrifuge (SS-34 rotor). The pellet was extracted three times with Tris buffer, collecting supernatants  $S_1$  through  $S_3$  and a fourth time with Tris buffer + Triton. The supernatant from the fourth extraction  $S_4$ , was used directly for polyacrylamide gel electrophoresis or was centrifuged on a sucrose gradient. The supernatants  $S_1$  to  $S_4$  were assayed for protein content and PCH activity. In some of the experiments, the harvested leaves were frozen and kept at -90°C for several weeks. The leaves were not thawed prior to homogenization but ground to a fine powder with a mortar and pestle while frozen on dry ice. The powder was transferred to a Waring blender, and all subsequent steps were the same as with fresh leaves.

The  $S_4$  is of high purity, but not very stable. It loses its activity within several hours at 4°C. The  $S_4$  preparation was used immediately for further experiments or made 50% with respect to glycerol (v/v) and kept at

-20°C. The  $S_4$  PCH was illuminated directly to produce  $S_4$  CH. Absorption spectra of the various preparations were measured at 22°C in a 1 cm cuvette using a Cary Model 14 spectrophotometer.

Gel electrophoresis. Gel electrophoresis was carried out following the Weber-Osborn (28) procedure as modified by Neville (20). SDS gels contained 10% and 5% in the separating and stacking gel, respectively. Two different sets of experimental conditions were used. The first set provided for the analysis of the peptide composition of the holochrome. In this set, the gel measured 0.5 cm in diameter, 8.0 cm high and contained 0.1% SDS. Electrophoresis was carried out in 0.1% SDS at room temperature for 1 hr at 1.5 ma/gel followed by 2 hrs at 3 ma/gel. Samples containing 0.05 - 0.1 mg protein in 8 M urea, 1%  $\beta$ -mercaptoethanol, 2% SDS and 10% glycerol were boiled for 2 min and loaded on the gels. After electrophoresis, the gels were stained overnight with 0.1% Coomassie brilliant blue (Bio-Rad Lab., California) in 5/1/5 methanol:acetic acid:water. Destaining was achieved in 5/1/5 methanol:acetic acid:water by diffusion. A trace of the stained gel was obtained by scanning at 600 nm using a Gelscan densitometer (Gelman Instrument Co., Ann Arbor). The second set of experimental conditions was used for locating the pigment on the gel. For these conditions, a gel column 1.3 cm in diameter and 8.0 cm high was used. The sample contained 1 mg protein in 8 M urea, 2% SDBS, 1%  $\beta$ -mercaptoethanol, 10% glycerol. It was incubated for 15 min at 22°C and loaded on the gel. After electrophoresis at 4°C for 4.5 hrs at 3 ma/gel followed by 4.5 hrs at 6 ma/gel, the gel was frozen and sliced into 3.8 mm sections. (Typically, two identical gels were combined.) Each section was dispersed by extrusion from a syringe through 125 mesh nylon bolting cloth (Turtox/Combisco, Chicago, Illinois). The gel particles were then resuspended in 0.05 M Tris buffer, pH 8.0 containing

1% SDBS incubated for 4 hrs at 4°C and freeze dried. The dry pellet of each tube was extracted with 90% acetone. PChlide and Chlide were detected by measuring their fluorescence in the 90% acetone extracts with a Hitachi (Perkin-Elmer) MPF-2A Fluorescence Spectrophotometer. All gels were calibrated with respect to molecular weight using purified proteins of known size. The proteins employed were: bovine serum albumin (67,000 daltons), ovalbumin (45,000), cytochrome c- (12,400), myoglobin (17,800), chymotrypsinogen (25,000), all purchased from Schwarz/Mann, and lysozyme (14,300), trypsin (23,000) purchased from Sigma. Protein was determined using the method of Lowry et al. (18).

Sources of chemicals for electrophoresis were: acrylamide, bis, TEMED, ammonium persulfate, SDS, all from Bio-Rad (California); urea (Ultra-pure) from Schwarz/Mann; SDBS from Research Organic/Inorganic Chemical Co., Sun Valley, California.

Sucrose gradient centrifugation. PCH homogenates prepared by polyethylene glycol precipitation and hydroxylapatite chromatography (Method 1) contained 0.05 M glycine buffer, pH 9.5, and 10% glycerol. One ml samples containing 10 mg/ml protein were loaded on 10% to 70% sucrose gradients. The gradients were centrifuged for 41 hrs at 41,000 rpm in the SW 41 rotor of a Beckman ultra-centrifuge (Model L-2) at 2°C. After centrifugation, the gradients were collected in 22 fractions of 0.6 ml each. Each fraction was analyzed by absorption or fluorometrically either for convertibility of PChilde to Chlide or, in preparations containing pigment which has been photoconverted prior to centrifugation, for absorption or fluorescence in the region 600-700 nm. Similar gradients containing molecular weight markers such as thyroglobulin (670,000 daltons), apoferritin (480,000),  $\gamma$ -globulin (160,000) and bovine serum albumin (67,000), all from Schwarz/Mann, were centrifuged in parallel with the homochrome gradients.

Protochlorophyllide holochrome determination. The relative amount of PCH is defined as activity in units/ml, measured as (A639)/cm path length before irradiation. The specific activity is expressed as the activity of PCH per g of protein. The degree of phototransformation is described in terms of the ratio of the absorbance at 678 nm (A678) of the irradiated sample to the absorbance at 639 nm (A639) of the sample before irradiation. These definitions are according to Schopfer and Siegelman (23).

## RESULTS

(1) Identification of the major holochrome polypeptide. Purification of the protochlorophyllide holochrome by column chromatography resulted in a complex mixture of proteins (7). Therefore, the procedure described by Akoyunoglou *et al.* (1) was followed as depicted in Materials and Methods. Supernatant S<sub>4</sub> was centrifuged in a 5 to 25% sucrose density gradient. PCH appeared in the gradient in a peak corresponding to a sedimentation coefficient of 18S, and the PChlide was phototransformable to Chlide. The peak was collected in three fractions. The central and the following fractions, designated B and C respectively, were analyzed by SDS polyacrylamide gel electrophoresis. The polypeptide composition of fraction C is shown in Fig. 1. Peaks C1, C2, and C3 were estimated to be of molecular weights 60 Kd, 40 Kd, and 13 Kd, respectively. We suspected that this preparation of PCH contained RuDP-Case. The latter constitutes up to 50% of the total protein present in etiolated bean leaf extracts (1, 15), and has a similar size to the holochrome. We subjected purified spinach RuDP-Case to electrophoretic analysis and found that peaks B1, C1, and peaks B3, C3 had very similar mobilities and molecular

weights to those of the large (58 Kd) and small (14 Kd) subunits of RuDP-Case respectively. Peaks B2 and C2, corresponding to 40 Kd molecular weight (Table I), did not appear in the analysis of RuDP-Case. The ratio of staining intensity of peaks B1 and B3 to B2 was larger than the ratio of C1 and C3 to C2. From this result we conclude that the three polypeptides do not belong to one protein. The 40 Kd polypeptide (peak 2) appeared to be enriched in fraction B compared to that from fraction C from the sucrose gradient. The ratio between the areas of peaks B1 and B3 (presumably the large and small subunits of RuDP-Case, respectively) to the area of peak B2 (shown later to be the polypeptide of PCH) is about 1:1. This indicates that PCH can be no more than 50% pure in fraction B.

To identify Peak 2 as the major polypeptide of PCH, an attempt was made to locate the pigment on the gels. A sample of irradiated  $S_4$  containing 200  $\mu$ g of protein (five times more than the usual amount) was analyzed on polyacrylamide gel. Following electrophoresis the gel was scanned at 675 nm. Two peaks of Chlide absorption were observed (Fig. 2). The major peak, D1, had a mobility of 0.4 corresponding to a molecular weight of 40 Kd. The minor peak, D2, had a mobility of 0.8 corresponding to a molecular weight smaller than 10 Kd. The mobility of the major peak D1 was very similar to the mobility of peaks B2 and C2 (Table I). These results suggest that peak D1 is composed of a 40 Kd polypeptide associated with the Chlide molecule. The minor peak D2 appears to be a proteolytic product of PCH.



(2) Pigment extraction from the gel after electrophoresis. In Many experiments it was difficult to measure the absorption of Chlide directly on the gel because of rapid diffusion of urea from the gel resulting in changes in light scattering by the gel. This problem was overcome by extracting the pigment from the gel slices and recording the fluorescence spectrum from each extracted fraction. A holochrome sample was prepared by treating the crude extract of etiolated leaves with polyethylene glycol followed by hydroxylapatite chromatography (Method 1). PCH was then photoconverted to CH absorbing at 680 nm. This preparation was denatured and dissociated into polypeptides by incubation in 2% SDBS, 8M urea, 1%  $\beta$ -mercaptoethanol. The absorption maximum shifted to a shorter wavelength and was located at 673 nm. Whether the incubation was carried out at 22°C for 15 min or by boiling for 2 min, there was only one polypeptide of 45,000 daltons with which the chlorophyllide pigment was associated. However, the highest yield of the pigment - polypeptide complex was obtained by incubation of CH for 15 min at 22°C in the denaturing reaction mixture. The sample was then loaded on a 10% polyacrylamide gel, and electrophoresis was carried out at 4°C in the large gels (1.3 x 8.0 cm). The gel was sliced into 3.8 mm sections. Each section was extracted with acetone, and its Chlide content was measured by fluorescence, as described in Materials and Methods. A schematic representation of the gel is shown in Fig. 3A. One major peak of Chlide fluorescence was observed on the gel. Its location corresponded to a mobility of  $0.28 \pm 0.08$  and molecular weight of  $45 \pm 10$  Kd as calculated from the calibration curve. In addition, there was a minor peak with mobility of 0.87 corresponding to a molecular weight of about 6 Kd.

A typical fluorescence spectrum of the pigment obtained from the gel after acetone extraction is shown in Fig. 4. In some experiments, the

fluorescence of both PChlide at 630 nm and Chlide at 666 nm occurred at the same location on the gel (Fig. 4). The pigments were characterized by comparison with known fluorescence spectra of PChlide and Chlide in acetone and by the excitation spectra that were measured for each sample. The location of Chlide fluorescence on the gel shows that the Chlide pigment is complexed with a polypeptide of molecular weight 45 Kd (Fig. 3A). This is in good agreement with the 40 Kd molecular weight obtained by direct scanning of the gel at 675 nm (Fig. 2) or from the polypeptide molecular weight determined by SDS polyacrylamide gel electrophoresis (Fig. 1). The holochrome homogenate prepared by Method 1 could not be analyzed for its polypeptide content by gel electrophoresis, because the gel did not destain completely after fixing and staining.

(3) The number of chromophores associated with the holochrome protein.

The number of PChlide molecules per protein molecule can be estimated from the ratio of absorbance at 639 nm (PChlide),  $A_{639} = 0.016 \text{ cm}^{-1}$ , to the amount of protein, 150  $\mu\text{g/ml}$ , in fraction B. The specific activity was found to be 106 units/g protein. Since fraction B was apparently no more than 50% pure, we conclude that the most purified holochrome preparation has a specific activity of at least 210 units/g protein. Based on known absorptivities (4), this is equivalent to 0.13 g of protein per  $\mu\text{mole}$  PChlide, which is about half the value reported by Schopfer and Siegelman for their preparation of highest purity (23). Thus, an aggregate of molecular weight of about 600 Kd contains at least 4 PChlide chromophores.

(4) Association of chlorophyllide molecule with the holochrome subunit prior to and after the dark spectroscopic shift. Figure 5 shows the absorption spectrum of CH homogenate (obtained by Method 1) 1 min after photoconversion at 15°C. The absorption maximum was typically at 678-680 nm.

After incubation of the CH homogenate at 15°C for 40 min, the absorption maximum shifted to 674 nm (Fig. 5). CH absorbing maximally at 680 nm and CH absorbing maximally at 674 nm were denatured and dissociated into polypeptides by incubation in 2% SDBS, 8 M urea and 1%  $\beta$ -mercaptoethanol for 15 min at 22°C. The absorption maxima, under denaturing conditions, were blue-shifted to 673 nm and 671 nm, respectively. Both Chl-protein complexes were loaded on parallel polyacrylamide gels, and electrophoresis was carried out at 4°C on large gels. The position of the Chlide on the gels was determined by acetone extraction and fluorescence measurements. The electrophoretic patterns obtained for the dissociated samples before and after the dark spectroscopic shift are shown diagrammatically in Fig. 3, and the results of one of two such experiments are presented in Table II.

Before the dark shift, the location of the pigment on the gel corresponded to a mobility of  $0.28 \pm 0.08$  and molecular weight of  $45 \pm 10$  Kd. However, after the dark shift, the pigment was found at a location corresponding to a mobility of  $0.63 \pm 0.03$  and a molecular weight of  $16 \pm 1$  Kd. In a sample (not shown) where the dark shift was allowed to proceed only part way and the absorption maximum was at 677 nm before denaturation (673 nm after denaturation), about half of the Chlide fluorescence was associated with a molecular weight of 45,000 and the other half with a molecular weight of 16,000 daltons.

These results suggest that a polypeptide of molecular weight 45 Kd, to which the Chlide molecule is attached, has been cleaved to yield a polypeptide of molecular weight 16 Kd. The reaction leading to the decrease in molecular weight of the 45 Kd polypeptide occurred during the same interval as the dark spectroscopic shift. Both were completed in about 40 min at 15°C and were blocked below 0°C.

A minor Chlide peak, consisting of 10% of the total Chlide on the gel and displaying a mobility of  $0.86 \pm 0.04$  and molecular weight smaller than 10,000 daltons, was also apparent in many experiments. It is probably a proteolytic product of the major subunit of the holochrome (45,000 daltons molecular weight).

In many experiments, PChlide ran as a free pigment, with mobility identical to the bromphenol blue dye. Apparently, the PChlide molecule is easily detached from the polypeptide.

(5) The absence of the dark spectroscopic shift in the  $S_4$  chlorophyllide holochrome.  $S_4$  supernatant was photoconverted and then incubated for 40 min at room temperature. It was then subjected to SDBS gel electrophoresis. No Chlide could be detected on the gel after electrophoresis using the acetone extraction and fluorescence method. This is not surprising, because the  $S_4$  preparation is highly labile and Chlide absorption is lost to a large extent within an hour at room temperature.

The absorption maximum of  $S_4$  CH immediately after photoconversion was located at 672 nm. The absorption maximum after partial photoconversion was also at 672 nm. No corresponding long wavelength 678 nm form and no dark spectroscopic shift were observed for the  $S_4$  holochrome preparation. The  $S_4$  supernatant was analyzed by SDS polyacrylamide gel electrophoresis for its protein composition. An identical electrophoretic pattern was seen for the following samples:

- 1) A dark sample at 4°C
- 2) a dark sample incubated for 40 min at 22°C
- 3) immediately after illumination at 4°C
- 4) an illuminated sample incubated for 40 min at 22°C.

Fig. 6 depicts the electrophoretic pattern for the dark sample at 4°C. Two peaks were observed on the gels. The major peak in each gel was a polypeptide of molecular weight 45 Kd, according

minor peak was a polypeptide of molecular weight 60 Kd, according to the calibration curve. These peaks have been tentatively identified as the PCH polypeptide and the large subunit of RuDP-Case, respectively, as obtained previously for peaks C2 and C1 (Fig. 1). These results suggest that  $S_4$  CH does not exhibit the dark spectroscopic shift, nor does it display the change in molecular weight of the 45 Kd Chlide polypeptide to a 16 Kd polypeptide.

Addition of glycerol to a final concentration of 50% had an effect on the absorption maximum of  $S_4$  CH. After photoconversion, the absorption maximum was found at 676 nm instead of 672 nm (not shown). In the presence of 50% glycerol the holochrome is more stable, and the photoactivity is retained for a much longer period. A similar effect of high concentration of sucrose on the spectroscopic properties and stability of holochrome homogenates was reported by Mathis and Sauer (19). The absorption maximum of the pigment may depend on the immediate environment of the pigment and thereby reflect a conformational change in the holochrome upon addition of glycerol to high concentration. The polypeptide composition of a high glycerol (676 nm) and a no glycerol (672 nm) form of the CH were identical and indistinguishable from that shown in Fig. 6 for a dark  $S_4$  sample. Therefore, a difference in the absorption maximum of the pigment does not necessarily reflect a change in molecular weight or composition.

(6) Dissociation of the chlorophyllide holochrome molecules of 600,000 molecular weight simultaneously with the dark spectroscopic shift. PCH was prepared by polyethylene glycol precipitation and hydroxylapatite chromatography (Method 1) and then centrifuged in a 10 to 70% sucrose gradient. Its distribution in the gradient is shown in Fig. 7. The photoconvertible holochrome sedimented as a homogenous peak with a sedimentation coefficient of 18S, based on the sedimentation velocity of two markers, thyroglobulin (sedimentation coefficient 19.2S) and apoferritin (sedimentation coefficient 17.6S). The

inactive PCH had an additional slower sedimenting shoulder. Approximately 70% of the inactive PCH had a sedimentation coefficient of about 18S, and 30% had a sedimentation coefficient smaller than 10S.

Illumination for 2 min at an intensity of 90 ft-c at room temperature photoconverted all active PCH to CH. The photoconverted sample was loaded on a sucrose gradient. CH appeared as a major peak with a sedimentation coefficient of 18S and a minor peak with a sedimentation coefficient smaller than 10S (Fig. 8).

CH was allowed to complete the dark spectroscopic shift for 40 min at 22°C and the sample was analyzed by sucrose gradient centrifugation (Fig. 9). The 18S CH macromolecule dissociated almost completely to yield a new pigment complex with a sedimentation coefficient smaller than 4.6S. The inactive PChlide also appeared with a sedimentation coefficient smaller than 4.6S. In a control experiment, PCH was incubated in the dark for 40 min at 22°C. The sample was analyzed by sucrose gradient centrifugation (Fig. 10). The photoconvertible holochrome sedimented as a homogenous 18S PCH peak. No dissociation into a small molecular weight PChlide - protein complex was observed on the gradient. Inactive PCH followed the same pattern. These results are summarized in Table III.

The sedimentation coefficient of PCH was measured by Boardman (3). It was found to be 18S in the analytical ultracentrifuge, and the molecular weight was calculated to be  $600 \pm 50$  Kd. Our result is in good agreement with the reported sedimentation coefficient based on the analytical ultracentrifuge measurements. We found that upon illumination and the commencement of the dark spectroscopic shift the 600 Kd CH macromolecule dissociates. The final product is a Chlide complex of molecular weight smaller than 70 Kd, corresponding to a sedimentation coefficient smaller than 4.6S. The appearance of a small molecular weight pigment-protein complex after photoconversion was also reported by Bogorad *et al.* (5).

## DISCUSSION

RuDP-Case constitutes up to 50% of the total protein contained in etiolated bean leaf extracts (1). RuDP-Case has very similar physical-chemical properties to the holochrome. It has a sedimentation constant of 18.5S (15), while PCH extracted from etiolated bean leaves has a sedimentation constant of 18S (3). Moreover, both proteins bind to DEAE in the pH range from 7.0 to 8.0, indicating a similar electrical charge distribution. Therefore it is very difficult to separate RuDP-Case and PCH by the usual column chromatography techniques (7). However, RuDP-Case is a soluble protein easily removed from mature chloroplasts by washing the thylakoid membranes with dilute buffers (26). PCH, on the other hand, is associated with the prolamellar body in etioplasts, probably through hydrophobic interactions, because a much greater proportion of PCH is liberated from the etioplast membrane using detergents like Triton X-100. Therefore, the long procedure of column chromatography (7) was replaced by a procedure that involved several successive washes of etioplast fragments followed by sucrose density gradient centrifugation. In the latter procedure, electrophoretic analysis of the purified PCH reveals the presence of three polypeptides. The mobilities and molecular weights of two of these peaks correspond to the mobilities and molecular weights of the large and small subunits of spinach RuDP-Case. The third peak is a polypeptide with a molecular weight of 45 Kd. Using either direct absorption measurements at 675 nm or pigment extraction, we discovered that PChlide, as well as Chlide immediately after photoconversion, is attached to the 45 Kd polypeptide. The polypeptide is obtained by dissociating the PChlide holochrome with SDS, urea and  $\beta$ -mercaptoethanol. This treatment denatures the protein and the 45,000 dalton subunit is unable to photoconvert. Assuming that PCH is made from one kind of polypeptide of 45,000 daltons, we can estimate that the 600 Kd holochrome contains twelve

to fourteen polypeptides of identical size. De novo synthesis of a polypeptide in the same molecular weight range was recently implicated in barley (2).

From measurements of absorption of PChlide-639 per g protein, we calculate the number of PChlide molecules per 600 Kd dalton protein to be about 4. Therefore, only some and not all of the holochrome's subunits are attached to PChlide pigment molecules. The smallest photoactive subunit of PCH from dark grown bean leaves was reported to have an apparent molecular weight of about 170,000 daltons (13). CD studies (12) are consistent with the presence of a single PChlide molecule per photoactive subunit of the holochrome. This result is in agreement with our findings of 4 PChlide molecules per 600 Kd molecular weight of holochrome.

A post-illumination dark shift in the absorption spectrum of dark grown bean leaves was discovered and characterized by Shibata (25). The 684 nm absorbing form of chlorophyll (C684), which resulted from the photoconversion of protochlorophyllide (P650) after a short illumination, transformed in about 10 to 15 min in the dark to a form of chlorophyll absorbing at 672 nm (C672). The sharp isosbestic point obtained for the C684 → C672 shift indicated conversion of one definite component to another. A corresponding shift in the position of the fluorescence maximum of chlorophyll (from 690 nm to 680 nm) was observed by Litvin and Krasnovsky (17). The origin of this dark shift is unknown.

A similar dark shift is reported in chlorophyllide holochrome in vitro. Immediately after the phototransformation the principal absorbance maximum is at 678 nm, owing to the newly formed chlorophyllide. The 678 nm peak shifts slowly toward shorter wavelength with a slight decrease in absorbance. At 25°, the shift is completed in 10 to 15 min. At lower temperature 12°, the shift requires 30 to 40 min for completion (23, and this work). We make the



assumption that both the in vivo and in vitro dark shifts are due to the same process.

Immediately after photoconversion, the newly formed Chlide holochrome appears to have the same polypeptide composition (45,000 daltons) as the native PChlide holochrome macromolecule. After completion of the dark shift, the chlorophyllide holochrome yields instead a 16,000 dalton polypeptide associated with chlorophyllide. These results indicate that the polypeptide of 45,000 dalton molecular weight has undergone a cleavage to yield a smaller chlorophyllide polypeptide of 16,000 daltons. The time for completion of the reaction and the fact that it is blocked at 0°C are also characteristics of the dark spectroscopic shift.

The most plausible mechanism for the reaction leading to the change in the molecular weight of the Chlide polypeptide is an enzymatic one. We assume that an enzyme present in the crude holochrome homogenate is responsible for the cleavage. The fact that  $S_4$  does not have the capability of carrying out this reaction can be explained by the postulate that this enzyme activity has been removed from the highly pure  $S_4$  supernatant. However, the possibility cannot be ruled out that PCH in  $S_4$  has been modified in such a way that, although it is photoconvertible, it does not undergo the cleavage reaction.

Henningsen et al. (13) isolated an active subunit of 63,000 daltons from barley leaves using the detergent saponin. Concomitant with the dark spectral shift from 678 to 672 nm a change in the apparent molecular weight of the chlorophyllide holochrome subunit from about 63,000 to 29,000 was observed. In holochrome extracted from bean leaves with a high concentration of saponin, the spectral shift was extremely slow, and a corresponding change in the molecular weight was not detected (13). Our experiments with  $S_4$ -chlorophyllide holochrome (Tris/Triton extract), show the absence of both the spectral shift

and the corresponding cleavage of the holochrome subunit. Apparently, the ability to carry out this reaction has been lost in this preparation.

The data of Table III demonstrate that, during the course of the dark shift, a major disintegration occurs at the macromolecular level. The 600,000 dalton chlorophyllide holochrome with a sedimentation coefficient of 18S dissociates to a chlorophyllide protein with a sedimentation coefficient smaller than 4.6S. This reaction has a specific light requirement, because it did not occur in a dark incubated sample. These results are in agreement with similar experiments conducted by Bogorad et al. (5). The observation that both active and inactive PChlide are associated with low molecular weight protein may mean that not all of the PChlide of a holochrome particle needs to be photoconverted to bring about the dissociation.

Butler and Briggs (6) have previously attributed the Shibata shift in leaves to a pigment disaggregation. Pigment disaggregation during the Shibata shift was also implied from the studies of Schultz and Sauer (24) and Mathis and Sauer (19) on the circular dichroism (CD) spectrum of chlorophyllide holochrome. They observed that a double CD signal, attributed to a dimer of chlorophyllide molecules, disappeared progressively during the course of the dark shift. It was suggested that a dimer of chlorophyllide molecules is dissociating into chlorophyllide monomers. Another feature of the CD spectrum of chlorophyllide holochrome immediately after photoconversion is a negative peak at 580 nm. This peak also vanishes in the course of the dark spectral shift (8, 19). These experiments have been interpreted as indicating a holochrome protein conformational change, which in turn affects the pigment-pigment interaction.

Our observations in this study together with the observations of others can be assembled in the following model. PCH with a molecular weight of 600 Kd

is photoconverted to a 600 Kd CH. The photolytic hydrogenation of two carbon atoms in ring IV of PChlide to produce Chlide may necessitate proton donation from the protein, together with a simultaneous change in configuration of the protein molecule. This conformational change in the protein leads to an unstable 600 Kd CH oligomer. The 600 Kd CH oligomer can be stabilized in vitro either in a high concentration of sucrose or in a buffered solution below 0°C. Otherwise, the unstable CH complex dissociates into a small molecular weight chlorophyll protein. The dissociation leads also to pigment disaggregation, which is manifested in the Shibata shift and the loss of the characteristic CD Chlide dimer signal. Concomitantly, the 45 Kd Chlide polypeptide undergoes cleavage resulting in a 16 Kd Chlide polypeptide. The 16 Kd Chlide polypeptide may then be incorporated in vivo into the developing chloroplast membrane.

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Table I

Electrophoretic mobilities of proteins from two fractions of the PHC peak  
after sucrose gradient centrifugation

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<u>Gel</u>	<u>Peak 1</u>	<u>Peak 2</u>	<u>Peak 3</u>
PCH { Fraction B	0.228	0.385	0.785
{ Fraction C	0.226	0.397	0.731
RuDP-Case	0.225	---	0.74

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Table II

The molecular weight, mobilities, and absorption maxima of the different  
Chlide-Polypeptides before and after the dark spectroscopic shift

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<u>Incubation time at 15°C</u>	<u>Absorption maximum</u>	<u>Mobility</u>	<u>Molecular weight</u>
1 min	679 nm	0.3 (100%)	40,000 daltons
40 min	674 nm	0.22 (5%)	50,000
		0.66 (90%)	15,000
		0.89 (5%)	<10,000

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Table III

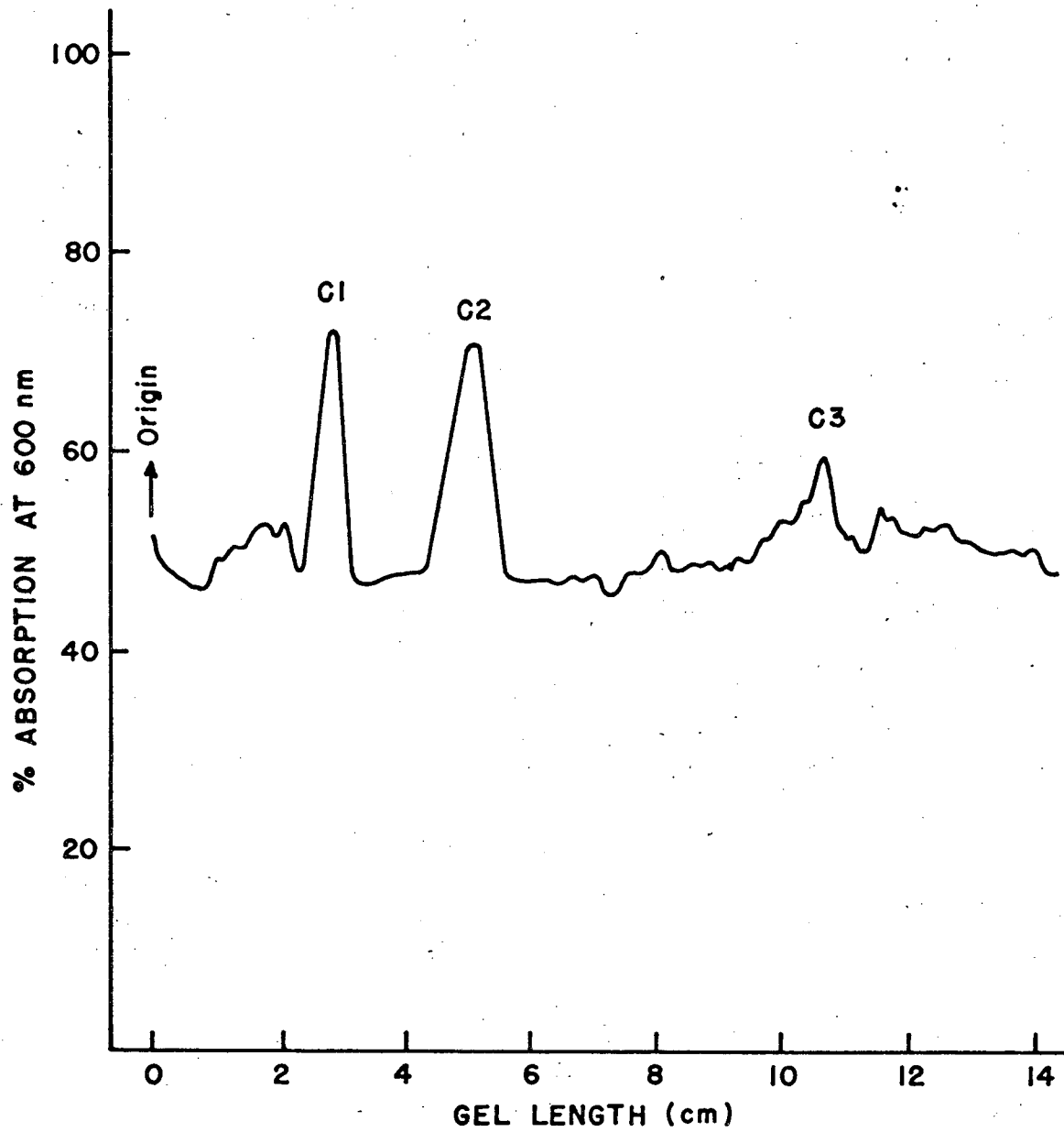
The relative mobility, sedimentation coefficients and molecular weights of pigment-protein complexes isolated by Method 1 and marker proteins

<u>Samples</u>	<u>Relative Mobility</u>	<u>S value</u>	<u>Mol. wt.</u>	<u>Pigment</u>
PChlide-holochrome	$0.62 \pm 0.03$	-	-	active PChlide
	$0.62 \pm 0.03$	-	-	inactive PChlide
	$0.43 \pm 0.02$ shoulder	-	-	inactive PChlide
Chlide-holochrome after 2 min at 22°C	$0.63 \pm 0.03$	-	-	Chlide
	$0.41 \pm 0.02$			
	$0.60 \pm 0.03$	-	-	inactive PChlide
	$0.45 \pm 0.02$			
Chlide-holochrome after 40 min at 22°C	$0.20 \pm 0.02$	-	-	Chlide
	$0.25 \pm 0.02$	-	-	inactive PChlide
PChlide-holochrome after 40 min at 22°C	$0.60 \pm 0.03$	-	-	active PChlide
	$0.60 \pm 0.03$	-	-	inactive PChlide
Thyroglobulin	$0.68 \pm 0.03$	19.2 S	670,000	-
Apoferritin	$0.60 \pm 0.03$	17.6 S	480,000	-
$\gamma$ -globulin	$0.40 \pm 0.02$	7.2 S	160,000	-
BSA	$0.37 \pm 0.02$	4.6 S	67,000	-

## FIGURE CAPTIONS

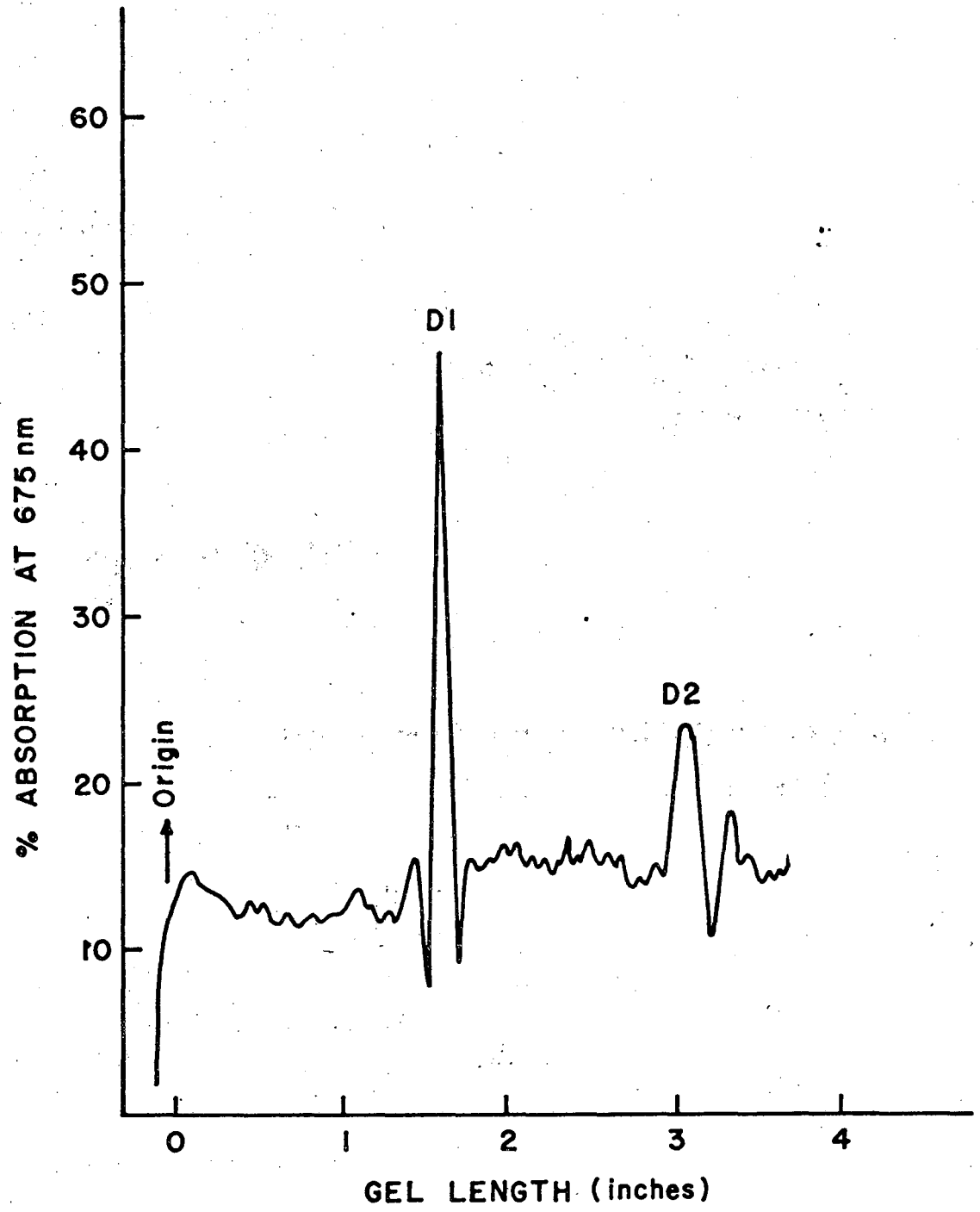
- Figure 1. SDS-polyacrylamide gel electrophoresis of PCH after purification by differential solubility and sucrose gradient centrifugation. The sample contained 40  $\mu\text{g}$  protein. The 10% stacking gel was stained by Coomassie blue and scanned at 600 nm. The peaks observed are marked C1 through C3.
- Figure 2. SDS-polyacrylamide gel electrophoresis of CH in an irradiated  $S_4$ -supernatant. The sample contained 200  $\mu\text{g}$  protein. The 10% stacking gel was scanned at 675 nm for Chl absorption before fixation. The peaks observed are marked D1 and D2.
- Figure 3. Schematic representation of SDBS-urea treated CH on 10% polyacrylamide gels before (A) and after (B) the dark shift. The shaded bands depict the position of the pigment, and the respective mobility and molecular weight associated with each band are indicated.
- Figure 4. A fluorescence emission spectrum of PChlide and Chlide in acetone. The sample was extracted from a 10% stacking gel. Bandwidth for emission and excitation was 10 nm. Fluorescence was excited at 430 nm. Control extraction was performed on a gel section that did not contain pigment.
- Figure 5. The absorption spectrum of CH at  $t=1$  min and  $t=40$  min after photoconversion at  $15^\circ\text{C}$ . The numbers 674, 679 nm refer to the absorption maxima.

- Figure 6. SDS-polyacrylamide gel electrophoresis of PCH in a dark kept  $S_4$ -supernatant. The sample contained 40  $\mu$ g protein and was run on 10% stacking gel. The gel was stained by Coomassie blue and scanned at 600 nm. The numbers 0.16 and 0.28 refer to the relative mobility of the two peaks on the gel.
- Figure 7. The distribution of photoconvertible PCH and inactive PCH from etiolated bean leaves on a 10 to 70% sucrose gradient. Active PCH was photoconverted and monitored by Chlide fluorescence at 685 nm. Inactive PChlide fluorescence was monitored at 637 nm. Sample was loaded in darkness at 4°C. The numbers 0.62, 0.43 refer to relative distances of migration on the gradient.
- Figure 8. The distribution of CH and inactive PCH on a 10 to 70% sucrose gradient. The sample was irradiated for 2 min at 22°C prior to centrifugation. Other experimental conditions are as in Fig. 7.
- Figure 9. The distribution of CH and inactive PCH on a 10 to 70% sucrose gradient. The sample was irradiated for 2 min at 22°C prior to centrifugation. Other experimental conditions are as in Fig. 7.
- Figure 10. The distribution of photoconvertible PCH and inactive PCH on a 10 to 70% sucrose gradient. The sample was incubated for 40 minutes at 22°C prior to centrifugation. Photoconverted PCH was monitored by Chlide absorption at 674 nm. Inactive PChlide absorption was monitored at 630 nm.



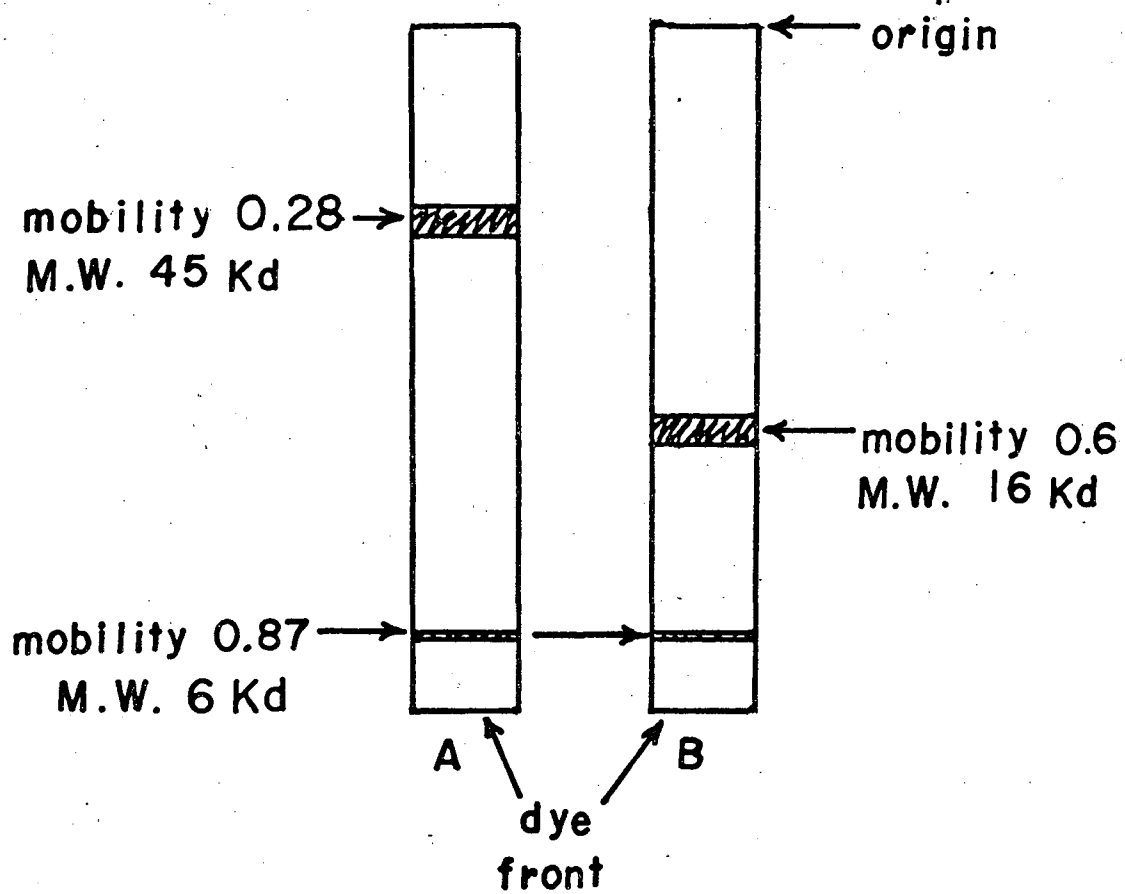
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Canaani & Sauer  
Fig. 1



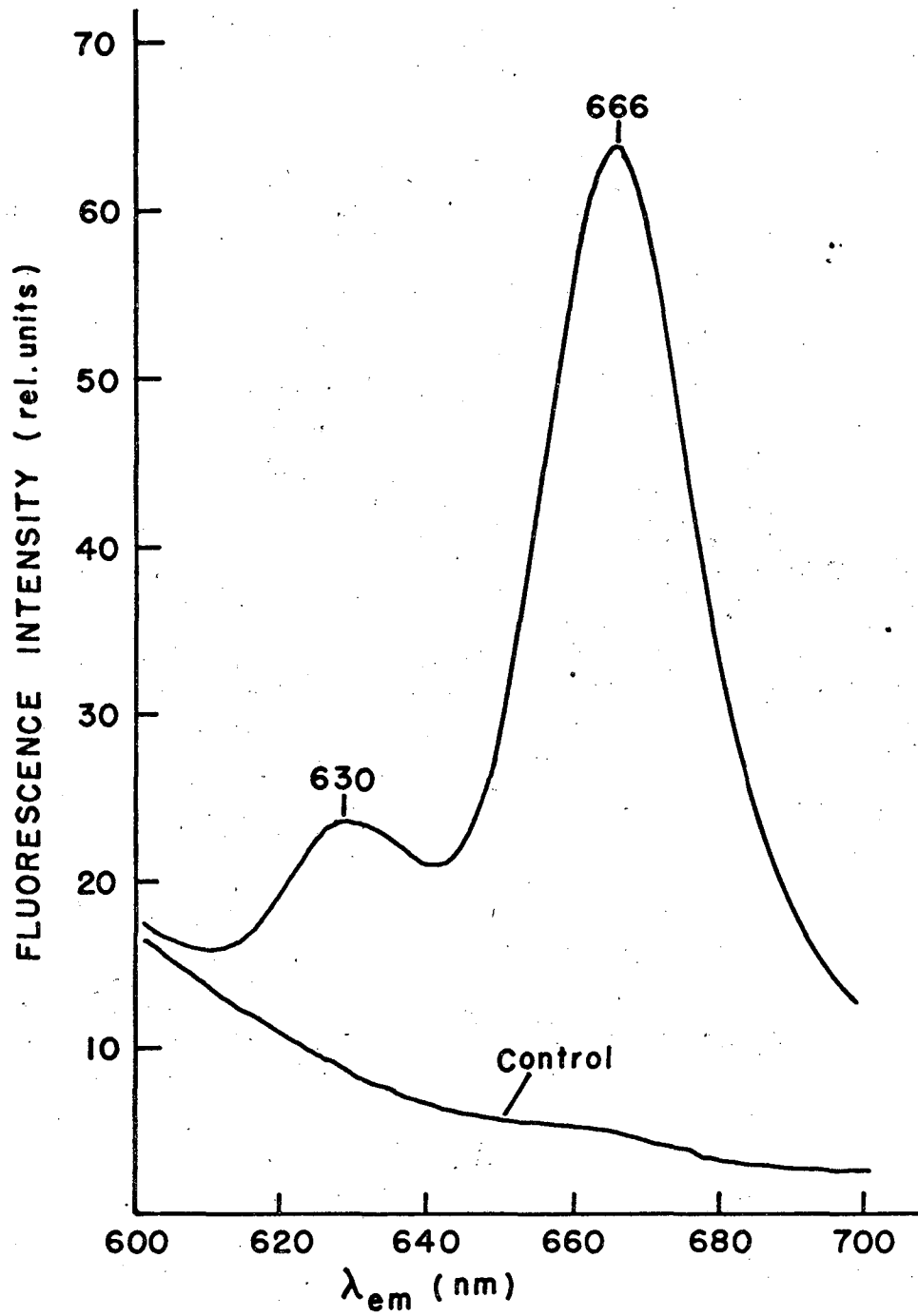
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Fig. 2



XBL757-5341

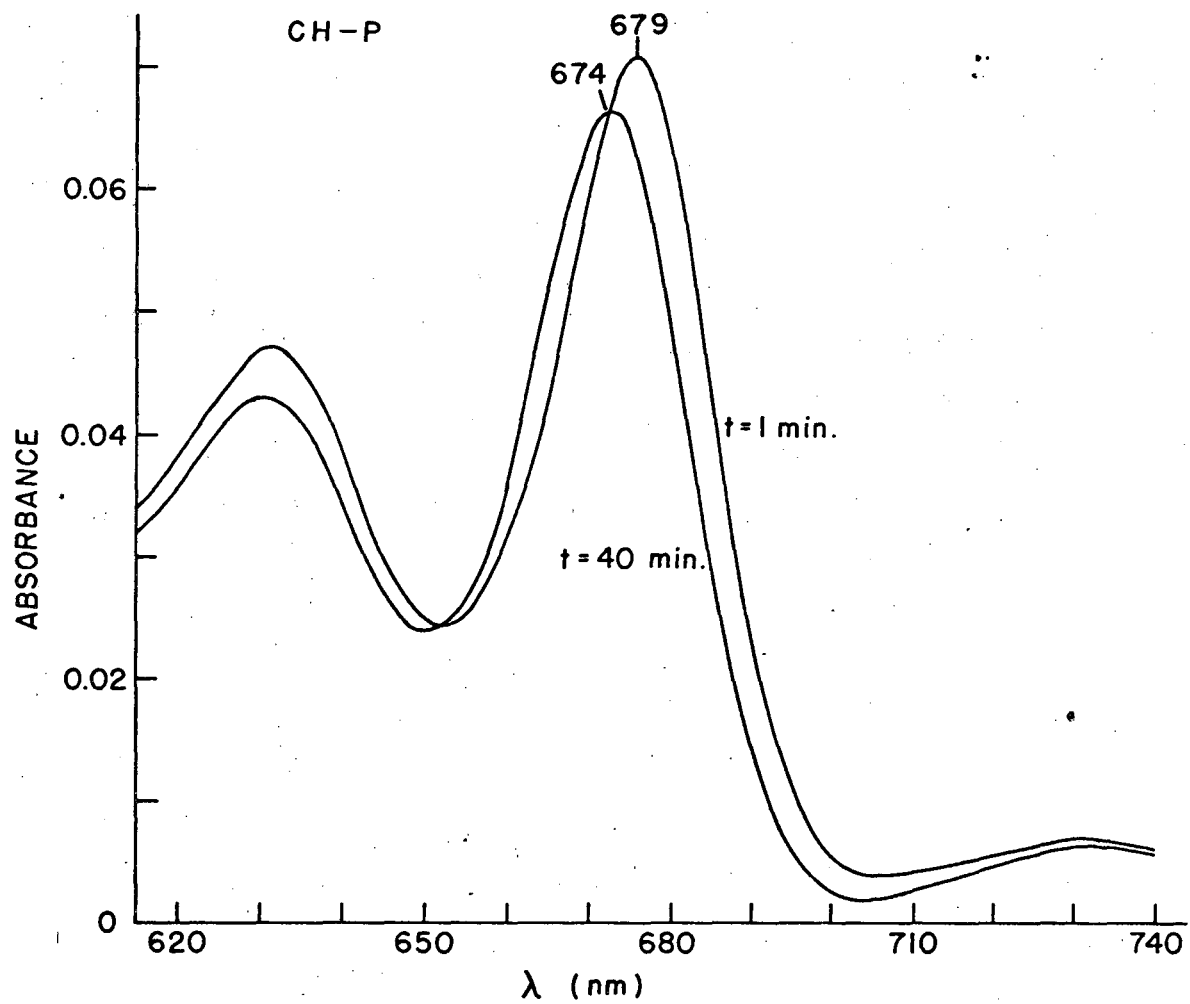
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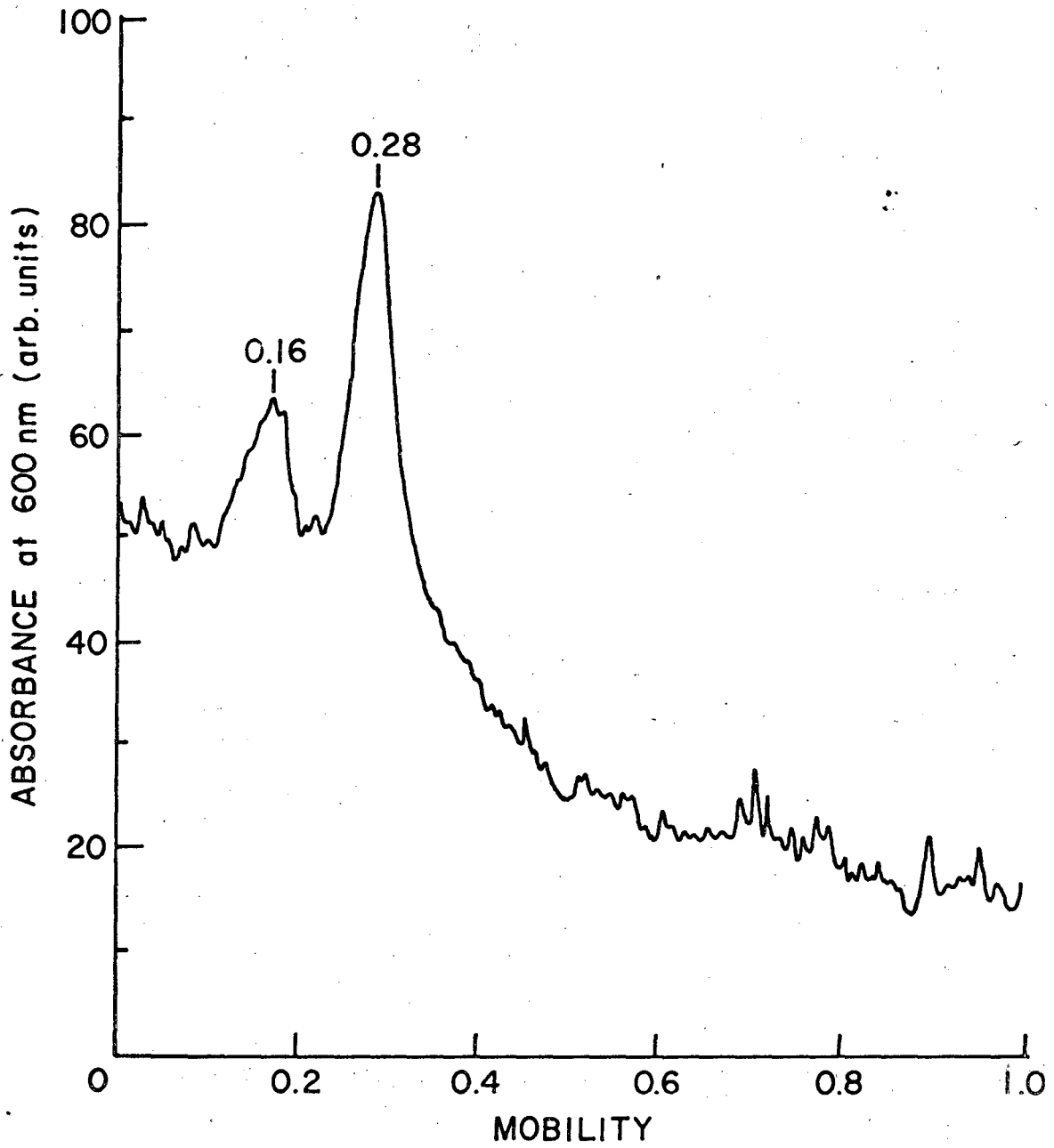
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Canaani & Sauer  
Fig. 4



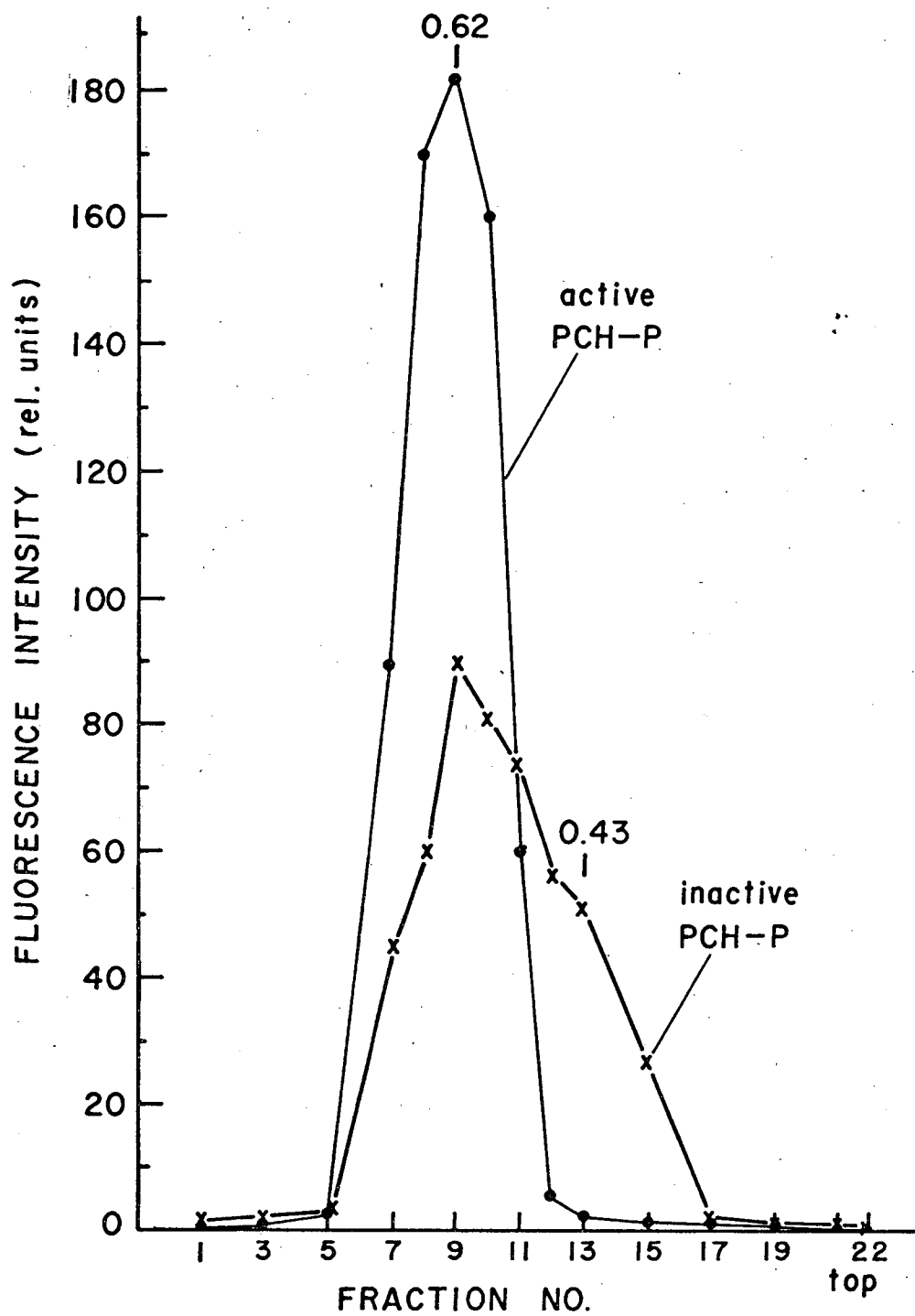


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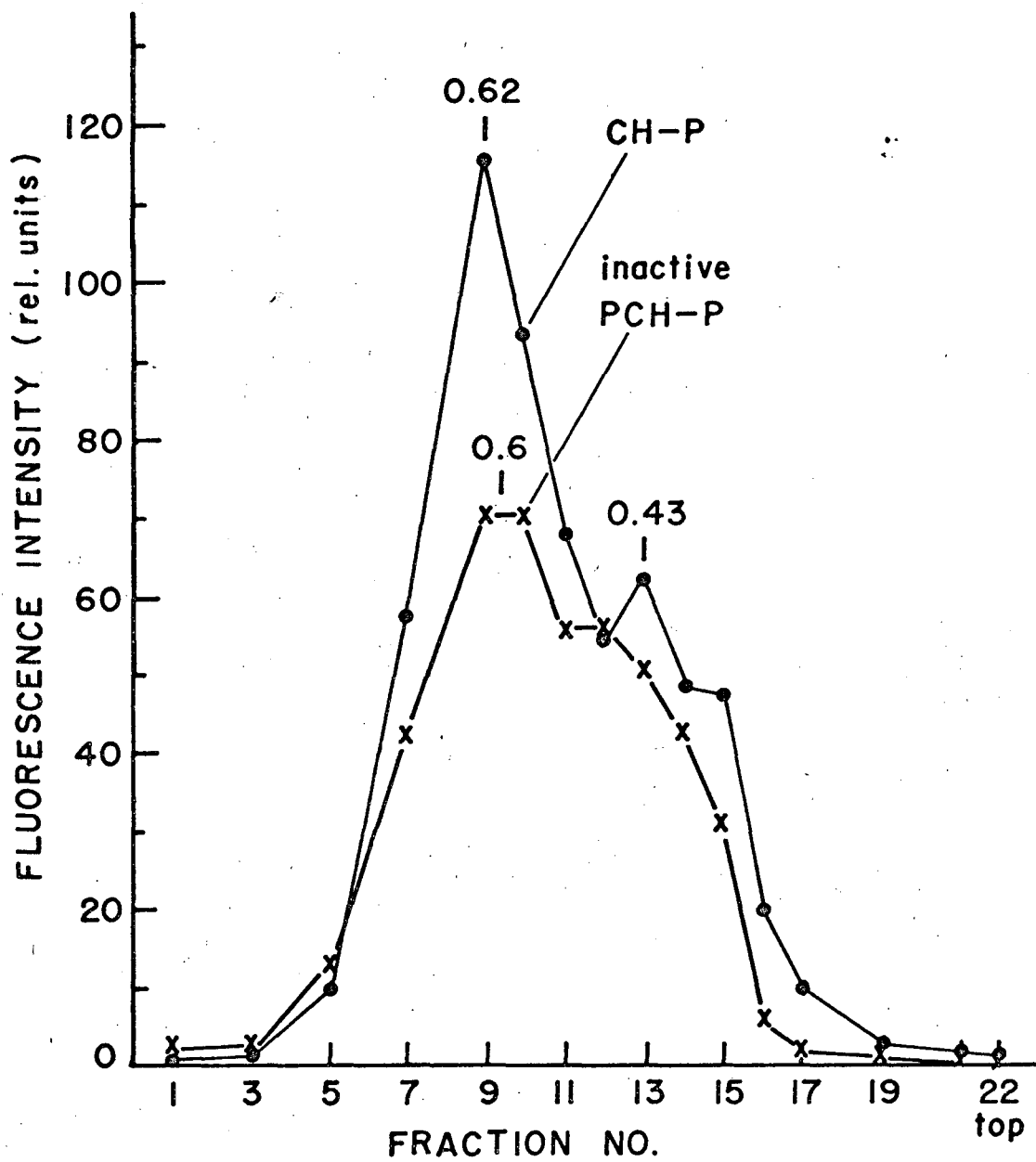
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Canaani & Sauer  
Fig. 6



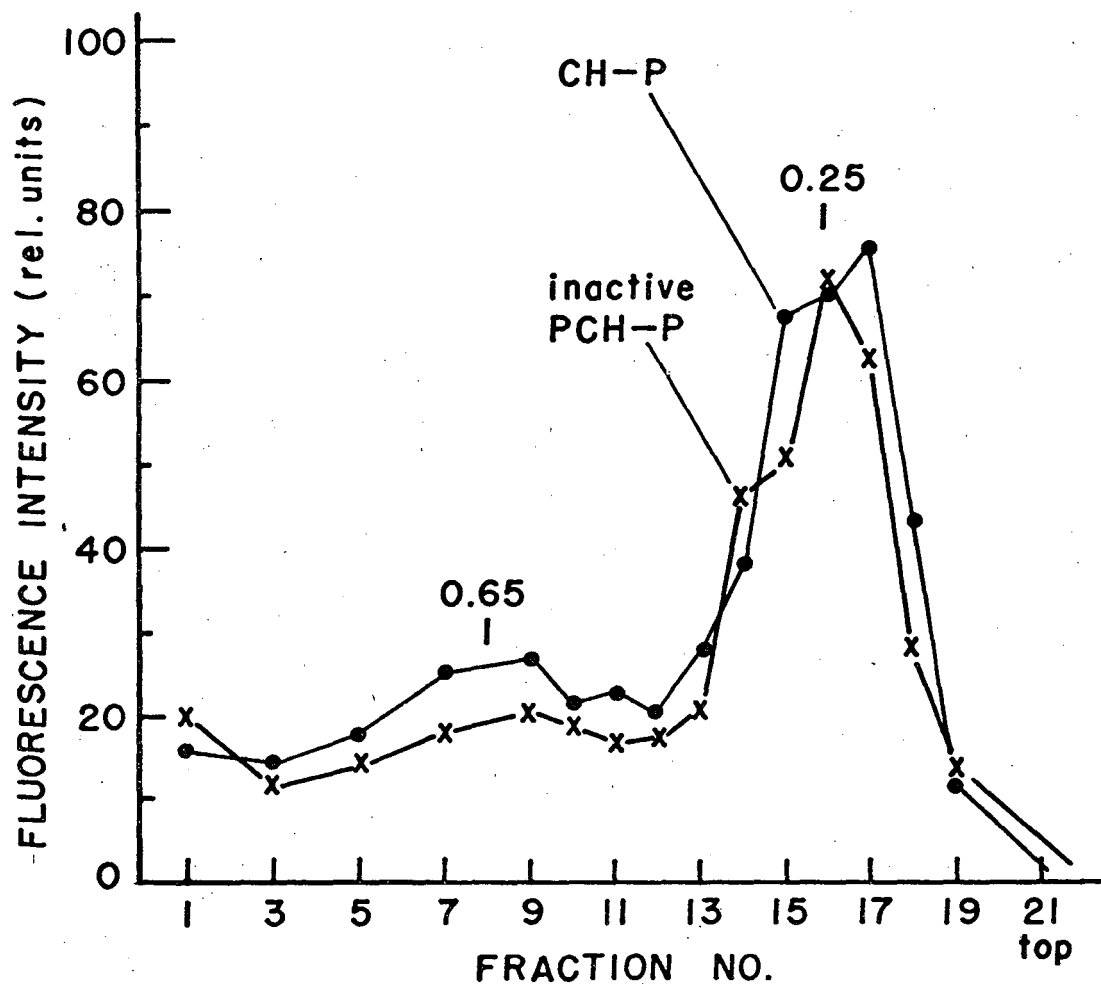
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Fig. 7



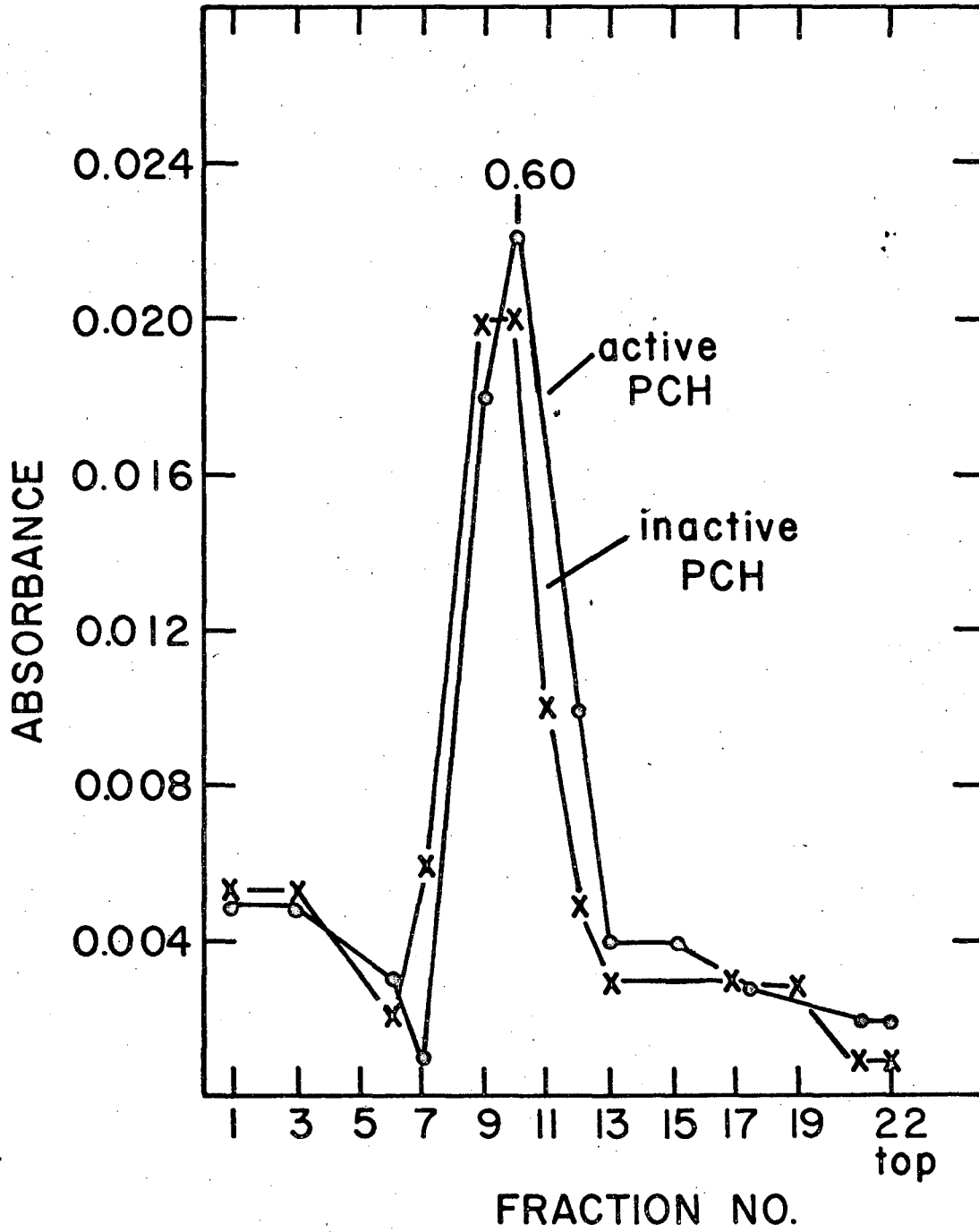
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Canaani & Sauer  
Fig. 8



XBL 757-5342

Canaani & Sauer  
Fig. 9



XBL 7611-9676

Canaani & Sauer  
Fig. 10

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