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Limited Fragmentation of Specifically Labeled Rhodopsins

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LIMITED FRAGMENTATION OF SPECIFICALLY LABELED RHODOPSINS

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ABSTRACT

Thermolysin splits rhodopsin in disc membranes into a pair of membrane-bound fragments. These fragments have amino acid compositions that are strikingly similar to each other and to that of opsin. The larger fragment, called F1, has the blocked amino terminus of opsin, the binding site for Con A, the site for galactosyl transferase galactose insertion, and the amino sugar content of opsin. The smaller fragment, called F2, has an alanine amino terminus, the accessible sulfhydryl group alkylated by IAENS, and the 11-cis retinal chromophore binding site. The estimated molecular weights of F1 and F2 are 25,000 ± 3,000 and 10,000 ± 2,000, respectively; these two fragments account for 90% of the amino acid residues contained in whole rhodopsin.

Thermolysin-cleaved rhodopsin retains the 500 nm absorbance band and its regenerability. The 500 nm absorbing species was purified by deoxycholate extract, hydroxylapatite chromatography, or Con A-agarose affinity chromatography. In all three cases, cleaved rhodopsin was a non-covalent complex of F1 and F2. Light dissociates F1 from F2 in detergent solution, altering these non-covalent forces.

Three other enzymes, papain, subtilisin, and chymotrypsin, cleave rhodopsin into fragments which resemble F1 and F2. The stretch of polypeptide connecting F1 and F2 is highly susceptible to proteolysis by enzymes of broad specificity. Guinea pig liver transglutaminase inserts amines into rhodopsin in disc membranes, at a stoichiometry of one mole amine per rhodopsin; the site(s) labeled by the transglutaminase-catalyzed reaction is located within the region of rhodopsin that is proteolytically-sensitive.

Energy transfer between a dansyl cadaverine probe, inserted by transglutaminase, and the 11-*cis* retinal chromophore indicates a distance of over 60 Å separating these sites in disc membranes. A new model of rhodopsin structure is presented which accounts for these observations.

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THESIS

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INTRODUCTION

Cells must respond to their environment in order to survive. A powerful generalization of modern biology is the theory that cell membranes contain specific receptors which serve both to detect stimuli, and, in the presence of a stimulus, to produce specific, controlled changes in the physiological state of the cell. Many membrane receptors contain protein as their major structural component. The working assumption of receptor biology is the transition between physiological states of the cell is caused by a transition between conformational states of the receptor protein. The aim of much receptor research has been to discover the three-dimensional structure of such conformational states so that physiological changes may be explained in terms of macromolecular chemistry (CIBA Foundation Symposium, 1970). It is far too premature to generalize about the structural features of membrane receptor proteins; we do not yet have a single detailed structure of a receptor molecule. The purpose of the research presented in this doctoral thesis is to advance our knowledge about the structure of the best characterized receptor protein, vertebrate rhodopsin.

The function of rhodopsin is to detect photons by absorption and to signal this event to the organism. The physiological mechanism which signals photon absorption to the CNS is probably a decrease in the extracellular resting (dark) sodium current which normally flows from the area of the inner segment of the retinal rod cell to that of the outer segment (Hagins et al., 1970). Rhodopsin is bound to an internal membrane system of the outer segment, but the extracellular sodium current is altered by a change in the sodium flux across the plasma membrane of the outer segment (Tomita, 1970; Korenbrot & Cone, 1972; Bownds & Brodie, 1975). On the basis of

capacitance measurements, it is generally accepted that the disc membranes, which contain rhodopsin, are electrically uncoupled from the plasma membrane (Penn & Hagins, 1972). It is not known how rhodopsin can affect the current flux across the plasma membrane. Most current hypotheses assume that a photon releases or produces a transmitter substance which diffuses to the plasma membrane and blocks sodium channels. There has been no convincing demonstration of the release, production or existence of such a putative transmitter. For this reason, there will be no attempt to interpret the literature or my results in terms of a transmitter model. Instead, a wholly structural review will be presented; the structure of rhodopsin must underly whatever functions this receptor protein performs.

My dissertation will begin with a critical summary of the published literature on the structure of bovine rhodopsin. The first part of Chapter I will provide an overview which can serve as a framework for the specific structural problems I have tried to solve. The second part of Chapter I is a more detailed review of those experiments which are more directly related to my own work. Chapters II-V comprise the experimental sections of this thesis. The experiments of Chapters II-IV are entirely my own work. The transglutaminase labeling experiments of Chapter V were done in collaboration with V. Iwanij and E. Reich of the Rockefeller University. Finally, Chapter VI will present a model of rhodopsin structure which accounts for my new observations.

CHAPTER I

A. GENERAL REVIEW OF RHODOPSIN STRUCTURE

Rhodopsin is the photoreceptor pigment of vertebrate retinal rod cells. Each rhodopsin pigment molecule consists of a retinal chromophore bound by a Schiff's base linkage to a lysine residue side chain of an apoprotein, called opsin (Bownds, 1967). The retinal group, when extracted from rhodopsin, is in the 11-cis conformation (Hubbard, 1958a; Rotmans et al., 1972a). Rhodopsin has a broad absorption spectrum with distinct maxima at 498 nm (α band), 350 (β band) and 278 nm (γ band) and with minima at 398 nm and 320 nm (Schichi et al., 1969). The extinction coefficient at 498 nm is 41,000 ± 1,000 (Table I). The absorption spectrum of rhodopsin is considerably broader and more red shifted than the spectra of free retinal isomers. The visual pigment also has an induced circular dichroism in the α and β bands; free 11-cis retinal is not circularly dichroic (Crescitelli et al., 1966; Schichi, 1970; Honig et al., 1973). Interestingly, the resonance Raman spectrum of rhodopsin is not significantly different than that of an 11-cis retinal protonated Schiff's base, indicating the vibrational structure of the chromophore is not altered upon protein binding (Mathies et al., 1976). The precise nature of the interactions between the protein and the chromophore are unknown. Much theoretical and experimental research has been done to elucidate the conformation of the chromophore in rhodopsin, and this work has been reviewed exclusively elsewhere (Honig & Ebrey, 1974; Honig et al., 1975). This review will concentrate on data relating to the conformation of the protein portion of rhodopsin. It is obvious, however, that the complete solution of the problem of rhodopsin structure will require information about the

chromophore conformation, about protein conformation, and about interactions between them.

As a receptor protein, rhodopsin requires the capacity for controlled conformational change in response to a stimulus. The most striking feature of this molecule is the sensitivity of its spectral properties to the action of light. A single absorbed photon, with a quantum efficiency of ~50%, causes rhodopsin to decay, through a well-defined series of meta-stable intermediate states, to the apoprotein opsin and free all-*trans* retinal (Wald, 1968); this process is called bleaching. No other single fate of the captured photon has an appreciable quantum efficiency. Since a dark adapted eye can detect a single photon with an efficiency of ~50% (Hecht, *et al.*, 1942), this photolytic decay process must be a part of the physiological sequence of events which signals photon absorption.

The decay process occurs through a series of dark reactions with complex kinetics (Abrahamson, 1973, 1975; Williams, 1975; Stewart *et al.*, 1976). A proposed reaction sequence is outlined in Fig. 1 (Wald, 1968). The electrical response of the rod cell has been observed to occur before the disappearance of metarhodopsin II, so that the physiological role of rhodopsin may be concluded at this stage. The sole action of light is to initiate the process, presumably by isomerizing the chromophore from the 11-*cis* to the all-*trans* conformation. Each of the characterized bleaching intermediate states can be prevented from further decay by low temperature; heat is probably required for an activation energy between steps of the cascade. Also, each of these meta stable intermediates can be reconverted to rhodopsin by absorption of a second photon (Wald, 1968). The opsin, produced as a final product of the cascade, can be regenerated to rhodopsin in the dark by adding

free 11-*cis* retinal (Hubbard & Wald, 1952-1953; Hubbard *et al.*, 1971). The physiological regeneration process probably involves chemical and/or photoreisomerization of the liberated all-*trans* retinal to the 11-*cis* conformation (Akhtar *et al.*, 1973; Schichi & Somers, 1974).

Precise structures of the various intermediates and of opsin are not known. Elucidating the conformations of the spectral intermediates is a particularly important problem since these light-induced conformational changes must underly the physiological response to photon capture.

The apoprotein portion of rhodopsin has been the object of considerable investigation. Rhodopsin is an integral membrane protein; this is an operational term and is defined to mean a protein that cannot be removed from a biological membrane by manipulations of ionic strength (Singer & Nicholson, 1972). Usually, the lipid bilayer structure of a membrane must be disrupted to release such a protein. The "integral" nature of such a protein does not imply any particular location for the molecule with respect to the cross-section of the lipid bilayer, but all integral membrane proteins studied to data at least partially extend into the hydrocarbon tail region of the phospholipid membrane (Coleman, 1973). Rhodopsin is bound to the retinal disc membranes. The discs are closed, flattened sacs which exist as part of an ordered stack of vesicles in the outer segment of retinal rod cells (Sjostrand, 1953; Fernandez-Moran, 1954; Nilsson, 1965). Rhodopsin is a major structural component of the disc and comprises about 40% of the dry weight and 80% of the integral protein content of bovine disc membranes (Daemen, 1973). Both of these per cent compositions are probably slightly higher in frog photoreceptors (Daemen, 1973).

Rhodopsin is not soluble in aqueous buffer solutions even after disruption of the lipid bilayer structure. Rhodopsin has been solubilized by organic solvents(including acidified methanol (Anderson, 1972), hexane (Montal & Korenbrot, 1973), 2-chloroethanol and 2-bromoethanol (Lewis et al., 1974) and by aqueous solutions of detergents (Table II). Some of the detergents used to solubilize rhodopsin are steroid-based detergents such as digitonin (Tansley, 1931; Hubbard, 1954) and cholate (Sugita, 1926; Henselman & Cusanovich, 1974); non-ionic detergents such as Emulphogene BC 720 (Schichi et al., 1969), Triton X-100 (Zorn & Futterman, 1971), Tween 80 (Zorn & Futterman, 1973), alkyl glucosides (Stubbs & Litman, 1975) and Ammonyx LO (Ebrey, 1971); cationic detergents such as CTAC (Bridges, 1957), CTAB (Bowness, 1959; Heller, 1968), DTAB (Hong & Hubbell, 1972), TTAB and TeTAB (Hong & Hubbell, 1973); and anionic detergents such as SDS (Cavanagh & Wald, 1969; Robinson et al., 1972; Heitzmann, 1972; Daemen et al., 1972; Papermaster & Dreyer, 1973). In general, organic solvents appear to produce considerable loss of structure including protein refolding, aggregation and chromophore extraction, though claims have been made for a certain degree of structural preservation in hexane plus calcium ion (Montal & Korenbrot, 1973). Detergent solutions usually preserve the absorbance and both the visible and UV circular dichroism of rhodopsin observed in disc membrane suspensions (Schichi, 1970; Rafferty et al., 1972; Stubbs & Litman, 1975) although some short chained ionic detergents are denaturing. Because of better structural preservation, the detergents have been the solubilizing agents of choice for most studies of rhodopsin. The steroid detergents appear to preserve features of the free opsin structure as well.

Detergent-solubilized rhodopsin has been purified away from other components of disc membranes by various types of fractionation procedures including differential extraction and centrifugation (Hubbard et al., 1971), ammonium sulfate fractionation (Henselman & Cusanovich, 1974), gel filtration (Heller, 1968; Robinson et al., 1972), isoelectric focusing (Huang et al., 1973), affinity chromatography (Steinemann & Stryer, 1973), ion-exchange chromatography (Schichi et al., 1969; Zorn & Futterman, 1973; Osborne et al., 1974), calcium phosphate chromatography (Bowness, 1959; Erhardt et al., 1966; Shields et al., 1967; Schichi et al., 1969), and hydroxylapatite chromatography (Robinson et al., 1972; Hong & Hubbell, 1972; Applebury et al., 1974). SDS-gel electrophoresis has been used under denaturing conditions to purify detergent-solubilized opsin (Trayhurn et al., 1975b). The most effective of these procedures to date has been hydroxylapatite chromatography of DTAB-rhodopsin or of Ammonyx LO-rhodopsin solutions. Both methods have produced rhodopsin solutions free of other proteins and which contain less than one mole of phospholipid per rhodopsin, yet retain the absorption and circular dichroism spectra of membrane-bound rhodopsin (Hong & Hubbell, 1972; Applebury et al., 1974).

The isolation of lipid-free rhodopsin has important implications because the role of lipid as a structural component of rhodopsin was a matter of much conjecture in the past. Disc membrane suspensions containing rhodopsin can be regenerated after bleaching by the addition of exogenous 11-cis retinal. However, partial lipid extraction caused a loss of regenerability, which could be restored by additon of phosphatidyl ethanolamine (but not phosphatidyl choline) (Zorn & Futterman, 1971). Steroid detergents, which are not good delipidating agents, produce disc membrane

extracts which are regenerable whereas extracts made with other detergents are not (Hubbard & Wald, 1953; Zorn & Futterman, 1973; Henselman & Cusanovich, 1974; Stubbs & Litman, 1975). These observations suggested that some phospholipid, probably phosphatidyl ethanolamine, was intimately associated with rhodopsin and was a necessary structural component of the molecule. This view is no longer tenable for several reasons. First, very small amounts of detergents, such as Emulphogene, have been shown to block regeneration in disc membranes and in digitonin solutions (Schichi, 1971). This effect is best explained not as displacement of "structural" lipid, but rather as binding to some key site, possibly the retinal binding site. Second, while no change is seen in the polypeptide region of the CD spectrum upon bleaching in disc membranes or in digitonin, there is a large change in the CD spectrum upon bleaching in stronger detergents (Rafferty et al., 1972; Stubbs & Litman, 1975). This observation is best explained by assuming that the 11-cis retinal binding energy is necesary to keep rhodopsin in its native conformation in stronger detergents, and that in solutions of these strong detergents, the loss of this stabilizing energy through bleaching leads to denaturation. This interpretation is supported by observations that rhodopsin in disc membranes is considerably more stable to denaturants such as heat than is opsin (Hubbard, 1958). Denaturation of opsin, not displacement of a "structural" lipid, probably prevents regeneration. Third, when hydroxylapatite purified rhodopsin in DTAB or Ammonyx LO solution, which contains less than 1 phospholipid per rhodopsin, is dialyzed into digitonin solution, regenerability is restored (Hong & Hubbell, 1972). No "structural" lipid is present in this species. If, however, the rhodopsin was bleached while still in DTAB or Ammonyx LO, structure is irreversibly lost and the protein aggregates upon dialysis; under

these conditions regenerability has not been restored (personal observation). In summary, these experiments lead to a view of rhodopsin with the following features: (1) the molecule consists solely of the apoprotein and chromophore, and contains no tightly-associated structural lipid; (2) rhodopsin is more stable to the denaturing effects of detergents than is opsin; and (3) the role of detergent as a solubilizing agent is to cover certain hydrophobic regions of the surface which are covered by the hydrophobic regions of the phospholipids in disc membranes.

Having defined rhodopsin as the complex of apoprotein plus chromophore, there are several additional structural questions about rhodopsin that will be examined. First, what is the covalent structure of rhodopsin? Second, what is the conformation of rhodopsin? Third, how does rhodopsin interact with its environment? This question involves a discussion both of the surface properties of rhodopsin and of the organization of rhodopsin in the disc membranes. Finally, how does the conformation of rhodopsin change upon photon capture? Each of these structural questions will be discussed under a separate heading in the pages that follow.

Covalent Structure of Rhodopsin

The insoluble nature of rhodopsin in aqueous solutions has complicated the molecular weight determination of this protein. Three methods appeared to have overcome this problem. First, quantitative amino acid analysis on column purified rhodopsin-detergent complexes place a maximum value of about 40,000 daltons of protein per retinal chromophore (Robinson et al., 1972; Daemen et al., 1972). Barring technical errors, this number should be regarded as a highly accurate maximum molecular weight. Second, opsin was solubilized in mixtures of 2-chloroethanol-pyridine or 2-bromoethanol-pyridine and subjected to a rigorous ultracentrifuge sedimentation-equilibrium determination of apoprotein molecular weight. This method indicates a value of 35,000 daltons (Lewis et al., 1974). The behavior of integral membrane proteins in such organic solvent mixtures is not known; conceivably artifacts such as oligomerization or abnormal solvent binding or particle interactions could have complicated the analysis of the sedimentation-equilibrium data. However, there is no proven flaw in the method, and the molecular weight obtained should be given reasonable credence. Third, low angle neutron scattering of hydroxylapatite-purified Ammonyx LO-rhodopsin solutions gave a value of 38,000 daltons of protein per retinal (Yeager, 1976). In this method, the solvent is contrast-matched to the detergent so that only the rhodopsin has an appreciable scattering-density contrast with the solvent. The scattering intensity at zero angle is a function of the molecular weight of those particles which have scattering-densities different from that of the solvent. Thus three independent methods define a reasonable range of 37,500 ± 2,500 for the molecular weight of rhodopsin. These data are summarized in Table I.

A number of investigators have determined the amino acid composition of rhodopsin, these data are summarized in Table III. In general, these compositions are in rough agreement, and indicate that rhodopsin has a high degree of hydrophobicity. This hydrophobicity explains the poor solubility of rhodopsin in aqueous solutions. However, rhodopsin is not a proteolipid; there is sufficient hydrophilicity to explain the equally poor solubility of rhodopsin in most organic solvent mixtures. These solubility properties have thwarted the use of traditonal methods of protein chemistry.

Purified rhodopsin does not have a reactive amino terminus (Heller, 1968) and its carboxy terminus has not been determined by any simple chemical method (Heller, 1968). Data are unavailable to determine rigorously the number of polypeptide chains per molecule. The molecular weight of rhodopsin is 37,500 ± 2,500. SDS-gel electrophoresis (Cavanagh & Wald, 1969; Robinson et al., 1972; Heitzmann, 1972; Daemen et al., 1972) suggest single polypeptide chain weights of this magnitude. It has not been shown that these "denaturing" conditions really separate the polypeptide chains of integral membrane proteins. It is probable, however, that rhodopsin consists of a single polypeptide chain.

Rhodopsin is a glycoprotein (Heller, 1968). The most recent determination places the sugar composition at 9 moles of mannose and 5 moles of N-acetyl glucosamine per molecule of rhodopsin (Platner & Kean, 1976). This is substantially higher than previous determinations (Heller, 1968; Heller & Lawrence, 1970) and the issue will not be entirely settled until the carbohydrate chain structure(s) are established.

Only two peptides of rhodopsin have been well characterized. One of these is a glycopeptide which contains a single oligosaccharide and accounts

for part of the sugar content (3 mannose and 2-3 N-acetyl glucosamine). The sequence reported is (Heller & Lawrence, 1970):

Met-Asx(CHO)-Gly-Thr-Glu-Gly-Pro-Asn-Phe

The second characterized peptide contains the retinal-lysyl Schiff's base reduced by sodium borohydride to form a secondary amine. This peptide, prepared by pronase digestion, is highly hydrophobic and was isolated by $CHCl_3$-MeOH chromatography on silica gel (Bownds, 1967a,b). It has not been purified to homogeneity, and apparently contains several peptides of overlapping lengths. Their full sequences are not known, but a partial sequence was reported as (Bownds, 1967a,b):

Phe (Phe, Ala, Lys)

No other peptide chemistry has been reported. The problem that has hindered much work is that the peptides of rhodopsin are more insoluble than the parent molecule. Extensive enzymatic or chemical cleavage usually produces material which is insoluble in aqueous solutions, in organic solvents, and in detergent solution. Traditional chromatographic and electrophoretic peptide separation methods leave greater than 90% of the amino acid residues at the origin. Further advances will require new techniques in protein chemistry.

Conformation of rhodopsin

Measurements of the conformation of rhodopsin have been restricted to the lowest resolution type of question: is the molecule spherical or elongated? The possibility of a non-spherical shape were first raised by measurements of fluorescence energy transfer efficiencies between fluorescent sulfhydryl alkylating agents as donors (which were assumed to react with a single, accessible cysteine residue called site A), and the 11-cis retinal group as an acceptor. The low efficiency of transfer between probes at site A and the retinal chromophore corresponded to a distance of over 75 Å (Wu & Stryer, 1972). Since a solid protein sphere of 40,000 daltons would have an expected diamter of 46 Å, this measurement suggested that rhodopsin must have a non-spherical shape to accommodate both sites in the same molecule. The most serious objections to this experiment are: (1) the fluorescence measurements were performed in digitonin solution, and the shape may be significantly different in digitonin than it is in the membrane; (2) the chemical characterization of the labeling site, particularly the homogeneous, covalent nature of probe binding, was not rigorously documented. Other objections to this work have been based on theoretical arguments about orientation anomalies between the dipole moments of the fluorescent donor and the 11-cis retinal acceptor (Abrahamson & Fager, 1973), but these objections were minimized by empirical measurements of donor mobility and the use of different donors at the same site (Wu & Stryer, 1972). The most important result of this work is that it led to serious consideration of non-spherical shapes for rhodopsin.

Dried digitonin solutions of rhodopsin appear to contain spherical micelles when examined by negative-stained electron microscopy (Abrahamson et al., 1974), but partially-dried digitonin-rhodopsin films can be oriented by shear forces, suggesting that the hydrated detergent-protein complex is elongated (Wright et al., 1972). Relaxation measurements of electrically perturbed detergent-rhodopsin solutions show at least two correlation times for the relaxation of the chromophore dipole (Wright, 1974; Wright, 1976). This may be interpreted as a mixture of a fast relaxation process over a restricted angular range superimposed upon a slower process over the full angular range, or by the relaxation of an asymmetric (i.e., non-spherical) particle. The least ambiguous measure of rhodopsin shape in detergent solution comes from analysis of neutron solution scattering at low angles from samples which have the detergent contrast-matched to solvent scattering density. The slope of the scattering intensity vs. scattering angle is used to determine a radius of gyration. The radius of gyration of rhodopsin in hydroxylapatite-purified Ammonyx LO solutions is about 23 ± 2 Å; a spherical shape would have a radius of gyration of only about 17 Å. This difference corresponds to an elongated shape for rhodopsin with axial ratios of about 3:1 for prolate-like particles (Yeager, 1976).

None of these measurements indicate what shape rhodopsin might have in the disc membrane. This is an important point. Rhodopsin in detergent solutions might retain its membrane-bound secondary structure but alter its native tertiary structure. Fluorescence energy transfer measurements could be performed on membranes if excess fluorescence reagent can be quantitatively removed; this has not been the case to date. The answer to this question will require future experimentation.

Two techniques have been applied to ascertain the secondary structure of rhodopsin, and the results obtained are contradictory. Circular dichroism spectra have been recorded from sonicated disc membranes and from purified rhodopsin in detergent (Crescitelli et al., 1966; Schichi, 1970, 1971a; Schichi & Shelton, 1974). The empirical features of such curves in the far UV suggest that rhodopsin is about 50% α-helical. The rate of tritium-hydrogen exchange has been examined on disc membrane suspensions, but not yet on purified rhodopsin (Downer & Englander, 1975). The observed kinetics suggest that rhodopsin has a very limited extent of internal hydrogen bonding of amide protons. Specifically, rhodopsin has only 30% of its amide hydrogen atoms internally hydrogen bonded compared with soluble proteins, such as myoglobin, which show 70% internal hydrogen bonding. A 30% internally hydrogen bonded structure is not compatible with a structure that is 50% α-helical. One possible explanation for this discrepancy is that some proteins, such as chymotrypsin, have structural features that produce CD spectra which overestimate α-helical content (Greenfield & Fasman, 1969). Rhodopsin might fall into this category. It is equally plausible that many of the hydrogen atoms observed by tritium exchange are not part of rhodopsin, but belong to some other component of the disc membrane. A note added in proof to a recent paper states that Raman spectroscopy indicates that opsin has "extensive α-helical structure," but this was not quantified (Rothschild et al., 1976). Until this controversy is settled, it is pointless to construct models which account for either type of data.

Surface Properties of Rhodopsin and the Organization of the Disc Membrane

Rhodopsin is an integral membrane protein. Part of the surface of rhodopsin must interact with the hydrophobic groups in the interior of a lipid bilayer membrane. Some portions of rhodopsin are also readily accessible to the aqueous region outside the lipid bilayer. Therefore, rhodopsin is a protein that exists in at least two types of environments, and the exposed surfaces of rhodopsin must be able to interact favorably with both. Closely related to the surface properties of rhodopsin are the questions of where the protein is located with respect to the lipid bilayer, and how the protein might perturb the local structure of the lipid portion of the membrane. The surface properties of the protein will define its short range interactions, and the organization of the protein in the membrane bilayer must be the product of these interactions. Experiments which approach any of these types of problems will provide information relevant to the others.

Three general experimental approaches have been used to examine the interaction of rhodopsin with its environment. The first of these methods is to study the detergent binding as a surrogate for phospholipid binding. The rationale for this type of experiment is the assumption that the most important structural feature of phospholipids is the amphiphilic nature of the molecule. As amphiphiles, detergents immediately adjacent to the protein should bind to the same types of surfaces as do phospholipids. The more long range preference of detergents form micelles rather than bilayers fashion to rhodopsin (Osborne et al., 1974; Clarke, 1975). Therefore, it is not legitimate to assume that all of the bound detergent is in contact

with the protein surface and one cannot directly calculate the amount of protein surface covered by detergent simply from the amount of the detergent bound. However, the information from this kind of calculation does place a maximum on the surface area of the rhodopsin covered by detergent. Triton X-100 covers a maximum of 76% of the surface of a spherical rhodopsin (Osborne et al., 1974). An elongated rhodopsin, the probable shape in detergent, would have a smaller percentage of its more extensive surface covered.

Neutron scattering from Ammonyx LO-rhodopsin complexes is a more elegant way of examining detergent binding to the surface of the protein. The radius of gyration of rhodopsin was obtained by neutron scattering from solutions for which the solvent and detergent scattering were contrast-matched. By performing similar experiments with solutions for which the solvent and protein (but not the detergent) are contrast-matched and with solutions for which neither detergent nor protein are contrast-matched to the solvent, the parallel axis theorem could be applied to measure the distance between the center of mass of bound detergent and the center of mass of the protein in a detergent-protein complex. The experimentally measured separation was greater than 20 Å, indicating that the surface regions which bind detergent must be highly asymmetric in their distribution (Yeager, 1976). This 20 Å difference between the center of mass of the detergent and the center of mass of the protein is the expected result if rhodopsin extends beyond the hydrophobic region of the lipid bilayer into the hydrophilic aqueous space on only one side of the membrane. It is not an expected result for a protein which is symmetrically located in the center of a lipid bilayer.

A second group of experiments probe the surface of rhodopsin which interacts with water by testing the chemical accessibility of specific sites

on the surface rhodopsin to water-soluble reagents. In the case of membrane proteins, it is assumed that if the modifying or binding reagent is confined by its solubility properties to the aqueous region outside the lipid bilayer, then any protein which group which is accessible must be on a surace of the molecule that is normally exposed to the aqueous region. The interpretation of such experiments requires that the solubility of the reagent absolutely restrict its location to the aqueous space. This condition is best fulfilled by water-soluble proteins. These macromolecules do not readily partition into membrane bilayers and are in fact highly restricted to the aqueous space. Antibodies directed against rhodopsin bind readily to antigenic determinants on disc membranes (Dewey et al., 1969; Blasie et al., 1969). However, these antibodies were not shown to be specific for rhodopsin and were not adsorbed against the non-rhodopsin antigenic determinants of the disc. Recent work with antibodies has not overcome these objections (Jan & Revel, 1974). There still has not been a convincing demonstration of the antigenic specificity of any antibody preparation and, therefore, there still has not been an adequately controlled test of the accessibility of rhodopsin antigens in disc membranes to antibody binding. Newer protocols offer some hope of obtaining and demonstrating highly specific antibodies (Papermaster et al., 1975).

The specific affinity of plant lectins for certain carbohydrate configurations has also been used to test for the accessibility of rhodopsin. Rhodopsin contains covalently attached mannose and N-acetyl glucosamine (NAG) (Heller, 1968). Detergent-solubilized rhodopsin can be bound to a column of Con A-agarose, and specifically eluted by addition of competing glucose. Fluorescent derivatives of lectins have been used to show that Con A and wheat germ agglutinin both bind to disc membranes in a manner that saturates

at 1 lectin monomer per rhodopsin. The binding is blocked by competing sugar. Furthermore, the two lectins show competitive binding, presumably due to steric hindrance (Steinemann & Stryer, 1973). These experiments suggest that some of the carbohydrate of rhodopsin is exposed to the aqueous region. However, the one to one stoichiometry could be fortuitous, and it was not demonstrated that the carbohydrate involved is attached to rhodopsin.

A more conclusive test of the accessibility of part of the carbohydrate of rhodopsin has been conducted with the enzyme galactosyl transferase. This enzyme catalyzes the transfer of a galactosyl group from UDP-galactose donor to an NAG acceptor. The carbohydrate of rhodopsin can be modified in disc membranes and the transferred galactose is associated with rhodopsin after SDS-gel electrophoresis, after Con A-agarose affinity chromatography, or after hydroxylapatite chromatography. The extent of galactose incorporation is greater than 1 mole of sugar per rhodopsin. This experiment does not prove that every rhodopsin is modified, but it does show that the carbohydrate of some rhodopsins is accessible to the aqueous space outside the lipid bilayer (Shaper & Stryer, in preparation).

Proteolytic enzymes have also been used as macromolecular probes of rhodopsin accessibility. Rhodopsin can be cleaved by proteolytic enzymes in disc membranes, but digestion is limited (Trayhurn et al., 1974a, 1974b; Saari, 1974; van Breugel et al., 1975). The fact that cleavage occurs demonstrates that some region of rhodopsin is accessible to the aqueous space. The limited degree of proteolysis can be interpreted as either partial protection by the lipid bilayer (i.e., lack of aqueous accessibility) or as

partial protection by protein folding. These possibilities cannot be distinguished. In summary. macromolecular probes have established that at least two sites (a galactose acceptor and an accessible peptide bond) are exposed to the aqueous space outside the membrane bilayer.

In addition to protein probes, a variety of small molecule reagents have been used to test the accessibility of rhodopsin to the aqueous space. These reagents usually have a finite partition into the hydrophobic region of the membrane, and are less reliable as indicators of location than the macromolecular reagents. This approach has been used for four types of reactive functions. First, the 11-cis retinal binding site has been probed with reducing agents. Sodium borohydride does not react with unbleached rhodopsin in disc membranes (Bownds & Wald, 1965; Akhtar et al., 1965), but claims have been made that sodium cyanoborohydride (Fager et al., 1972) and dimethyl borane (Hall, 1975) will reduce the Schiff's base between the 11-cis retinal and the amino group of a lysine residue in the rhodopsin molecule. However, the need to resort to hydrophobic reducing agents suggests that the Schiff's base is not accessible to reagents in the aqueous space. A second class of functional group probes which have been used are sulfhydryl-modifying reagents. The number of sulfhydryl groups which can be modified in rhodopsin in disc membranes depends on the reagent used (de Grip et al., 1973c; De Grip et al., 1975), but only two sulfhydryls can be modified without altering the spectral properties (and presumably conformation) of rhodopsin. Small reagents have also been used to test the accessibility of the carbohydrate of rhodopsin to the aqueous space. In light of the accessibility of the carbohydrate group(s) to macromolecules, it is not surprising that sodium periodate readily modifies the carbohydrate in disc membranes (Renthal et al., 1973). The last class of sites which have been modified are the amino

groups of rhodopsin. This has proved to be a non-specific modification, since many amino groups of rhodopsin readily react with various hydrophilic reagents in disc membranes (de Grip et al., 1973b; Raubach et al., 1974). The only unsuccessful attempt at amino group modification was attributed to a mucopolysaccharide coat that could be removed by washing at low ionic strength (Dratz et al., 1972; Dratz & Schwartz, 1973). The extent and ease of amino group modification suggests either that much of rhodopsin is located in the aqueous region outside the lipid bilayer or that rhodopsin has many of its hydrophilic residues segregated in a small portion of the molecule.

Other experiments have attempted to answer the general question of how rhodopsin is organized within the disc membrane. Is rhodopsin monomeric or oligomeric in the membrane? Neutron scattering showed that rhodopsin molecules are monodisperse in detergent solution, i.e., each 38,000 dalton rhodopsin was in a separate micelle from the other rhodopsins (Yeager, 1976). Nevertheless, it is possible that detergent solubilization has disrupted physiological interactions between membrane-bound rhodopsins, and that the normal structure of rhodopsin could be oligomeric. X-ray diffraction from strucures in the plane of the frog disc membrane show no regular repeating unit, but rather show a random distribution of rhodopsins on the surface of the membrane (Blaurock & Wilkins, 1969, 1972; Blasie & Worthington, 1969; Chabre, 1975). This result is not consistent with an oligomeric arrangement of rhodopsin molecules in the plane of the membrane.

Freeze-fracture electron microscopy has been used to observe rhodopsin both in disc membranes (Clark & Branton, 1960; Raubach et al., 1974) and in reconstituted phospholipid vesicles (Hong & Hubbell, 1972, 1973; Chen & Hubbell, 1973; Hubbell, 1975). The size and number of intramem-

braneous particles has been interpreted to indicate an aggregated rhodopsin structure. This possible discrepancy with the interpretation of the diffraction data is currently under investigation (Corless, manuscript in press, 1976). For the moment, the diffraction experiments are presumably more reliable indicators of the lateral arrangement of rhodopsins, and an oligomeric model should be discounted.

Is rhodopsin bound in a rigid lattice in the disc? Rhodopsin is free to rotate about an axis perpendicular to the plane of the membrane (Brown, 1972; Cone, 1972). Lateral diffusion within the plane of the membrane of frog rhodopsin has also been measured by following the migration of unbleached rhodopsin molecules into a zone of bleached molecules (Poo & Cone, 1973, 1974; Leibman & Entine, 1974). The diffusion coefficients have been used to calculate viscosities; the membrane has an average viscosity of 0.7 - 6.0 poise based on rotational diffusion and 2.0 - 4.0 poise based on lateral diffusion measurements (Poo & Cone, 1973, 1974). Motions perpendicular to the plane of the membrane and rotations about an axis parallel to the plane of the membrane have not been observed to occur. These experiments are probably the best evidence that rhodopsin is not locked into a rigid matrix, but is in a fluid mosaic-type structure.

How uniform is the viscosity of the lipid region? Cytochrome oxidase has been shown to immobilize a boundary of lipids within a more fluid sea (Jost et al., 1973). Rhodopsin does not immobilize spin-labeled lipids within a certain region, bur rather generally immobilizes all of the spin-labeled lipids in a bilayer (Hong & Hubbell, 1972). This effect probably correlates with the long range lipid arrangements detected by freeze-frac-

ture of vesicles containing rhodopsin (Chen & Hubbell, 1973). On the contrary, titrations of the extent of fluidity in the lipid by tempo partition suggest that there are patches of lipid that are more immobilized than others (Pontus & Delmelle, 1975a). Proton NMR is also reported to detect two classes of lipids (Dratz, 1975), but carbon-13 NMR does not confirm this observation (Millet et al., 1973). The degree of uniformity of the lipid viscosity is still an unresolved question.

In addition to these investigations of the arrangement of molecules in the plane of the membrane, there has been much research on the organization of components in the direction perpendicular to this plane. Freeze-fracture electron microscopy, X-ray diffraction and neutron diffraction all provide strong evidence that the lipid portion is indeed arranged as a bimolecular leaflet. Because 80% of the integral membrane protein is rhodopsin, perturbations in the bilayer pattern have been used to assign the location of this protein. In intact discs (or rods), only the outer leaflet of the bilayer contains "bumps" demonstrable by freeze-fracture (Raubach et al., 1974). The inner leaflet is smooth and may display "pits" upon etching (Chen & Hubbell, 1973). This asymmetric distribution between the two leaflets of the bilayer is lost in reconstituted bilayers (Hong & Hubbell, 1972, 1973). The simplest interpretation of this data is that rhodopsin is located in the lipid bilayer on the outer leaflet of the disc, and that it may extend partly into the inner leaflet to produce "pits."

Diffraction experiments offer a complementary approach. The natural stacking of discs in the rod is too disordered to obtain sufficient resolution to locate the protein by X-ray diffraction. Different laboratories have proposed electron density profiles that place rhodopsin in every

conceivable location in the membrane (Gras & Worthington, 1969; Blaurock & Wilkins, 1969, 1972; Corless & Longley, 1970; Corless, 1972; Webbs, 1972; Chabre & Cavaggioni, 1973; Schwartz et al., 1975). Some investigators have overcome the problem of disorder by artificially ordering discs in centrifugal fields (Blasie et al., 1965; Blasie & Worthington, 1969; Blasie, 1972) or by ordering whole rods in magnetic fields (Chabre, 1975; Chabre & Cavaggioni, 1975). Recently, centrifugally-stacked, water-washed discs give enough orders of diffraction to produce resolution at 7 Å. The interpretation of these data suggests that much of rhodopsin is outside the lipid bilayer on the outer face of the disc, and that the remaining portion of rhodopsin extends into the bilayer (Santillan & Blasie, 1975).

Neutron diffraction patterns of intact rod cells have been obtained at much lower resolution. The neutron scattering experiment is less subject to phasing problems because of the higher contrast among the various components (lipid, protein, water) of the retinal rod system and because of the ability to assign phases by D_2O-H_2O substitution. Interestingly, the interpretation of neutron scattering experiments is in rough agreement with the 7 Å resolution X-ray structure: much of rhodopsin is outside of the bilayer on the outside face of the disc membrane (Yeager, 1976). The remaining portion of the protein is seen to perturb the outer leaflet of the bilayer. Both kinds of diffraction experiments are subject to ambiguities. The X-ray patterns may have errors in choice of phases and the neutron patterns include too little of the total scattering to be certain of a unique solution. However, these methods are essentially independent, and so the similarity of results lends credibility to both interpretations.

In summary, it appears that a considerable portion of the rhodopsin molecule is accessible to the aqueous phase (chemical modification experiments) and that rhodopsin is found on only the outside face of the disc membrane (freeze-fracture experiments). The rhodopsin molecule extends well beyond the hydrophobic region of the outer leaflet of the bilayer into the aqueous medium (X-ray diffraction, neutron diffraction) on the outside face of the disc membrane. Rhodopsin may reach into the inner leaflet (freeze-fracture), but probably does not extend across the bilayer in a symmetric fashion (solution neutron scattering). Rhodopsin exists as a monomeric protein in disc membranes (X-ray scattering), possibly immobilizing some lipids more than others (spin-label experiments). Rhodopsin is free to move in the plane of the bilayer (lateral and rotational diffusion experiments), but motions in other directions are limited.

Conformational Changes After Photon Absorption

What changes in the conformation of rhodopsin are produced by photon absorbtion? Several events have been observed to occur upon bleaching in detergent solution that do not occur in membranes. Changes produced by bleaching in non-steroic detergents include alterations in the CD spectra (Schichi, 1971; Rafferty et al., 1972; Stubbs & Litman, 1975) and in spin-label mobility (Pontus & Delmelle, 1975b; Hubbell, 1975). The bleached protein aggregates and elutes earlier from gel filtration columns (Heller & Ostwald, 1972); bleaching also produces a decrease in detergent binding (Osborne et al., 1974). All of these changes are indicative of a large conformational change in some detergent solutions. These alterations are accompanied by loss of regenerability by 11-cis retinal. None of the above changes occur in steroid detergents or in disc membranes and so it is unlikely that these changes are physiologically important.

There is a second set of changes that occur both in non-steroid detergents and in digitonin, but not in disc membranes. The most prominent of these is the appearance of one or two newly titratable sulfhydryl groups upon formation of metarhodopsin II (Wald & Brown, 1952; Erhardt et al., 1966; Ostroy et al., 1966; Heller, 1968; Zorn & Futterman, 1971; de Grip et al., 1973c; Kimble & Ostroy, 1973). There have been some claims that the appearance of sulfhydryl groups is associated with sulfhydryl-disulfide exchange, but convincing evidence has not been presented (Williams, 1975, 1976). (The cysteine-cystine content of rhodopsin is itself a controversial question, but most recent data suggests 6 cysteines and 2 cystines per rhodopsin (de Grip et al., 1973c).) In digitonin, but not in

other degergents, pressure shifts the metarhodopsin I-metarhodopsin II equilibrium, indicating that there is a large change in volume during this transition (Lamola et al., 1974). Perhaps the same change occurs at a different step with other detergents.

The conformational changes which occur in disc membranes upon photon absorption are the hardest to detect. The most obvious indication of a change is the shifting of absorbance maxima as rhodopsin decays through the series of spectral intermediates to produce opsin and all-trans retinal. There is no difference between the ultraviolet CD spectrum of rhodopsin and of opsin in disc membranes (Schichi, 1971); the ultraviolet CD spectra of the metastable intermediates have not been reported. There is no change in the strength of the induced circular dichroism of the retinal chromophore through the formation of metarhodopsin II (Waggoner & Stryer, 1971). Bleaching produces a small change (one or two) in the total number of rapidly exchangeable protons, but there is no significant change in the overall rate of tritium exchange (Downer & Englander, 1975). There is no change in the mobility of exogenous spin-labels (Pontus & Delmelle, 1975b). There is no change in the accessibility of opsin to macromolecular probes such as lectins (Steinemann & Stryer, 1973) or proteases (Saari, 1974; van Breugel et al., 1975). There is no change in the number of titratible sulfhydryls (de Grip et al., 1973c). Some groups claim that an opsin phosphorylation site appears upon bleaching (Bownds et al., 1972; Kuhn & Dreyer, 1972; Frank et al., 1973; Kuhn et al., 1973, Weller et al., 1975; Chader et al., 1975), but one group has reported that both opsin and rhodopsin are equally well phosphorylated and attributes this discrepancy of observations to the presence of a light-activated phosphatase (Miller & Paulsen, 1975). In general, most methods do not detect evidence of a significant conformational change upon bleaching.

There are three solid pieces of evidence that a light-induced protein conformational change does indeed occur in disc membranes. First, the Schiff's base linkage between retinal and lysine becomes accessible to reduction by sodium borohydride (Bownds & Wald, 1965; Akhtar et al., 1965). Second, there is a proton taken up during bleaching (Broda & Victor, 1940; Radding & Wald, 1956; Matthews et al., 1963; Erhardt et al., 1966; Wong & Ostroy, 1973; Ostroy, 1974). Both of these changes occur in detergents as well as in disc membranes and both occur during the conversion of metarhodopsin I to metarhodopsin II. This step is a pseudoequilibrium at 4° C and the relative concentrations of metarhodopsins I and II can be altered by adjusting the pH. Third, there is an alteration in the physical organization of disc membranes detectable by X-ray diffraction (Blasie, 1972; Chabre, 1975; Santillan & Blasie, 1975), freeze-fracture electron microscopy (Chen & Hubbell, 1973; Mason et al., 1974) and optical birefringence (Liebman et al., 1974). None of these observations is readily interpreted in terms of detailed protein conformation. These data are summarized in Table IV.

A plausible explanation for the paucity of evidence of protein conformational change to accompany the large changes in absorption spectra is that much of the protein rearrangement is in the tertiary structure but not its secondary structure. That is, a small shift of atoms at the site of chromophore binding leads to large movements of segments of rhodopsin, but not to movements within the segments. Many of the techniques used to probe for alterations in conformation depend upon changes in secondary structure and upon concomitant changes in the environment of reporter groups. Motion largely restricted to tertiary rearrangements would not be readily

detected. There is ample precedent for such types of conformational change. Hemoglobin undergoes very large changes in tertiary and quaternary structure between the deoxygenated and oxygenated forms, but little change in secondary structure occurs (Perutz, 1970). The actual solution of this conformational problem awaits future investigation and is the ultimate aim of rhodopsin research.

B. SPECIFIC REVIEW: PROTEOLYSIS AS A PROBE OF RHODOPSIN STRUCTURE

Proteolytic enzymes have been used to study the structure of several water-soluble proteins. In general, a tightly-packed protein structure is highly resistant to attack to proteolytic enzymes, while a more unfolded conformation, with the same sequence, is much more susceptible to degradation. For example, the proteolytic enzyme subtilisin will readily cleave only one particular peptide bond in native ribonuclease A; the rest of the structure is resistant to further cleavage. The same enzyme will degrade denatured ribonuclease A into pieces no larger than tripeptides (Markland & Smith, 1971). Proteolytic activation of digestive enzymes such as chymotrypsin from a proenzyme depends on the limited extent of cleavage in native forms (Blow, 1971). In fact, the existence of region that is highly susceptible to proteolysis by enzymes of different specificity has often been the first clue that this portion of the protein has a special structure. The hinge region of skeletal muscle myosin, which connects the part of the molecule that forms the cross-bridge (heavy meromyosin) to the part of the molecule forms the thick filament (light meromyosin) was first discovered by its high degree of susceptibility to proteolysis (Szent-Györgyi, 1960). Similarly, the hinge region of antibodies, which connects the antigenic binding part of the molecule (F_{ab}) to the portion of the molecule that mediates immunologic responses such as complement fixation (F_c), was also initially demonstrated as a proteolytically-sensitive region (Porter, 1959). In the case of both of these proteins, subsequent measurements, including X-ray diffraction, have demonstrated the identity of the protease-sensitive region as a molecular hinge (Huxley, 1971; Edelman, 1973).

Proteolytic enzymes have been used to study integral membrane proteins as well. In many cases, these enzymes have been used as probes for accessibility of sites in membrane proteins to macromolecules confined to the aqueous space. A positive result is clearly interpretable: if the membrane protein can be partially degraded by a proteolytic enzyme, the membrane protein must be partially exposed to the aqueous space outside of the bilayer. Lack of proteolysis does not imply that the membrane protein is excluded from the aqueous region outside of the bilayer. Membrane proteins, like water-soluble proteins, are probably resistant to proteolysis unless they are partly unfolded.

The most interesting results obtained from proteolysis of membranes has been the demonstration that certain membrane proteins can be split into distinct hydrophilic and hydrophobic domains. In the cases of cytochrome b_5 from liver endoplasmic reticulum (Spatz & Strittmatter, 1971) and glycophorin in the human red blood cell (Marchesi et al., 1972), proteolytic enzymes release from the membrane intact segments of the protein with hydrophilic properties. In cytochrome b_5, this hydrophilic region contains the catalytic portion of the enzyme. In both cases, a hydrophobic core is left bound to the membrane. Accessibility to phosphorylating enzymes has shown that a portion of the remaining glycophorin actually extends across the bilayer into the aqueous space on the intracellular side of the bilayer (Marchesi & Shapiro, personal communication). There has been an expectation, among some workers, that all integral membrane proteins would be partitioned by proteolytic cleavage into hydrophobic and hydrophilic domains. It is probable that some other proteins will show this pattern, but there is no reason to expect it to be a general principle of structure for membrane proteins.

Recently, there has been a well-documented case of a different pattern of proteolytic cleavage for an integral membrane protein. The Ca^{++} pump protein of sacroplasmic reticulum membranes from skeletal muscle appears to have a molecular weight of 120,000, according to SDS gels, and is replaced, upon cleavage, by two membrane-bound peptides of about 55,000 and 45,000 daltons, respectively (Migala et al., 1973; Inesi & Scales, 1974; Stewart & MacLennan, 1974; Louis et al., 1974; Thorley-Lawson & Green, 1975). These fragments probably arise from a single split of the original polypeptide chain. Immunoanalysis has shown that each of these fragments contains different, non-overlapping antigenic determinants (Stewart, 1975). Sarcoplasmic reticulum membranes with an enzymatically-cleaved Ca^{++} pump still function to accumulate Ca^{++} at the expense of ATP. Both of these fragments are integral membrane proteins; that is, neither can be readily separated from the membrane. Upon prolonged proteolysis, the two initial fragments are each split into smaller pieces whose parent-precursor relationships were also established by immunoanalysis. Even at this later stage of degradation, little, if any, of this membrane protein is released from the membrane as soluble peptides.

Proteolytic analysis has added important information to molecular biology. Such an approach could contribute to our understanding of the structure of rhodopsin. Wald and Radding had used chymotrypsin on digitonin-extracted rhodopsin in 1958, but no means existed to analyze the products (Radding & Wald, 1958). On the basis of the rate of appearance of amino groups, they made the ingenious deducation that a slow, initial phase of proteolysis corresponded to limited cleavage at susceptible sites, and a more rapid, second phase of proteolysis, which was accompanied by

loss of 500 nm absorbance, was the result of degradation of more restricted bonds.

The advent of SDS-polyacrylamide gel electrophoresis made it possible to reexamine the action of proteases on rhodopsin by permitting rapid separation of the proteolytic products. The staining patterns of gels showed differences in the position of the major bands before and after proteolytic cleavage. The first experimental account of rhodopsin cleavage was presented by Trahurn, Mandel and Virmaux (1974a). These authors reported that papain produced a shift in the major Coomassie blue staining band of 0.1-0.15% SDS extracts of bovine disc membrane centrifugal pellets. They observed apparent molecular weights of 38,000 and 25,000 for opsin and their cleaved fragment respectively. These apparent molecular weights depend on the conditions of sample preparation and electrophoresis; such numbers are not comparable between research groups. No analysis was presented of what material was released from the membrane or remained in the SDS-extracted pellet.

In a second report (Trayhurn et al., 1974b), the same authors showed that their 25,000 dalton fragment was PAS positive and therefore presumably retained at least some of the carbohydrate content of rhodopsin. The rhodopsin in papain-cleaved disc membranes retained 500 nm absorbance and was regenerable after bleaching by exogenous 11-cis retinal. These authors attributed the 500 nm absorbance to a complex of their carbohydrate bearing fragment and 11-cis retinal. They did not demonstrate such an association by purifying the putative complex. When Trayhurn et al. isolated their fragment by preparative SDS-gel electrophoresis, they found it had an amino acid composition similar to that of whole rhodopsin isolated by the same method. Since the 280 nm absorbance of the membrane was unchanged

by papain cleavage, they assumed that all of the tryptophan residues in rhodopsin were retained by their fragment; this was not examined by quantitative alkaline hydrolysis of their purified fragment.

A more extensive account of proteolytic treatment of rhodopsin in bovine disc membranes was presented by Saari (1974). This author showed that thermolysin, subtilisin and chymotrypsin all produced changes in the electrophoretic mobility on SDS-gels of the major Coomassie blue staining protein of disc membrane pellets. Saari did not analyze the supernatant for released material, but he did dissolve the complete disc membrane pellet for his SDS-gel analysis. Thermolysin converted the major Coomassie blue staining band from an apparent molecular weight of 35,000 to one of 29,000. Subtilisin and chymotrypsin did the same, but subsequently degraded the 29,000 apparent molecular weight band to a series of smaller pieces, ranging in apparent molecular weights from 20,000 to 25,000. All except the smallest subtilisin fragment was PAS positive. Trypsin failed to cleave rhodopsin.

Saari confirmed that proteolysis of rhodopsin in disc membranes did not cause loss of 500 nm absorbance or its regenerability. He used Con A-agarose affinity columns to purify CTAB-solubilized thermolysin-cleaved rhodopsins, demonstrating that cleaved-rhodopsins do retain at least part of the carbohydrate content of whole rhodopsin. Saari used the cleaved-rhodopsins which he had purified to obtain amino acid compositions, and found they were similar to whole rhodopsin. He did not measure the size of cleaved rhodopsin. Furthermore, he did not analyze the products of his column purifications on SDS-gels. This was an important oversight. Saari, too, argued that the 500 nm absorbing species was a complex of his

carbohydrate-bearing fragment and 11-cis retinal, but he never demonstrated that his Con A column had purified such a complex. Finally, Saari noted the variable appearance of a Coomassie-staining band at less than 20,000 apparent molecular weight.

About the same time these reports were being made, a third group, van Breugel, Bonting, and Daemen, presented some preliminary communications to suggest that pronase extensively cleaved rhodopsin in bovine disc membranes into many small fragments of less than 10,000 daltons without loss of 500 nm absorbance (Daemen et al., 1974). They have more recently indicated that this result was caused by a failure to arrest pronase degradation in SDS, and that much of the proteolysis followed solubilization. This group now reports that pronase, chymotrypsin and subtilisin cleave rhodopsin in disc membranes into a large fragment with little release of material from the membrane (van Breugel et al., 1975). These fragments appear to be similar to the ones Saari reported. The properties of these proteolytic enzymes are summarized in Table V and a brief summary of the various reported results with rhodopsin is presented in Table VI.

Van Breugel et al. (1975) tried to locate the 11-cis retinal binding site by using sodium borohydride reduction of the chromophore in disc membranes. Previous experiments, some by this group, have shown that borohydride reduction conducted on disc membranes is subject to considerable loss of specificity through transimination of the retinal to other amines (Daemen et al., 1971; Fager et al., 1972; de Grip et al., 1973a; Rotmans et al., 1974). Every band on their gels exhibited N-retinyl fluorescence, including opsin, their large fragments, and a faintly staining band at 15,000 apparent molecular weight. This ubiquity of fluorescence suggests that the

reduction did not specifically bind the chromophore to the physiological binding site.

Van Breugel et al. also reported that rhodopsin molecules in digitonin, Triton X-100, Emulphogene BC 720 or CTAB detergent solutions or in disc membranes pretreated by phospholipase C are susceptible to extensive pronase degradation. These results suggest that either the phospholipids provide steric protection of additonal susceptible bonds or that they stabilize a conformation which is less susceptible to proteolytic attack.

This summary establishes the framework for my own experimental work. The prevailing interpretation, given by other workers, implied that rhodopsin is a protein similar in structure to human red blood cell glycophorin. According to this view, a substantial portion of the molecule extends into the hydrophilic aqueous space, outside the membrane, where it is readily clipped off by a variety of proteolytic enzymes. A core, containing the retinal binding site, and, interestingly, some hydrophilic carbohydrate groups, is left protected in the membrane. As was pointed out, much of this interpretation was inferential and none of the work was quantitative. The following chapters outline a chain of evidence that leads to a very different conclusion.

CHAPTER II

THERMOLYSIN CLEAVES RHODOPSIN IN DISC MEMBRANES INTO TWO LARGE FRAGMENTS

Rhodopsin in disc membranes has been shown to be susceptible to proteolytic cleavage by a number of enzymes (Trayhurn et al., 1974a, 1974b; Saari, 1974; van Breugel et al., 1975). The work on thermolysin cleavage of rhodopsin presented in this chapter is the first quantitative account of such work. These data comprise one of the most extensive characterizations of fragments produced by proteolytic degradation of any membrane protein, and are consistent with only one simple hypothesis: thermolysin splits each rhodopsin polypeptide to generate two large membrane-bound fragments.

A. Methods

Bovine retinas were obtained from Hormel. Thermolysin was purchased from Calbiochem. Ammonyx LO was a gift from the Onyx Chemical Company. SDS was purchased from BDH Poole (Gallard Schlessinger), Triton X-100 from Eastman, CTAB from Analabs. Hydroxylapatite, acryalmide and bis acrylamide came from BioRad. N-(iodoacetamidoethyl)-1-aminonaphthalene-5-sulfonic acid (IAENS) and ^{14}C-galactose-labeled rhodopsin in disc membranes were both gifts from Dr. Joel Shaper. Dansyl chloride in acetone and dansyl amino acid standards were purchased from Pierce, and Chen-Chin polyamide plates from Gallard Schlessinger. Sodium borohydride, dithiothreitol, β-mercaptoethanol, and dansyl hydrazide were obtained from Sigma. All of the radioactive isotopes and Aquasol liquid scintillation cocktail were supplied by New England Nuclear. All other chemicals were reagent grade.

Disc membrane preparation

The bovine retinal disc membranes used in these studies were prepared from the frozen bovine retinas by a modification of the sucrose flotation method (McConnell, 1965; Bownds, 1967a). All steps were conducted at 4° (on ice) and under dim red light. Fifty frozen retinas were thawed and gently suspended by stirring in 80 ml of 40% sucrose (weight/volume) in 0.067 M sodium phosphate buffer, pH 6.5. The suspension was poured into two 50 ml polyethylene centrifuge tubes and spun for 15 min at 17,000 rpm in a Sorvall SS-34 rotor. The supernatant, containing hemoglobin, was discarded. The retinal pellets were resuspended in 60 ml of buffered 40% sucrose and transferred to a 250 ml Erylenmeyer flask. The contents of

of the flask were rapidly swirled for up to 1 min to detach the rod cell outer segments from the rest of the retina. Care was taken not to cause foaming of the suspension. The contents of the flask were returned to the two 50 ml centrifuge tubes and 3 ml of sucrose-free buffer (0.067 M sodium phosphate, pH 6.5) were carefully layered over both sucrose phases. The tubes were spun at 17,000 rpm in the SS-34 rotor for 30 min. The detached rod outer segments, which floated to the interface and adhered to the side of the tube, were removed by suction through an 18 gauage needle into a 5 ml glass syringe. The harvested rods from both tubes were combined in one centrifuge tube, diluted with sucrose-free buffer to a volume of 40 ml, and spun at 17,000 rpm for 20 min. The supernatant was discarded leaving a pellet greatly enriched for rod outer segment disc membranes. These rods were further purified by three additional flotations to the interface between phosphate-buffered 40% sucrose and sucrose-free buffer. Following the first flotation, the harvested interface was hand homogenized with a size C Thomas tissue grinder to detach inner segment contaminants. The final harvest was washed three times with sucrose-free phosphate buffer, and stored in the dark, frozen as a pellet.

The absorption spectrum of each outer segment preparation was recorded with a Cary 15 spectrophotometer. An aliquot of suspended membranes in 0.067 M sodium phosphate, pH 6.5, was mixed with an equal volume of 1.4% CTAB in the same buffer and briefly centrifuged to reduce scatter. The absorbance ratio of A_{270}/A_{500} typically varied between 2.5 and 2.9 and averaged 2.7 for all membranes used in this study. These ratios were obtained without regeneration of bleached protein, and indicate that the membranes are at least as pure as those produced by other

procedures from frozen retinas. The typical yield was 9-10 nmoles of rhodopsin/frozen retina.

Regeneration of 500 nm absorbance

Regeneration of the 500 nm absorbance of rhodopsin was performed by addition of a 1-2 fold molar excess of 11-cis retinal over rhodopsin (Hubbard et al., 1971). One volume of 11-cis retinal in ethanol was added to one hundred volumes of disc membranes suspended in 0.067 M sodium phosphate, pH 6.5, containing 1 mM β-mercaptoethanol or 1 mM dithiothreitol. The membranes may be partly or completely bleached prior to the addition of retinal. The addition of 11-cis retinal and all subsequent steps were performed under dim red light. The reaction was allowed to proceed at room temperature for up to 3 hrs, and was terminated by addition of hydroxylamine to a final concentration of 10 mM. The extent of regeneration, assayed by comparing the change in 500 nm absorbance (ΔA_{500}) in a detergent-solubilized sample of membranes produced by bleaching both before and after regeneration, is given by

$$\% \text{ regeneration} = \frac{\Delta A_{500} \text{ regenerated}}{\Delta A_{500} \text{ initial}} \times 100$$

Thermolysin cleavage of rhodopsin

Thermolysin cleavage of rhodopsin in disc membranes was performed by adding thermolysin to an unbleached disc membrane suspension at a concentration of 5 mg rhodopsin per ml of 10 mM tris-acetate, pH 7.4, containing 5 mM calcium chloride (Saari, 1974). All procedures were performed under

dim red light at room temperature. Thermolysin depends on calcium ion for activity. To avoid the precipitation of calcium-phosphate, the disc membranes, which were stored in sodium phosphate buffer, were equilibrated with 10 mM tris acetate buffer prior to proteolysis. The weight ratio of thermolysin to rhodopsin was 1:50. Proteolysis was terminated by adding sodium EDTA to a final concentration of 20 mM and by centrifuging the reaction mixture at 17,000 rpm for 30 min. The supernatant was carefully removed from the membrane pellet and both fractions were analyzed as described below. Occasionally, bleached disc membranes or 0.5% Triton X-100 solutions of purified rhodopsin were substituted for unbleached disc membranes at the same protein concentration.

AENS labeling

AENS-rhodopsin was produced by adding 2 mg/ml of IAENS to a suspension of disc membranes at a concentration of 5 mg rhodopsin/ml of 0.067 M sodium phosphate buffer, pH 6.5 or 7.0 (Hudson & Weber, 1973; Wu & Stryer, 1972). The rhodopsin was allowed to react 2 hrs at room temperature in the dark, and the labeling was stopped by removal of the reagent from the membranes by centrifuging 5 x in phosphate buffer containing 5 mM cysteine or 5 mM dithiothreitol. Membranes containing AENS-rhodopsin were subsequently treated in the same manner as unmodified membranes.

N-retinyl labeling

N-retinyl opsin or thermolysin-produced opsin fragments were produced by sodium borohydride reduction of the retinal chromophore to its protein binding site (Hubbard et al., 1971). This was accomplished by adding 10 mM sodium borohydride to rhodopsin or thermolysin-cleaved rhodopsin in 5% Triton X-10(

or 2.5% SDS solution at a concentration 1-2 mg rhodopsin/ml and by exposing the borohydrdide-containing sample to light for 2 min at room temperature. The 500 nm absorbance of rhodopsin in 0.5% Triton X-100 solution is constant in the dark, indicating that structure of rhodopsin is preserved. However, the presence of this non-ionic detergent causes considerable aggregation of opsin during subsequent SDS-polyacrylamide gel electrophoresis. Little aggregation of opsin was produced during reduction in SDS. Although the 500 nm absorbance of rhodopsin is lost in 2.5% SDS solution at room temperature in several minutes, even when the sample is kept dark, the retinal group could still be reduced at its binding site in SDS solution if sodium borohydride was added within one minute of solubilization of the membrane. The specific reduction of the retinal group to its binding site was lost if borohydride was added more than 15 min after solubilization. Except for the absence of the Triton-produced high molecular weight fluorescent aggregates, the fluorescent band pattern of SDS-polyacryalamide gels was the same for samples reduced in SDS as it was for samples reduced in Triton X-100. Reduced samples were subjected to SDS-polyacryalmide gel electrophoresis as described below.

Periodate-dependent labeling

Sodium periodate was used to create reactive aldehyde groups on rhodopsin that could be subsequently labeled (Renthal et al., 1973). Disc membranes were treated with 0.1 M sodium acetate buffer, pH 5.0, at 4° under dim red light. Periodate will oxidize vicinal diol groups, as in saccharides, to dialdehydes under such conditions. Oxidation was terminated by addition of 1.5% sodium arsenite and removal of periodate from the membranes by centrifugation. The oxidized membranes were labeled either

with a ten-fold molar excess of dansyl hydrazide over rhodopsin in 0.1 M sodium acetate, pH 5.0, or with 2.5 mM sodium borohydride in 0.1 M sodium phosphate, pH 7.0. These labeling reactions were stopped by removal of the reagents from the membranes by centrifugation. Labeled membranes were subsequently treated in the same fashion as unmodified membranes.

SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis was performed with 0.6 cm diameter tube gels by the method of Weber and Osborn (1969), by the method of Fairbanks et al. (1971), or by the SDS-polyacrylamide gradient slab-gel method of Laemmli (1970). Most experiments used the method of Weber and Osborn modified as follows: tube gels containing 0.2% SDS, 0.11 M sodium phosphate, pH 7.2, 10% acrylamide monomer, and 0.25% bis acrylamide were polymerized by Temed-ammonium persulfate catalysis. Samples for electrophoresis were prepared by mixing up to 2.5 mg protein/ml of 10 mM tris-acetate, pH 7.4 with an equal volume of 5% SDS, 5% β-mercaptoethanol, 10% sucrose and 0.5 mM EDTA in the same buffer (Papermaster & Dreyer, 1974). The mixture was incubated at 37° for 30 min. A 25 to 100 µl aliquot was immediately applied to a gel and subjected to electrophoresis at 4-10 mamps/gel. Electrophoresis was continued until 5 µl of 0.025% bromophenol blue tracking dye migrated 8-10 cm. Gels were fixed and stained with Coomassie brilliant blue (Fairbanks et al., 1971).

Fluorescence gel scanning

Gels containing fluorescent-labeled polypeptides were fixed by shaking in 25% isopropanol, 10% acetic acid and scanned in an apparatus designed and constructed by Mr. Mark Yeager and Mr. Gerald Johnson. Excitation wavelengths were chosen by a monochromator and emission was recorded through Corn-

ing glass filters. N-retinyl fluorescence was excited at 333 nm and AENS fluorescence was excited at 360 nm; emission from both labels was detected through a 3-72 filter. The gels were subsequently stained with Coomassie brilliant blue (Fairbanks et al., 1971)

Radioactive gel slicing and counting

Gels containing radioactive samples were frozen and sliced with a template containing evenly spaced razor blades, either before or after fixation and staining. Each gel slice was dissolved by incubation at 50° C in 0.5 ml of 30% hydrogen peroxide for at least 3 hrs. The dissolved gel sample was counted in 10 ml Aquasol using a Unilux IIA Nuclear Chicago liquid scintillation counter.

Quantitation of cleavage products

Quantitation of cleavage products was performed by several methods. The number of residues released from the disc membranes by proteolysis was assessed by 6 N HCl hydrolysis for 24 hrs at 110° in vacuuo of membrane centrifugal pellets and supernatants followed by amino acid analysis (Spackman et al., 1958) on a Durrum D-500 one column microanalyzer. One drop of 5% phenol was added to the hydrolysis tubes to prevent chlorination of the tyrosines. Following hydrolysis, the samples were evaporated to dryness under vacuum and dissolved in buffer for application to the amino acid analyzer. The distribution of amino acid residues remaining in the membrane pellets was assessed by 6N HCl hydrolysis of an aliquot of the material applied to an SDS gel as well as hydrolysis of excised, fixed and stained gel bands. Hydrolyzed acryalmide formed a lump upon cooling, which was extracted 3 times with 6N HCl to remove trapped amino acids.

The pooled extracts were evaporated to dryness under vacuum and dissolved for amino acid analysis. The analysis was stopped after the elution of lysine to avoid the huge ammonia peak resulting from the hydrolysis of acrylamide. The more rapidly eluted amino acids give reasonable quantitative estimates of the number of residues in each band.

Polypeptides were eluted from macerated slices of unfixed gel bands by 3 times extraction in 10 volumes of 0.2%SDS (Weber et al., 1972). The pooled extracts were concentrated 10 times by lyophilization. The number of amino groups could be directly determined by alkaline hydrolysis with 2N sodium hydroxide for 30 mins in an autoclave (Hirs et al., 1965), followed by fluorescamine quantitation. The procedure for fluorescamine quantitation involves dilution and pH adjustment to pH 9.0 of the sample with sodium borate buffer, and reaction of the sample with an equal volume of 0.2 mg/ml fluorescamine in acetone. The relative fluorescence, which is proportional to the number of amino groups in the sample, was measured with a spectrofluorimeter. The samples were excited with light at 375 nm and the emission was observed at 490 nm.

Complete amino acid analysis

The extracted polypeptides corresponding to opsin and to the major cleavage products were subjected to complete amino acid analysis (Weber et al., 1972). The SDS was removed from concentrated samples by dilution with 9 volumes of acetone. After 3 hrs of precipitation in the cold, the samples were centrifuged and the SDS-containing supernatant was separated from the polypeptide pellet. The acetone extraction was repeated once more, and the polypeptide pellet was washed with deionized water once to remove salt. The salt-free pellet was hydrolyzed with 6N HCl as described above and subjected to amino acid analysis.

Amino terminal analysis

SDS-polyacryalmide gel purification of polypeptides was also used to test for amino termini of rhodopsin and the major thermolysin-cleavage products. Whole or cleaved disc membranes were dissolved in 7% SDS in 0.1 M sodium phosphate pH 7.0, at a concentration of 5 mg rhodopsin/ml. One tenth volume of dansyl chloride in acetone (10 mg/ml) was added and the mixture was incubated at 37° overnight. The dansylated polypeptides were subjected to SDS-polyacrylamide gel electrophoresis as described above, and the fluorescent bands were extracted as usual. The acetone precipitated, water-washed polypeptides were hydrolyzed in 6N HCl for 10 hrs at 110° *in vacuuo* and evaporated to dryness. Dansyl amino acids were identified by thin-layer chromatography on polyamide plates using the following solvent systems: I - 1.5% formic acid; II - benzene-acetic acid 9:1; and III - ethyl acetate - methanol - acetic acid (18:1:1) (Woods & Wang, 1967). The residue following hydrolysis was applied to a corner of a 5 cm x 5 cm plate in 50% pyridine, and subjected to chromatography in solvent system I. After drying the plate, chromatography was conducted in the perpendicular direction using solvent system II. When necessary for resolving certain pairs of dansyl amino acids, solvent system III was subsequently used in the same direction as solvent system II. Identifications of amino termini was made by comparing the migration of dansylated products with that of dansylated amino acid standards.

Hydroxylapatite chromatography of rhodopsin

Hydroxylapatite purification of rhodopsin and modified-rhodopsins was conducted by a step-gradient modification of the Applebury *et al.* (1974)

procedure developed by Mr. Mark Yeager. All steps were performed at 4° under dim red light. Disc membranes were extracted overnight with 3% Ammonyx LO in 10 mM imidazole-chloride, pH 7.0 containing 10 mM dithiothreitol. The rhodopsin-rich extract was applied to a hydroxylapatite column equilibrated with 1% Ammonyx LO in 10 mM imidazole chloride, pH 7.0 containing 2 mM dithiothreitol. The column was washed with 1% Ammonyx LO in 35 mM sodium phosphate, pH 7.0 2 mM dithiothreitol until the absorbance of the eluate at 280 nm returned to baseline. This step removed the phospholipids and much opsin. The rhodopsin was eluted by changing the column buffer to 1% Ammonyx in 150 mM sodium phosphate pH 7.0, 2 mM dithiothreitol. The 500 nm absorbing fractions were pooled as purified rhodopsin. The ratio of absorbance at 280 nm to that at 500 nm was usually less than 1.9.

Amino sugar analysis

Amino sugar composition was determined using a hydroxylapatite purified modified-rhodopsin which contained ^{14}C-galactose covalently attached the carbohydrate portion of rhodopsin by the action of the enzyme galactosyl transferase (Shaper & Stryer, in preparation). Disc membranes containing ^{14}C-galactose-rhodopsin were a generous gift of Dr. Joel Shaper. Aliquots of hydroxylapatite purified ^{14}C-galactose-rhodopsin were counted in 10 mls of Aquasol on a liquid scintillation counter to determine the number of radioactive counts per mole of rhodopsin. Other aliquots of the ^{14}C-galactose-rhodopsin were extracted with 6 volumes of ethanol-ether (3:1) to remove detergent. The precipitated protein was re-extracted one time with ethanol-ether (3:1) and washed with water to remove salt. The salt-free precipitate was hydrolyzed with 6N HCl at 110° C *in vacuuo* for 24 or 48 hrs or with 4N HCl at 100° C in vacuuo for 4, 6, 8, 10 or 12 hrs

The hydrolyzed samples were evaporated to dryness under a vacuum and analyzed for amino acids (in the case of 6N HCl hydrolysates) or amino sugars (in the case of 4N hydrolysates). 50 µl aliquots of the samples applied for analysis were counted in duplicate in 10 ml of Aquasol. The number of amino sugars were determined at various times of hydrolysis. The interpolated maximum amino sugar content was used to quantify the total amino sugar content of rhodopsin by comparing the ratio of amino sugars to the radioactivity with the specific activity of the ^{14}C-gal-rhodopsin determined above.

Amino sugar determinations on the products of thermolysin-cleavage were conducted on ^{14}C-galactose-containing polypeptides extracted from SDS-polyacrylamide gels. In this case, only enough material was obtained for one 6N HCl hydrolysis at 110° C for 24 hrs in vacuuo and for one 4N HCl hydrolysis at 100° C for 8 hrs in vacuuo.

B. Results

SDS-gel analysis shows two major cleavage products

The Coomassie blue staining pattern of the membrane proteins following SDS-acrylamide gel electrophoresis was altered when retinal disc membranes were treated with thermolysin. The single major protein band, identified as opsin in Fig. 2, was replaced by two new bands of higher electrophoretic mobility, called F1 and F2. The kinetics of appearance of F1 and F2 are the same as those of the disappearance of opsin; this pattern did not change any further with longer times. F1 and F2 appear to be fragments derived from the cleavage of rhodopsin by thermolysin. A second result which can be seen in Fig. 2 is that the small amount of aggregated material which did not enter the gel in the untreated membranes has disappeared completely within 15 min after adding thermolysin. This aggregated material does not exhibit N-retinyl fluorescence following sodium borohydride reduction, and thus may represent proteins in retinal disc membranes other than rhodopsin. Bleaching of retinal disc membranes prior to thermolysin treatment does not alter the products, rate, or extent of cleavage. Fragments with the same electrophoretic mobilities as F1 and F2 are produced when rhodopsin in Triton X-100 solution is cleaved with thermolysin (Fig. 3).

There is a linear relationship between the electrophoretic mobility of water-soluble proteins on an SDS-polyacrylamide gel and the logarithm of their molecular weights. By calibrating a gel experiment with standard proteins of known molecular weights, the molecular weight of an unknown protein can be estimated from its electrophoretic mobility. The theoretical basis for this relationship involves both the refolding of all soluble pro-

teins in SDS to some hydronomically equivalent form and the relatively constant weight to weight binding of SDS monomer by these proteins (Reynolds & Tanford, 1970). Integral membrane proteins do not necessarily refold in SDS and show micellar SDS binding rather than monomeric SDS binding. SDS-gels can be used to analytically separate such proteins, but should be used to estimate their molecular weights only with extreme caution. SDS-gel electrophoresis performed under the conditions of Weber and Osborn (1969) shows apparent molecular weights of 38,000, 30,000, and 18,000 for opsin, F1 and F2 respectively. SDS-gel electrophoresis performed under the conditions of Fairbanks, Steck and Wallach (1971) shows apparent molecular weights of 35,000, 25,000 and 12,000 for these same fragments. Molecular weights as low as 10,000 can be seen for F2 in some SDS-gel systems. Thus there are empirical as well as theoretical reasons not to rely upon apparent SDS-gel molecular weights for rhodopsin and its fragments.

Recently, the products of thermolysin cleavage have been examined on the Laemmli (1970) SDS-gel system which uses a continuous gradient of acrylamide to improve resolution. Thermolysin treatment of disc membranes produces a very rapid, but very slight shift of opsin to a new position of higher electrophoretic mobility. This shift was first described by W. Hubbell and interpreted by him to be the release of about 4,000 daltons of protein (personal communication). Following this shift, thermolysin cleavage produces two closely spaced bands at the expected position of F1 and three closely spaced bands at the expected position of F2. This resolution of F1 and F2 into different bands may indicate multiple cleavage sites, may indicate pre-existing microheterogeneity in the charge or composition

of the opsin chain, or may indicate differential SDS binding and variations in the degree of protein unfolding. While F1 and F2 will be referred to as homogeneous polypeptides, it is quite possible that both of these bands contain a number of closely related peptides differing slightly in length.

In three experiments, thermolysin proteolysis liberated into the supernatant about 25% of the amino acid residues initially bound to the retinal disc membrane pellet as determined by amino acid analysis. There was no macromolecular species detectable by SDS-acrylamide gel analysis of the supernatant, and most of these residues probably arise from the degradation of proteins other than opsin. In three determinations by quantitative amino acid hydrolysis of fixed and stained gel bands, F1, F2 and residual opsin accounted for greater than 70% of the amino acid residues in the SDS solubilized membrane pellet applied to an SDS-acrylamide gel. (Most of the remaining residues were accounted for by the aggregates at the top of these heavily overloaded gels.) It was not feasible to analyze every slice of an SDS gel because of the limited access to an amino acid analyzer. The acrylamide releases far too much ammonia under alkaline or acid hydrolysis conditions to permit analysis by some general amino group quantitation. This problem was circumvented by the following experiment: Unfixed gels, containing opsin or cleaved opsin, were completely sliced and each slice was completely extracted with 0.2% SDS solution. The alkaline hydrolysate of each extract was assayed for amino groups by fluorescamine quantitation. Only the gel slices containing F1 and F2 showed any significant number of amino groups, and the number of amino groups in F1 plus F2 roughly corresponded to the number lost by opsin

in the undigested control. Both the determinations from fixed and stained gels and the determination from extracts of unfixed gels indicate that F1 contains about 2.5 ± 0.5 times as many amino acid residues as does F2.

Thermolysin-cleaved rhodopsin retains 500 nm absorbance and its regenerability

Thermolysin-treated retinal disc membranes still appear red under room light. Detergent extracts of proteolyzed membranes compared to untreated controls show that the absorption spectrum of rhodopsin is unchanged and that no absorbance at 500 nm was lost during thermolysin treatment (Fig. 4A). Furthermore, thermolysin-treated retinal disc membranes, which have been bleached by light show approximately the same capacity for regenerating the rhodopsin absorption spectrum following addition of 11-*cis* retinaldehyde as do unproteolyzed disc membranes (Fig. 4B). Thus, thermolysin-cleaved rhodopsin retains the 500 nm absorption band and is regenerable.

Opsin, F1 and F2 have similar amino acid compositions

Reproducible amino acid compositions from opsin, F1 and F2 were obtained with polypeptides extracted from unfixed SDS-acrylamide gels. The location of these bands was detected by prelabeling the SDS solubilized proteins with a fluorescent reagent such as dansyl chloride or fluorescamine (which would alter the lysine content) or more commonly with IAENS (which would alter the cysteine content). These composition results are summarized in Table VII. It is striking that F1 and F2 have compositions which closely resemble each other and that of whole opsin. Minor differences may be seen in the mole percentages of leucine and lysine.

Tryptophan and cysteine cannot be determined after 6N HCl hydrolysis. Reproducible cysteine compositions were not obtained from dithiothreitol reduction and alkylation iodoacetic acid because of variability in the extent of alkylation and inadequate resolution of carboxymethylcysteine from aspartic acid on the Durrum D-500 amino acid analyzer. Reproducible cysteine compositions were not obtained from performic acid oxidation, possibly because of other material present in extracts from SDS gels. No attempt was made to determine the tryptophan content by alkaline hydrolysis. Because the gel bands were localized by fluorescent markers, it was not possible to estimate the tryptophan content by fluorescence. The cysteine and the tryptophan compositions might be of particular interest if either residue showed significant asymmetric distribution between F1 and F2. This line of investigation was not pursued further.

F1 contains all of the amino sugar of rhodopsin

The only amino sugar detected upon hydrolysis of opsin is glucosamine. Rhodopsin was initially reported to have an N-acetyl glucosamine content of 3 moles hexosamine/mole rhodopsin (Heller, 1968; Heller & Lawrence, 1971). A more recent report has been published which estimates the glucosamine content as 5 moles (Plantner & Keen, 1976). In the following experiments, a radioactive covalently-bound marker, ^{14}C-galactose, was used to standardize determinations of the glucosamine content of rhodopsin as indicated in the Methods section. The glucosamine content was 5-7 moles of hexosamine/mole of rhodopsin in two determinations by a 4N HCl hydrolysis time course. It was not tested whether the glucosamine is N-acetylated.

There was insufficient material isolated by SDS-gel electrophoresis to perform a complete time course of hydrolysis as was done with hydroxyl-

apatite-purified opsin. However, the number of moles of hexosamine/opsin and the number of moles of hexosamine/opsin fragment were compared under the same hydrolysis conditions. In such experiments, F2 never gave any indication of glucosamine content. All of the ^{14}C galactose bound to opsin appears to remain bound to F1 and the ratio of the number of moles of glucosamine on F1 to the radioactivity equaled the ratio of the number of moles of glucosamine on opsin to the radioactivity, suggesting that all 5-6 moles of glucosamine in opsin are bound to F1.

F1 has a blocked amino terminus; F2 has an alanine amino terminus

The dansyl chloride method of amino terminus determination was modified for use with rhodopsin and its proteolytic fragments. In this variation, the proteins were first dansylated in SDS solution and then electrophoresed on SDS acrylamide gels. The dansyl fluorescence is used to locate the bands, which can be exised, extracted and hydrolyzed as for amino acid composition. This method was tested with lysozyme; as expected, only lysine showed a free α amino group in this model study. Hydrolysis of dansylated opsin and dansylated F1 did not produce any detectable α amino dansyl derivatives, confirming the observations of others that opsin has a blocked amino terminus. Contrariwise, hydrolysis of dansylated F2 yielded a single α amino dansyl derivative which co-migrated on polyamide plates in 3 solvent systems with dansyl-alanine. Thus F1 appears to retain the unreactive amino terminus opsin, while F2 has a free amino terminus produced by proteolysis.

N-retinyl group is on F2

The Schiff's base linkage between a lysine residue and the aldehyde of the retinal chromophore can be reduced by sodium borohydride in deter-

gent solutions of metarhodopsin II, one of the rhodopsin bleaching intermediates. The result product, a secondary amine derivative called N-retinyl-opsin, is fluorescent and can be detected on SDS-acrylamide gels by fluorescence gel scans. The efficiency of the sodium borohydride reduction of metarhodopsin II is not known; an unspecified percentage of the retinal can dissociate or trans-iminate before reduction takes place, producing fluorescent retinol or other fluorescent N-retinyl compounds, respectively. Retinol and N-retinyl phosphatidyl ethanolamine migrate at the front of the SDS gels; transimidation to other lysines of opsin would produce fluorescent products which would co-migrate with the N-retinyl-opsin derivative that has retinal reduced at the physiological chromophore binding site. Whole rhodopsin solutions in Triton X-100, which were borohydride reduced, exhibited fluorescence at the position of opsin, at the electrophoretic front, at the positions of several opsin aggregates, and at the origin. Subsequent staining of these gels with Coomassie blue showed that substantial aggregation of opsin had taken place. Duplicate experiments in the absence of borohydride indicated that Triton X-100 was responsible for aggregation. Thermolysin-cleaved rhodopsin solutions in Triton X-100 which were borohydride reduced, exhibited fluorescence at the position of F2, at the electrophoretic front, and at the origin. There was no fluorescence at the position of F1. Therefore, it is safe to conclude that F1 does not contain the retinal binding site, and it is probable that most (if not all) of the reduced chromophore attached to F2 is at the physiological site. However, it is necessary to show that fluorescent F1 has not selectively aggregated and produced the fluorescent band at the origin. This was achieved by taking advantage of the observation that SDS

treatment of rhodopsin in the dark caused slow loss of 500 nm absorbance. Addition of sodium borohydride to SDS solubilized whole rhodopsin yielded a product which had most of the N-retinyl fluorescence bound to opsin, as seen by scans of the SDS-acrylamide gel electrophoretic pattern. Some fluorescence was near the electrophoretic front, but no fluorescent aggregates were created. (Since a small amount of Coomassie blue staining aggregates was seen at the origin, this scan suggests that the aggregated material does not arise from rhodopsin.) Reduction of thermolysin-cleaved rhodopsin in SDS produced a single, large fluorescent peak at the position of F2 and a smaller amount of fluorescence near the electrophoretic front. No fluorescence was seen at the position of F1, and this time no fluorescent aggregates were produced (Fig. 5). The SDS reduction technique gives the same general result as the reduction performed in Triton X-100: F2 shows all of the N-retinyl protein fluorescence. However, since there are no longer any fluorescent aggregates, it is unequivocal that F1 does not contain any N-retinyl groups. F2 must contain the physiological binding site of 11-cis retinal.

AENS group is mostly on F2

Wu and Stryer (1972) investigated the use of fluorescent sulfhydryl modifying reagents as spectroscopic probes for rhodopsin structure. The only sulfhydryl-reagents they found which were successful in covalently reacting with rhodopsin in disc membranes were the fluorescent iodoacetamide derivatives of Hudson & Weber (1973). The reagent N-(iodoacetamidoethyl)-1-aminonaphthalene-5-sulfonic acid (IAENS) modifies a sulfhydryl group, called site A by Wu and Stryer (1972), to produce an AENS-rhodop-

sin derivative. The stoichiometry of labeling was now reexamined both by quantitative AENS fluorescence measurements and by incorporation of radioactive AENS. Con A-agarose purified and hydroxylapatite purified rhodopsins consistently contained 0.3-0.4 mole AENS/mole retinal.

Thermolysin treatment of AENS-modified rhodopsin in disc membranes showed no changes in the products, rate of extent of cleavage compared to unmodified rhodopsin as analyzed by Coomassie blue staining patterns of SDS-polyacrylamide gels, except that F2 stained more intensely after AENS modification. This observation is unexplained. Fluorescence gel scans of fixed SDS-gels loaded with AENS-modified ROS disc membranes have a major fluorescent peak at the position of opsin, some fluorescent aggregates at the origin, some fluorescence at the dye front, and occasionally a few minor fluorescent bands in the region of the gel just behind opsin. Following thermolysin treatment, fluorescence gel scans reveal a shift of fluorescence from the higher molecular weight regions, from the origin and from the position of opsin to the positions of F1 and F2. The fluorescence intensity at the position of F2 is 8-10 times as great as that at the position of F1 (Fig. 6). The appearance of the fluorescence at the position of F2 occurred at the same rate as the disappearance of fluorescence at the position of opsin and coincided with the shift of Coomassie blue staining from the position of opsin to that of the fragments. The magnitude of the fluorescence intensity on the fragments is approximately equal to that lost from the opsin position. Some fluorescence still appears at the front.

Amino acid analysis of hydrolysates of AENS-opsin and AENS-F2 revealed the presence of carboxymethyl cysteine, the expected product from

hydrolysis of IAENS alkylation of a cysteine sulfhydryl group. No other carboxymethyl amino acids were seen. Hydrolysates of AENS F1 did not contain any carboxymethyl amino acids. F1 is probably labeled by non-covalent interactions between AENS and opsin, while the accessible sulfhydryl group, called site A, is located in F2.

^{14}C-galactose group is on F1

A galactosyl group can be transferred from UDP-galactose to one or two of the N-acetyl glucosaminyl groups found in the carbohydrate moietie(s) of rhodopsin in the disc membrane in a reaction catalyzed by the enzyme galactosyl transferase (Shaper & Stryer, in preparation). Following thermolysin treatment, the position of the radioactivity on SDS-gel loaded with ROS disc membranes shifts from opsin to F1 (Fig. 7). No sign of radioactivity is exhibited by F2. The amount of radioactivity on F1 quantitatively accounts for the amount lost from opsin. Thus F1 contains the carbohydrate acceptor site for galactose incorporation. This is consistent with the observation that all the N-acetyl glucosamine is also contained on F1.

The quantitative transfer of galactose radioactivity from ^{14}C-gal-opsin to ^{14}C-gal-F1 is an important result. It was used as part of the demonstration that all of the N-acetyl glucosamine of opsin is retained by F1. It can also be used to calculate the ratio of the number of amino acid residues found in F1 compared to that in opsin. Hydrolysates of SDS-polyacrylamide gel purified ^{14}C-gal-opsin and ^{14}C-gal-F1 derived from the same membrane were quantitatively analyzed. The number of residues in a known volume of hydryolysates was standardized to the number of radioactive

disintegrations per minute. Three determinations place the size of F1 at 7 % of that of opsin with an estimated ± 10% error, attributable to the relatively low specific activity of the ^{14}C-gal label.

A similar attempt was made to determine the size of F2 using ^{14}C-AENS-opsin and ^{14}C-AENS-F2. This probe is not quantitatively transferred from opsin to F2 (i.e., at least 10% of the AENS fluorescence is found on F1), making this calculation subject to greater error. F2 appears to contain 30% ± 10% the number of residues in opsin. Since F1 contains about 2.5 times as many residues as F2, F1 probably weighs 25,000 ± 3,000 daltons and F2 weighs 10,000 ± 2,000.

Periodate-dependent reactive groups are on both fragments

Thermolysin treatment was used to cleave disc membranes which had been treated for various periods of time with sodium periodate and then labeled with tritiated sodium borohydride or with dansyl hydrazide. Both probes showed the same results: there is a basal, pre-periodate level of labeling restricted to F1 and periodate treatment induces considerable labeling of both F1 and F2 above this baseline (Fig. 8). Periodate treatment is usually considered to oxidize carbohydrate diols to dialdehydes; subsequent hydride reduction or hydrazone formation are regarded as tests for the presence of carbohydrate. There is no chemical evidence of the presence of carbohydrate on F2, yet there is definite periodate-dependent labeling. Also, it is not known what group reacts during the labeling of F1 prior to periodate oxidation. This experiment does serve to warn that the specificity of protein modification must be checked in each case, and reagents cannot be assumed to react only in an expected fashion.

C. Discussion

Thermolysin cleaves rhodopsin in retinal disc membranes into two large membrane-bound fragments. The properties of these fragments are summarized in Table VIII. Both fragments appear simultaneously as opsin is degraded. Thermolysin-cleaved rhodopsin retains its 500 nm absorbance even in detergent solution. Cleaved rhodopsin in disc membranes also retains regenerability of 500 nm absorbance with 11-cis retinal after photobleaching. The thermolysin produced fragments, called F1 and F2, have molecular weights estimated as 25,000 ± 3,000 and 10,000 ± 2,000 respectively. These molecular weight determinations were based on quantitative amino acid analysis standardized to the size of whole rhodopsin by use of covalently attached radioactive labels as explained in the results section; they do not depend on electrophoretic mobilities which are subject to significant error for membrane proteins. The amino acid compositions of both fragments are quite similar. The largest differences are between mole percent lysine and leucine. The mole percentages of tryptophan and cysteine were not determined.

About 25% of the amino acid residues in disc membranes are released upon thermolysin proteolysis. The disc membranes contain about 50,000 daltons of amino acid residues per rhodopsin as determined by amino acid analysis and 500 nm absorbance (after correcting for partial bleaching). This number is in agreement with previous reports (Heitzmann, 1972). Since rhodopsin probably has a molecular weight of less than 40,000, over 20% of the amino acid residues in the disc membranes are in proteins other than opsin. SDS-gel analysis suggests that these other proteins are probably degraded

more completely and rapidly. Thus the bulk of the released amino acids probably arises from proteins other than rhodopsin, and very few residues are released from rhodopsin. Of those residues which remain in the membrane, over 70% are contained in F1 and F2; no other position on a gel except the origin shows any appreciable amount of amino acid residues. As indicated above, estimates of the molecular weights of F1 and F2 suggest that F1 is about 2.5 times as large as F2. Since the position of F1 on a gel contains about 2.5 times as many amino acids as does the position of F2, F1 and F2 are produced in equimolar amounts.

These data rule out several interpretations for the relationship of F1 and F2 to whole rhodopsin. First, there are not enough unaccounted amino acid residues to permit a model in which some 38,000 dalton rhodopsin polypeptide chains are degraded to F1 (25,000 ± 3,000 daltons) while a roughly equal number of 38,000 dalton rhodopsin polypeptide chains are degraded to F2 (10,000 ± 2,000 daltons). F1 and F2 must arise from the same chain. There are also insufficient unaccounted amino acid residues for all the chains to be degraded to F1 and half of the F1 chains to be subsequently degraded to F2. It may be possible for all of the rhodopsin chains to be degraded to F1 and about one third of the F1 chains to be split into two "F2-sized" chains which have the same electrophoretic mobility. It is much more likely, and more consistent with the quantitative data, that F1 and F2 represent the two pieces arising from the cleavage of a single rhodopsin polypeptide chain. In such a model, the limits of the quantitation are broad enough to be equally consistent with a single, well-defined cleavage site at a particular peptide bond, or with a number of closely spaced sites such that an alanine is always left as the amino ter-

minal residue of F2. It is also possible that an additional cleavage could occur at the carboxy terminus of the F2 chain, possibly accounting for the small rapid shift in the electrophoretic mobility of rhodopsin noted on high resolution acrylamide-gradient gels prior to F1-F2 cleavage.

The sequential model of the two "F2's" arising from one F1 differs in some important predictions from the simultaneous model of one F1 and one F2 arising from different ends of the same polypeptide. Specifically, the sequential model would insist that any chemical marker bound to F1 would probably appear on one of the "F2's" and that every chemical marker on either F2 would have to be found on F1. The same predictions would be made by the sequential model in which an F1 is degraded to a single F2, though this model was inconsistent with the amino acid accounting. The simultaneous model predicts that markers should be found on either F1 or F2 but rarely (if ever) on both unless the marker is contained within a region of closely spaced cleavage sites. The observations described in the results section indicate that the blocked N-terminus and the sugar groups of rhodopsin are exclusively located on F1 while both the retinal binding site and the accessible sulfhydryl which is IAENS alkylated are located on F2. Hence, neither fragment can be wholly contained in the other. The only simple explanation consistent with all of the data is that F1 arises from the amino end while F2 arises from the carboxy end of the same rhodopsin polypeptide chain.

A number of additional points should be raised with regard to the results described in this section. The amino sugar compositions presented indicate there are about 5-6 moles of glucosamine/retinal. The 9 amino acid glycopeptide isolated by Heller and Lawrence (1971) contained a single

oligosaccharide moiety with no more than 3 moles of N-acetyl glucosamine. If both these results are correct, then F1 (and rhodopsin) must contain at least two oligosaccharide groups to account for all the amino sugar.

Another interesting observation is that F1 and F2 are produced by thermolysin cleavage of opsin as well as of rhodopsin in disc membranes This result is one further argument against a major conformational difference between rhodopsin and opsin. It is also interesting that delipidated rhodopsin in detergent solution is cleaved by thermolysin into F1 and F2 and not completely degraded (Fig. 3). This result is in contradiction with the reports of van Breugel et al. (1975) who reported extensive proteolysis of rhodopsin in Triton X-100 using pronase. Proteolysis of any protein requires that there be a susceptible bond which is sufficiently exposed for binding to the active site of the enzyme. Protein folding and lipid binding are both capable of limiting proteolysis. Detergent molecules probably occupy the normal lipid binding sites and afford the same protection. Therefore, the observation that proteolysis of rhodopsin in detergent is limited merely indicates that major unfolding of the protein has not taken place. A limited degree of proteolysis in degergent with any integral membrane protein should not be a major surprise.

Finally, the results in this section indicate that proteolysis does not separate rhodopsin into distinct hydrophobic and hydrophilic (i.e., water-soluble) domains, as in the case for some other integral membrane proteins. In rhodopsin, both fragments are equally hydrophobic and remain membrane-bound. The Ca^{++} pump of sarcoplasmic reticulum is another integral membrane whose proteolytic fragments remain membrane associated (Migala et al., 1973). Perhaps a number of integral membrane proteins

which have functions that involve dynamic changes across membranes will show splitting into membrane-bound fragments as characteristic proteolysis patterns.

CHAPTER III

THERMOLYSIN-CLEAVED RHODOPSIN IS A NON-COVALENT COMPLEX OF THE TWO FRAGMENTS

The results in the previous chapter establish that rhodopsin is cleaved by thermolysin into two fragments, and that cleaved-rhodopsin retains 500 nm absorbance and regenerability. Is the 500 nm absorbing entity simply F2 with a bound 11-cis retinal group, or is it a complex of both fragments, held together by non-covalent forces? The experiments which follow strongly indicate that cleaved-rhodopsin is a non-covalent complex of two chains, and that this complex responds to light in a most interesting way.

A. Methods

Deoxycholate differential extraction

Sequential deoxycholate extraction of whole or thermolysin-cleaved disc membranes was carried out by suspending disc membranes containing 4 mg of rhodopsin in 2 ml of 100 mM sodium chloride, 20 mM tris-acetate, pH 7.4 solution and centrifuging at 17,000 rpm in a Sorvall SE-12 rotor for 15 mins. The supernatant was set aside and the procedure was repeated on the residual membrane pellet using the same buffer containing 0.1% sodium deoxycholate. The supernatant of this centrifugation was set aside as the 0.1% sodium deoxycholate extract, and the whole procedure was repeated sequentially with 0.15%, 0.2% and 0.4% sodium deoxycholate solutions. The absorbance spectrum of each supernatant was recorded with a Cary 15 spectrophometer. An aliquot of each extract was mixed with an equal volume of 5% SDS, 10% sucrose, 5% β-mercaptoethanol and applied to an SDS-gel for electrophoresis.

Hydroxylapatite chromatography

Hydroxylapatite chromatography of whole or thermolysin-cleaved disc membranes was conducted as described in Chapter II, except that 1% Ammonyx LO 150 mM sodium phosphate, pH 7.0, 2mM dithiothreitol buffer did not elute cleaved rhodopsin. A third buffer, containing 1% Ammonyx LO, 250 mM sodium phosphate, 2 mM dithiothreitol was required to remove 500 nm absorbing material from the column. The 500 nm absorbing fractions were pooled, a spectrum was recorded, and the fractions were dialyzed for 2 days vs. Ammonyx LO-free buffer. The dialysate, containing little Ammonyx LO, was

adjusted to a final concentration of 2.5% SDS, 2.5% β-mercaptoethanol and 5% sucrose. This mixture was incubated at 37° for 3 mins and an aliquot was applied to an SDS-gel for electrophoresis. The dialysis step was necessary to avoid precipitation produced by directly mixing SDS and Ammonyx LO containing solutions. This treatment did not prevent Ammonyx-induced aggregation of opsin on the SDS-gels.

Con A-agarose chromatography

Con A-agarose was synthesized as described by Steinemann and Stryer (1973). The buffer used for Con A-agarose affinity chromatography contained 2 mM dithiothreitol in 0.05 M sodium acetate, pH 5.0, 1 mM calcium chloride, 1 mM manganese chloride. Whole or thermolysin-cleaved disc membranes were extracted in the dark with this buffer containing 5% Triton X-100 at a concentration of 5 mg/ml rhodopsin, and the rhodopsin-rich extract was applied to a Con A-agarose column under dim red light at room temperature. The column was washed extensively under the same buffer containing 0.5% Triton X-100 until the absorbance at 290 nm returned to baseline. (Triton X-100 has an absorbance maximum near 270 nm, so 290 nm was chosen to maximize the ratio of tryptophan to Triton X-100 absorbance.) In some experiments, the rhodopsin-containing column was exposed to room lights or a flash produced by a Honeywell flash gun. In such experiments, the column was washed further until the 290 nm absorbance again returned to baseline. Material that was successfully bound to the affinity column eluted by addition of the same buffer containing 0.5% Triton X-100 and 0.1 M α-methyl-mannoside. Triton X-100 was the detergent used in these experiments because this nonionic detergent was compatible with subsequent SDS-gel analysis of the polypeptides in various eluted fractions. In some experiments, 1.4% CTAB or 1%

Ammonyx LO detergent solutions of Triton X-100 were used instead to ensure that the observed elution patterns were not dependent on the presence of Triton X-100.

Aliquots were taken from the eluted fractions and their concentrations were adjusted to 2.5% SDS, 2.5% β-mercaptoethanol, and 5% sucrose. The pH was adjusted to 7.0 by addition of 5N sodium hydroxide. These samples were applied directly to SDS gels and subjected to electrophoresis as described previously. When disc membranes containing ^{14}C-galactose-rhodopsin were subjected to Con A-agarose affinity chromatography, aliquots of each eluted fraction were assayed for radioactivity by liquid scintillation counting in Aquasol.

Alkaline hydrolysis and ninhydrin quantitation

Fractions were assayed for amino acid residues by alklaine hydrolysis and ninhydrin quantitation (Hirs et al., 1956). Aliquots of 0.2-0.3 ml were placed in polyethylene tubes and 1.0 ml of 2.5N sodium hydroxide was added. The samples were hydrolyzed by heating the unstoppered tubes in an autoclave for 30 min. The number of liberated amino groups was measured by forming the ninhydrin adduct. Each sample was neutralized with 1.0 ml of 30% acetic acid, and reacted with 2.0 ml of ninhydrin reagent (125 ml 4N sodium acetate, pH 5.5, 10 g ninhydrin, 1.5 hydrindantin, and 375 ml methyl cellosolve) by heating in a boiling water bath for 20 min. The tubes were cooled, vortexed for 1 min to oxidize excess ninhydrin and mixed with 5 ml of 50% ethanol to preserve color. The number of amino groups were determined by measuring the absorbance at 570 nm; the absorbance values were standardized with leucine solutions of known concentrations.

B. Results

Deoxycholate-extracted cleaved rhodopsin contains F1 and F2

Detergent solution extracts of thermolysin-cleaved disc membranes retain 500 nm absorbance. Deoxycholate, a steroid detergent, can be used for differential extraction of rod outer segment disc membranes. Solutions of 0.1% deoxycholate extract some 280 nm absorbing material, but not rhodopsin. Sequential 0.15% and 0.20% deoxycholate solutions extract 500 nm absorbing rhodopsin and leave a substantial pellet of residue without 500 nm absorbance. Finally, 0.40% deoxycholate solubilizes the residue. By SDS-gel analysis, only the 0.15% and 0.20% extracts contain opsin, as expected from the spectral properties. The behavior of thermolysin treated disc membranes is similar to that of untreated membranes: only the 0.1% and 0.15% deoxycholate solutions extract significant amounts of 500 nm absorbing material. SDS acrylamide gels show that these solutions of thermolysin-cleaved rhodopsin contain both F1 and F2 (Fig. 9).

Hydroxylapatite-purified cleaved rhodopsin contains F1 and F2

The elution behavior of thermolysin-cleaved rhodopsin was compared to that of whole rhodopsin from a hydroxylapatite column. Whole rhodopsin does not elute from hydroxylapatite with 1% Ammonyx LO in 35 mM sodium phosphate buffer. 1% Ammonyx LO in 150 mM sodium phosphate buffer elutes whole rhodopsin, but does not elute thermolysin-cleaved rhodopsin from hydroxylapatite. Cleaved rhodopsin is eluted with a solution containing 250 mM sodium phosphate. A sample of the cleaved-rhodopsin eluted with 250 mM sodium phosphate was transferred to SDS solution and analyzed by SDS-gel electrophoresis. It contained both F1 and F2. Neither fragment was eluted with the 150 mM sodium phosphate buffer.

Con A-agarose-purified cleaved rhodopsin contains F1 and F2

The elution behavior of thermolysin-cleaved rhodopsin was also compared with that of whole rhodopsin using Con A-agarose affinity columns. In the dark, Triton X-100 solutions of both cleaved and whole rhodopsin molecules adhered to the column, and could be displaced in 0.1 M α-methyl-mannoside, a competing sugar. Once again, the fractions containing thermolysin-cleaved rhodopsin were shown to contain both F1 and F2 when subjected to SDS-gel electrophoresis (Fig. 10). Thermolysin-cleaved rhodopsin, which was Con A-agarose purified, contained 90% as many amino acid residues as whole rhodopsin according to ninhydrin quantitation of alkaline hydrolsates.

The experiment which used Con A-agarose affinity chromatography to purify cleaved-rhodopsin was particularly intriguing. Chemical analysis of F2 gave no indication of amino sugar on this fragment. Therefore, either F2 contains a Con A binding oligosaccharide without any amino sugar, or it has adhered to the Con A column through non-covalent interactions with a sugar-containing molecule. Three different methods of purification did not separate F1 from F2 in cleaved rhodopsin. The 500 nm absorbing species must be a non-covalent complex of the two fragments.

Light dissociates F1 from F2

It was logical to test the possibility that the non-covalent interaction between F1 and F2 might be sensitive to light. Cleaved-rhodopsin was loaded on a Con A-agarose column in the dark and washed until the absorbance returned to the baseline. The column was flashed with light, bleaching the rhodopsin. Material rapidly eluted from the column (Fig. 11A). SDS-gel analysis indicated that this material had the same electrophoretic mobility as F2 (Fig. 11B). In other experiments, conducted with specifically

labeled cleaved-rhodopsin molecules, the light-eluted material was shown to exhibit AENS fluorescence but not ^{14}C-galactose radioactivity, the same properties exhibited by F2. Finally, the amino acid composition of the lighte-eluted material resembled F2; it showed the higher lysine and lower leucine mole percent characteristic of this fragment. Thus, the action of light was to release F2 from the Con A column. F2 does not have a Con A binding site.

Subsequent elution of the bleached column with competing sugar released material that had the same electrophoretic mobility as F1 (Fig. 11). In experiments with specifically labeled cleaved-rhodopsin molecules, this material contained ^{14}C-galactose, but very little AENS fluorescence. Its amino acid composition showed the lower lysine and higher leucine mole per cent characteristic of F1. Thus F1 contains the Con A binding site of rhodopsin.

In control experiments, whole rhodopsin was shown to have the same elution behavior before and after bleaching. When cleaved-rhodopsin was bleached and subsequently loaded on the column, only F1 adhered to the column. F1 could be eluted from the column by α-methyl-mannoside in the usual manner.

The number of amino acid residues in the fractions eluted by light and in the fractions eluted by α-methyl-mannoside were compared to estimate the relative sizes of F1 and F2. Alkaline hydrolysis and ninhydrin quantitation of column fractions showed that the material eluted by sugar contained three times as many residues as those eluted by light. This is a maximum ratio of the size of F1 compared to that of F2 because both residual amounts of whole rhodopsin and amounts of bleached cleaved-rhodopsin will

increase the number of residues eluted by sugar but not the number of those eluted by light. Therefore, the 3 to 1 ratio of residues in F1 to F2 is in very good agreement with the 2.5 to 1 ratio obtained from gel slices.

C. Discussion

The only simple interpretation of these experiments is that there are non-covalent interactions between F1 and F2, and that cleaved-rhodopsin is a complex of the two fragments. The action of light is to disrupt these forces in detergent solution. It is reasonable to assume that these same non-covalent interactions occur in whole rhodopsin. The dramatic dissociation produced by light is harder to interpret. Whole rhodopsin loses some of its secondary structure upon bleaching in detergents such as Triton X-100. Therefore, photodissociation of F1 and F2 might be an exaggeration of a physiologically relevant conformational change or it might be consequent to the unfolding of opsin in detergent solution.

Consider for a moment the possibility that the photodissociation of F1 and F2 is similar to a physiological change that follows the absorption of a photon by rhodopsin. In disc membranes, bleaching produces little change in the secondary structure of rhodopsin. However, if F1 and F2 are two tightly-folded domains which interact non-covalently along two juxtaposed surfaces, considerable motion could occur between these regions, with little accompanying change within the secondary structure of either domain. There is ample precedent in molecular biology for such types of conformational change. The motion between hemoglobin subunits upon oxygenation occurs with little change in secondary structure (Perutz, 1971) and in primitive vertebrate hemoglobins, these changes involve association and dissociation of subunits.

Two domains could move over large distances by swinging about a connecting hinge. Molecular hinges are known to occur in immunoglobulins (Edelman, 1973) and in skeletal muscle myosin (Huxley, 1971). If rhodop-

sin has a hinge region, it would be an important structural feature. Our knowledge of hinges from immunoglobulins and myosin provide a crucial test for such a model: hinges are highly susceptible to proteolysis by enzymes or different specificity. The experiments in Chapter IV will test this prediction.

CHAPTER IV

RHODOPSIN CONTAINS A REGION WHICH IS HIGHLY SENSITIVE TO PROTEOLYSIS

The features of thermolysin proteolysis of rhodopsin have been well documented in the last two chapters. The experiments which follow address the question: is the action of thermolysin on rhodopsin unique to this enzyme or does rhodopsin have a region that is highly sensitive to a variety of proteolytic enzymes? The existence of such an unfolded, flexible region connecting F1 and F2 would contribute to the plausibility of physiologically important motion between these two domains.

A. Methods

Papain, α-chymotrypsin, and TPCK trypsin were obtained from Worthington. Subtilisin Carlsberg came from Sigma. All other chemicals were reagent grade.

Proteolysis was conducted much as described with thermolysin in Chapter II. Proteases were added to disc membrane suspensions, 2.5-5 mg rhodopsin/ml 10 mM tris-acetate, pH 7.4, under dim red light at room temperature. The weight ratios of protease to rhodopsin were 1:50 for papain, 1:200 for subtilisin, and 1:20 for trypsin and chymotrypsin. Papain reactions were supplemented with 2 mM EDTA nad 5 mM dithiothreitol. Proteolysis was terminated by adding a specific inhibitor and centrifuging the membranes to remove the enzyme. The inhibitors used were 20 mM iodoacetamide for papain and 2 mM phenyl-methyl sulfonyl fluoride for the three serine proteases. Following proteolysis, the membrane pellets were quite flocculent and particular care was taken in removing the supernatant so as not to disturb the pellet. SDS polyacrylamide gel electrophoresis and Con A-agarose chromatography were performed as described previously except that β-mercaptoethanol was excluded from the SDS-gel sample preparation after papain treatment to prevent activation of additional enzyme.

B. Results

Papain cleaves rhodopsin into FP1 and FP2

Papain, like thermolysin, cleaves rhodopsin in retinal disc membranes into two large membrane-bound fragments without loss of 500 nm absorbance. This pair of fragments has very similar electrophoretic mobilities to F1 and F2; these fragments have been named FP1 and FP2 respectively (Fig. 12). FP1, like F1, contains the galactose group incorporated into rhodopsin by galactosyl transferase. FP2, like F2, contains the N-retinyl group produced by sodium borohydride reduction of retinal to its binding site (Fig. 13), and contains most of the AENS fluorescence produced by alkylation of the accessible sulfhydryl group of rhodopsin by IAENS. In the dark, papain-cleaved rhodopsin adheres to Con A-agarose until eluted by α-methyl-mannoside. Papain-cleaved rhodopsin purified by Con A-agarose chromatography, contains both FP1 and FP2. When papain-cleaved rhodopsin was bound to a Con A-agarose column in the dark, and exposed to light while still attached to the column, FP2 was eluted. FP1 could then be eluted by a competing sugar (Fig. 14). By the criteria of electrophoretic mobility, chemical marker localization, and elution behavior from a Con A-agarose affinity column, papain-cleaved rhodopsin is very similar to thermolysin-cleaved rhodopsin.

Occasionally, papain cleavage of rhodopsin did not stop after production of FP1 and FP2. FP1 would partially disappear and was replaced by a band of greater electrophoretic mobility, called FP1'. FP1 and FP1' have apparent molecular weights of 30,000 and 25,000 respectively. FP1' bears the ^{14}C-galactose incorporated by galactosyl transferase but not N-retinyl

or AENS fluorescence. Therefore, FP1' probably arises from limited degradation of FP1.

Subtilisin cleaves rhodopsin into FS1 and FS2

Subtilisin, like thermolysin, cleaves rhodopsin in retinal disc membranes into two large membrane-bound fragments without loss of 500 nm absorbance. This pair of fragments have very similar electrophoretic mobilities to F1 and F2; these fragments have been named FS1 and FS2 respectively (Fig. 15). FS1, like F1, contains the galactose group incorporated into rhodopsin by galactosyl transfer. FS2, like F2, contains the N-retinyl group produced by sodium borohydride reduction of retinal to its binding site (Fig. 13) and contains most of the AENS fluorescence produced by alkylation of the accessible sulfhydryl group of rhodopsin by IAENS. In the dark, subtilisin-cleaved rhodopsin adheres to Con A-agarose until eluted by α-methyl-mannoside. Subtilisin-cleaved rhodopsin purified by Con A-agarose chromatography, contains both FS1 and FS2. When subtilisin-cleaved rhodopsin was bound to a Con A-agarose column in the dark, and exposed to light while still attached to the column, FS2 was eluted. FS1 could then be eluted by a competing sugar (Fig. 16). By the criteria of electrophoretic mobility, chemical marker localization, and elution behavior from a Con A-agarose affinity column, subtilisin-cleaved rhodopsin is very similar to thermolysin-cleaved rhodopsin.

Occasionally, subtilisin-cleavage of rhodopsin did not stop after production of FS1 and FS2. FS1 would partially disappear and was replaced by a band of slightly greater electrophoretic mobility, called FS1'. FS1 and FS1' have apparent molecular weights of 30,000 and 25,000 respectively. FS1' bears the ^{14}C-galactose incorporated by galactosyl transferase but not

N-retinyl or AENS fluorescence. Therefore, FS1' probably arises from limited degradation of FS1.

These results show that papain and subtilisin gave very similar cleavage patterns as thermolysin; presumably these enzymes of different specificities are acting at the same region. Subtilisin is the most active enzyme of the three; it produces cleavage at a ten-fold greater rate than either thermolysin or papain. This may be related to the fact that subtilisin is also the enzyme with the lowest specificity (Table V).

Chymotrypsin cleaves rhodopsin into FC1, FC1' and FC2

Chymotrypsin cleaves rhodopsin in retinal disc membranes without loss of 500 nm absorbance. This reaction is somewhat slower than with the other proteolytic enzymes; at equivalent concentrations of enzyme, opsin is cleaved only about one tenth as quickly with chymotrypsin as it is cleaved with thermolysin or papain. On SDS-gels, the opsin band is replaced by a heavily staining band of apparent molecular weight 23,000 (FC1') and the two faintly staining bands at 30,000 (FC1) and 20,000 (FC2) (Fig. 17). The 20,000 (FC2) band is particularly broad, extending from apparent molecular weights of 22,000 to 18,000. At longer times of proteolysis, it becomes narrower and most intense in the 18,000 region. ^{14}C-galactose incorporated into rhodopsin by galactosyl transferase was attached to FC1 and FC1', but not to FC2. All of the N-retinyl (Fig. 18) and most of the AENS fluorescence were attached to FC2. Chymotrypsin-cleaved rhodopsin adhered to a Con A-agarose affinity column in the dark and could be eluted with 0.1 M α-methyl-mannoside. Chymotrypsin-cleaved rhodopsin contained FC1, FC1' and FC2. When chymotrypsin-cleaved rhodopsin was bound to a Con A-agarose column in the dark, and then exposed to light, FC2 was eluted.

Subsequent addition of 0.1 M α-methyl-mannoside eluted both FC1 and FC1' (Fig. 19). FC1 probably contains FC1' as part of its structure. The initial width of the FC2 band compared to F2 and the lack of a clear precursor-product kinetic relationship between FC1 and FC1' complicates the interpretation of these results. In contrast to papain and subtilisin, chymotrypsin may not cleave rhodopsin in exactly the same region of the polypeptide chain as does thermolysin, but does produce fragments which resemble F1 and F2 by the criteria of chemical marker localization and elution behavior from a Con A-agarose affinity column.

Trypsin does not cleave rhodopsin in disc membranes

Trypsin does not cleave rhodopsin in disc membranes at a significant rate. No further investigation was undertaken.

C. Discussion

Thermolysin, papain and subtilisin all appear to cleave rhodopsin in the same region to produce pairs of fragments with highly similar electrophoretic mobilities and marker localizations (Table IX). These results suggest there is a region of the rhodopsin chain structure that is highly susceptible to proteolysis. W. Hubbell (personal communication) has found that at least eight other proteolytic enzymes show a preference for this same site. Trypsin, the enzyme with the highest specificity, does not cleavage rhodopsin at this region. Chymotrypsin does produce some fragments with similar electrophoretic mobilities and marker localizations as those produced by other enzymes, but it has a much slower rate of cleavage and it appears to cleave rhodopsin at a different site. These differences are probably due to the high specificity of chymotrypsin compared to thermolysin, subtilisin, and papain. The region of rhodopsin which is sensitive to proteolysis by several different enzymes probably contains few, if any, basic or aromatic amino acids.

The fragments produced by thermolysin appear relatively homogeneous (i.e., F2 has a single amino terminal residue) and the amino acid residue accounting proved that these fragments are the only major products of cleavage. F2 may have variable lengths because of the removal of residues from the C-terminal and, as suggested by the slight molecular weight shift observed on Laemmli gels. F1 and F2 have been studied chemically. The pairs of fragments produced by the other enzymes appear similar to F1 and F2 on the basis of marker localization, Con A-agarose elution behavior, and electrophoretic mobilities. The subtilisin and papain fragments pro-

duced at early times probably have ends that can be further digested; possibly this nibbling leads to more homogeneous lengths at longer times of proteolysis. The chymotryptic fragments are even less well-defined because they are less similar to F1 and F2 than are the papain- and subtilisin-produced fragments. In summary, there appear to be two domains of rhodopsin that are resistant to the action of proteases of different specificities. The stretch of polypeptide connecting these regions is protease-sensitive to enzymes of low specificity. This is the structural feature predicted for a molecular hinge.

CHAPTER V

TRANSGLUTAMINASE LABELS RHODOPSIN IN THE REGION THAT IS HIGHLY SENSITIVE TO PROTEOLYSIS

The previous chapter presented evidence that rhodopsin has a region highly sensitive to a variety of proteases differing in specificity. In this chapter, an enzyme, guinea pig liver transglutaminase, was used to insert spectroscopic probes into rhodopsin in disc membranes in a highly specific manner. Most interesting, the probes appear to attach to the portion of rhodopsin that is sensitive to proteolytic enzymes. Fluorescence spectroscopic techniques were used to study the dynamic properties of this important part of the protein molecule.

The reaction catalyzed by transglutaminase (Fig. 20) involves the substitution of exogenous primary amines for the ammonia group in the γ-carboxamide linkage of a glutamine residue (Clarke et al., 1959). The enzyme requires that the glutamine be part of a peptide chain, and probably requires that the glutamine be located in a loosely-folded region on the surface of the protein. Transglutaminase demonstrates little specificity for the structure of exogenous primary amine. Dutton and Singer (1975) first used transglutaminase on membrane protein systems. They studied mouse erythrocyte membranes and sarcoplasmic reticulum membranes of rabbit skeletal muscles. These authors demonstrated that transglutaminase can modify membrane proteins and have advocated its use as a general membrane labeling technique. Iwanij and Reich (1976) have had extensive experience with transglutaminase-catalyzed labeling of plasma membranes of cell lines in culture. Both groups used this reaction as an assay for ac-

cessibility of membrane proteins to the aqueous space outside the lipid bilayer. It is an excellent technique for this application because, unlike lactoperoxidase-catalyzed iodination, transglutaminase must bind to the protein in order to produce labeling.

This work is the first to use the specificity of an enzymatic reaction to incorporate spectroscopic probes into a membrane protein. This is an important advance. In the past, labeling reactions have depended on the lower specificity of organic chemical groups. In complex systems, such as biomembranes, this lower specificity has led to heterogeneous labeling. The heterogeneity of labeling sites on rhodopsin was demonstrated in Chapter II: neither periodate-catalyzed hydrazide binding nor IAENS cysteine-alkylation were quantitative or wholly specific. The spectroscopic measurements made with any probes must be regarded with caution unless chemical specificity can be shown.

Three kinds of information were obtained from inserting fluorescent reporter groups. First, the fluorscent lifetime and the emission maximum of the probe provide information about the polarity of its environment. Is the probe in water or has it found a hydrophobic pocket? Second, if the probe is located near a suitable acceptor group, an estimate of the distance between the fluorescent donor and the acceptor can be made from the efficiency of dipole-dipole energy transfer. In practice, the efficiency of transfer is best determined by comparing the lifetimes of the fluorescent donor in the presence and absence of acceptor. The efficiency E is defined as

$$E = 1 - \frac{\tau(\text{presence of acceptor})}{\tau(\text{absence of acceptor})}$$

For rhodopsin, Wu and Stryer (1972) considered energy transfer from extrinsic fluorescent probes to the intrinsic 11-cis retinal chromophore. The process of bleaching was assumed to remove the chromophore acceptor without altering the environment, and hence lifetime, of the fluorescent donor. This is a good assumption for those cases in which the fluorescence emission maximum of the donor does not change upon bleaching. The efficiency of transfer may then be estimated as

$$E = 1 - \frac{\tau_d}{\tau_b}$$

where τ_d = lifetime in the dark, in the presence of 11-cis retinal and τ_b = lifetime after bleaching, in the absence of 11-cis retinal.

Förster related the distance between a fluorescent energy donor and an energy acceptor and the efficiency of transfer by the equation:

$$r = R_o (E^{-1} - 1)^{1/6}$$

where R_o is the distance at which transfer efficiency is 50%, and E is the measured transfer efficiency. R_o can be calculated from the expression:

$$R_o = (J K^2 Q_o n^{-4})^{1/6} (9.79 \times 10^3)$$

where K^2 is the orientation factor for dipole-dipole transfer approximated as 2/3 in those cases where the probe has rotational mobility, Q_o is the quantum yield of donor in the absence of acceptor, n is the refractive index of the

medium (taken as 1.4), and J, the spectral overlap integral, is given by

$$J = \frac{F(\lambda)\ \varepsilon(\lambda)\ \lambda^4\ d\lambda}{F(\lambda)\ d\lambda}$$

where $F(\lambda)$ is fluorescence intensity at wavelength λ, and $\varepsilon(\lambda)$ is extinction coefficient of acceptor at that wavelength. Förster's theory has been tested in model systems to confirm that E is proportional to r^{-6} (Stryer & Haugland, 1967).

The third type of information which can be obtained from a fluorescence spectroscopic experiment is the mobility of the probe (Stryer, 1968). The sample is excited by a nanosecond light-pulse, polarized in the y direction, photoselecting those probes whose axis of absorption has a component in the y direction at the time of the pulse. The intensity of the fluorescence in the parallel y direction (F_y) is compared to the intensity of fluorescence in the perpendicular x direction (F_x) as a function of time. These parameters can be used to define an emission anisotropy:

$$A(t) = [F_y(t) - F_x(t) / (F_y(t) + 2F_x(t))]$$

For a rigid sphere the decay of emission anisotropy (A(t)) with time is given by

$$A(t) = A_o\ e^{-t/\phi}$$

where A_o is the emission anisotropy at the moment of excitation and ϕ is the rotational correlation time. In practice, the raw emission anisotropy is calculated directly from the decay curves of the parallel and perpendicular fluorescence. The correlation time is then estimated by comparing the raw emission anisotropy with computer-generated simulations dependent upon a mean fluorescent lifetime, and a choice of correlation times. This procedure is necessary because of the exciting light pulse has a finite duration (Yguerabide, 1972).

The estimated correlation time gives an indication of the mobility of the probe. Usually, there is a very rapid motion over a very narrow angular range that is not detectable because it occurs during the exciting light pulse; the anisotropy for probes bound to macromolecules starts well below that for free probe in 100% glycerol. Probes bound rigidly to proteins having a molecular weight of 40,000 typically have correlation times on the order of 15 nanoseconds; highly mobile (or free) probes have correlation times less than 1 nanosecond. Correlation times of 5 nanoseconds are suggestive of movements of small domains of proteins or of restricted movements of the probe. Fluorescence anisotropy measurements are an important technique which can provide evidence of dynamic processes not available from steady-state measurements.

A. Methods

Transglutaminase modification of disc membranes

Transglutaminase was purified from guinea pig livers by the procedure of Connellan et al. (1971). ^{3}H and ^{14}C-putrescine and ^{3}H-glycine methyl ester were purchased from New England Nuclear. Unlabeled putrescine, dansyl cadaverine, cadaverine, and fluorescein isothiocyanate were obtained from Sigma; ethyl orange from Eastman and N-(aminoethyl)-5-aminonaphthalene-1-sulfonic acid was a gift from Dr. Joel Shaper. Bovine serum albumin essentially fatty acid free, came from Sigma. All other chemicals were reagent grade.

Transglutaminase-catalyzed insertion of probes into rhodopsin was carried out by adding one part transglutaminase to ten parts rhodopsin by weight in a suspension of disc membranes at 5-10 mg rhodopsin per ml of 100 mM tris acetate, pH 7.4 containing 5 mM dithiothreitol, 10 mM calcium chloride and 10-20 mM amine substrate. The membranes were shaken at 37° in the dark for up to 8 hrs. The labeling was stopped by adding 50-100 mM sodium EDTA, pH 7.4, and by centrifuging the membranes to remove enzyme. Labeled membranes were subsequently treated in the same manner as unmodified membranes. The amine substrates used in most experiments were putrescine and dansyl cadaverine. In a few experiments, N-(aminoethyl)-5-aminonaphthalene-1-sulfonic acid, ethyl orange cadaverine (synthesized by the reaction of ethyl orange sulfonyl chloride with cadaverine), or fluorescein cadaverine (synthesized by the reaction of fluorescein isothiocyanate with cadaverine), were used instead. These substrates are shown in Fig. 21. Fluorescein cadaverine formed an insoluble precipitate with calcium ion. Since transglutaminase depends on calcium ion. Since transglutaminase depends on calcium ion for activity, the calcium chloride concentration in these experiments was

raised to 20 mM. Dansyl cadaverine and N-(aminoethyl)-5-aminonaphthalene-1-sulfonic acid were not completely soluble at 10 mM, and were added as saturated suspensions. Occasionally, bleached disc membranes or rhodopsin in 0.5% Triton X-100 solution was substituted for unbleached disc membranes at the same protein concentration.

The rate of ^{3}H-putrescine incorporation into rhodopsin and distribution of ^{3}H-putrescine label following proteolysis was determined by slicing and counting stained Laemmli (1970) SDS-polyacrylamide gradient slab gels. Autoradiography was performed by the method of Bonner and Laskey (1974) with an exposure time of 2-6 days at -80° C. The stoichiometry of ^{14}C-putrescine labeling was determined by hydroxylapatite purification of modified rhodopsin as described previously. The rate and extent of dansyl cadaverine incorporation was assessed by fluorescence scans of SDS-gels prepared by the method of Weber and Osborn (1969) as modified in Chapter II. Gels containing dansyl cadaverine-rhodopsin were excited with light of 360 nm and the emission was viewed through a 3-73 Corning glass filter.

Proteolysis of labeled rhodopsins was conducted as described in Chapters II and IV except that 5-10 fold higher concentrations of proteolytic enzymes were used to accelerate the slower reaction rates. Con A-agarose chromatography was performed as described previously.

Preparation of samples for spectroscopy

Samples of dansyl cadaverine-rhodopsin in disc membranes for spectroscopy were prepared in the dark by washing labeled membranes 6-10 times with 1% bovine serum albumin

in 0.1 M sodium acetate buffer, pH 5.8 until no additional dansyl could be extracted, and then by washing 3 times with 10 mM tris acetate, pH 7.4 and 3 times with deionized water. The water-washed pellet was sonically dispersed in 80 mM sodium phosphate, 2 mM dithiothreitol by five or six 2 min bursts of 100 watts of power at 4° C with a Model W185D sonifer cell disrupter (Heat systems - Ultrasonics Inc.). The supernatant was briefly centrifuged at low speed to remove large particles and diluted 1:3 for fluorescence lifetime measurements. The supernatant was diluted 1:30 for recording emission and excitation spectra, so that the optical density throughout the wavelengths observed was less than 0.03.

Fluorescence spectroscopy conditions and techniques

Lifetimes and anisotropy curves were measured at 4° by the method of Yguerabide (1973). The sample was excited through a 7-37 glass filter and the emission viewed through a 3-70 filter. Excitation and emission spectra were recorded at room temperature and corrected for variability with wavelength of the detection system (Stryer, 1968). The quantum yield for dansyl cadaverine-opsin in disc membranes was estimated from comparison with that of pure dansyl cadaverine in dioxane, which has a very similar fluorescence lifetime. 1-Anilino-8-naphthalene sulfonate (ANS) in absolute ethanol was used as a quantum yield standard of 0.37 (Stryer, 1965).

B. Results

Transglutaminase inserts amine substrates into rhodopsin

Guinea pig liver transglutaminase catalyzed the insertion of various amine substrates into rhodopsin. ^{3}H-putrescine labeled rhodopsin in disc membranes in the presence of transglutaminase; no reaction occurred without the enzyme (Fig. 22). Under the conditions described in the Methods section, the amount of putrescine incorporated saturated after 3-4 hrs (Fig. 23). Addition of fresh enzyme or putrescine did not increase the labeling of rhodopsin by more than 10%, demonstrating that the limiting factor in the reaction is the number of available glutamine residues. The stoichiometry of ^{14}C-putrescine incorporation at saturation was 1.0 ± 0.1 putrescines per hydroxylapatite purified rhodopsin based on the ratio of the radioactivity to the absorbance (Fig. 24).

Dansyl cadaverine also served as a substrate for a transglutaminase-catalyzed reaction. Fluorescence gel scans showed that transglutaminase inserted dansyl cadaverine into rhodopsin, and that this reaction also saturated in less than 3 hrs. Again, no reaction occurred in the absence of transglutaminase. Fluorescence measurements showed that the stoichiometry at saturation was 1.0 ± 0.4 dansyl cadaverines per hydroxylapatite-purified rhodopsin. The degree of uncertainty in this value arises from uncertainty in the quantym yield of rhodopsin-bound dansyl cadaverine in 1% Ammonyx compared to the free dansyl cadaverine; both the lifetimes and emission maxima of these groups differed. Prior labeling of disc membranes with ^{14}C-putrescine reduced the degree of dansyl cadaverine incorporation to less than 10% of that in control membranes (Fig. 25). It appears that both pu-

trescine and dansyl cadaverine are inserted into the same site of rhodopsin and both label at a stoichiometry of 1 mole amine/mole rhodopsin.

No difference in the rate or in the extent of labeling was seen with transglutaminase-catalyzed insertion of putrescine or dansyl cadaverine when the disc membranes were bleached prior to reaction (Fig. 23). Transglutaminase does not sense a conformational change in the labeling site upon photon capture. Hydroxylapatite purified rhodopsin in 0.5% Triton X-100 solution was also labeled by these probes in the presence of transglutaminase. The site appears to be accessible to labeling in detergent solution.

Four other amines have been incorporated into rhodopsin by transglutaminase, but the products have not been thoroughly characterized. These probes are: fluorescein cadaverine, N-(aminoethyl)-5-aminonaphthalene-1-sulfonic acid, ethyl orange - cadaverine and glycine methyl ester. The structure of these probes is given in Fig. 21. As has been observed for other systems, transglutaminase-catalyzed labeling shows little specificity for the amine substrate (Clarke et al., 1959).

Subtilisin cleaves rhodopsin and then excises the amine label from the fragments

Several interesting observations were made by treating transglutaminase-catalyzed labeled-rhodopsin with proteolytic enzymes. First, rhodopsin labeled with putrescine or dansyl cadaverine is cleaved much more slowly by proteolytic enzymes than is unlabeled rhodopsin. In fact, dansyl cadaverine completely blocks thermolysin cleavage under the conditions normally used for proteolysis. Furthermore, this inhibition is reciprocal. Transglu-

taminase-catalyzed labeling of cleaved-rhodopsin proceeds much more slowly and to a lesser extent than the labeling of whole rhodopsin. In both directions, the mutual inhibition between transglutaminase modification and enzymatic-proteolysis is at least ten-fold.

The distribution of transglutaminase-inserted labels following proteolysis helps to assign the location of the labeling site. Immediately following subtilisin cleavage of rhodopsin, about 50% of the ^{3}H-putrescine label is attached to FS2 and 50% is located in the position of FS1. Longer exposure of the cleavage products to subtilisin results in the excision of the label from both fragments with only very small concomitant changes in electrophoretic mobilities (Fig. 26).

Complementary results were produced by proteolysis with dansyl cadaverine-labeled rhodopsin. Immediately after subtilisin cleavage of dansyl cadaverine-rhodopsin, all of the dansyl fluorescence was in the position of the larger fragment. Longer exposure of the cleavage products to subtilisin led to excision of the dansyl label from this fragment without an appreciable change in its electrophoretic mobility (Fig. 27). Since subtilisin cleaves rhodopsin much more rapidly than it excises the dansyl label, considerable amounts of dansyl cadaverine-labeled FS1 is present at early times. At longer times of proteolysis, only unlabeled FS1 is present. Both labeled and unlabeled FS1 could be bound to Con A-agarose and were eluted by 0.1 M α-methyl-mannoside, but not by light. Thus the excision of the dansyl cadaverine label does not remove the Con A binding site from the large fragment.

Similar results were obtained with papain (Table X).

The dansyl cadaverine label shows a mobile portion of rhodopsin in Ammonyx LO solution

Dansyl cadaverine-rhodopsin is readily purified by hydroxylapatite chromatography in 1% Ammonyx LO. This protein-detergent complex can be studied by several fluorescence techniques to obtain information about the conformation of rhodopsin. The fluorescent lifetime of the dansyl probe does not appear homogeneous; that is, the decay curve plotted in exponential form does not approximate a straight line (Fig. 28). This means the probe is in at least two environments. Free dansyl cadaverine in 1% Ammonyx LO also shows more than one lifetime while the same sample in glycerol or dioxane is quite homogeneous. Perhaps both the free and the protein-bound dansyl groups are moving between different environments within the detergent micelle. Alternatively, the protein-bound dansyl could be attached to more than one site. The average lifetime of the dansyl group on rhodopsin is 10.1 nsec. This lifetime is much longer than that of dansyl in an aqueous environment, suggesting that the dansyl group is either covered with Ammonyx LO or that it is located in a nonpolar crevice of the protein.

When rhodopsin is bleached, the lifetime of the dansyl group changes (Fig. 28). It now appears to be nearly homogeneous with a value of 16.4 nsec. This change in lifetime can be attributed to two processes. First, the environment of the probe may change upon bleaching, secondary to a protein conformational change. There probably is a shift in environment in going from dansyl cadaverine-rhodopsin to dansyl cadaverine-opsin in Ammonyx LO because there is a shift in the fluorescence emission maxima. The second process which can change the lifetime is dipole-dipole energy transfer. In rhodopsin, the lifetime of the dansyl group could be shortened by energy transfer to the 11-*cis* retinal group. Once the rhodopsin is bleached,

this transfer process could no longer take place, so the lifetime would increase. The change in efficiency of energy transfer can be related to the distance between the dansyl fluorescent donor and the 11-cis retinal energy acceptor. However, because the lifetimes are also influenced by changes in the dansyl environment, it is not feasible to use the change in lifetime to determine unequivocally the transfer efficiency in this experiment.

The mobility of the dansyl reporter group can be examined in dansyl cadaverine-rhodopsin by examining the decay of emission anisotropy (Fig. 29). The decay curve was used to estimate a correlation time of about 5 nanoseconds for the dansyl probe, suggesting that the dansyl group is moving as part of a mass of approximately 5,000-10,000 molecular weight. Much of this mobility is lost upon bleaching. The resulting decay of anisotropy curve now suggests at least two correlation times, of approximately 5 and 75 nanoseconds, possibly indicating that the motion seen in unbleached rhodopsin is now restricted to a limited angular range, so the probe moves as part of the whole detergent-protein complex as well. The conformation of bleached opsin in 1% Ammonyx Lo is not physiological; so the importance of the dansyl mobility in the bleached protein is uncertain. In contrast, unbleached rhodopsin in detergent does have a conformation similar to the native one in the intact membrane as deduced by CD and resonance Raman spectroscopy. Therefore, the high degree of mobility of the dansyl group in dansyl-cadaverine rhodopsin may be pertinent to understanding the conformation of rhodopsin.

The distance between the dansyl cadaverine-label and the 11-cis retinal chromophore is 60 Å in disc membranes

Dansyl cadaverine rhodopsin was also examined in disc membranes. Excess dansyl cadaverine readily partitions into the hydrophobic regions of the lipid bilayer. This material was nearly completely removed by washing the membranes with solutions of 1% bovine serum albumin so as to reduce the non-covalently bound dansyl fluorescence to undetectable levels.

The fluorescence lifetimes of the dansyl group in dansyl cadaverine-rhodopsin and dansyl cadaverine-opsin were measured in disc membranes (Fig. 30). Before bleaching, the sample showed a heterogeneous fluorescence decay curve with an average lifetime of 13.7 nanoseconds. After bleaching, the lifetime of the dansyl probe was a homogeneous 18.5 nanoseconds. Interestingly, the unbleached decay fits a composite of 80% of a 12.5 nanosecond lifetime and 20% of an 18.5 nanosecond lifetime. Since disc membranes from Hormel retinas typically contain 15-20% opsin, these data suggest that dansyl cadaverine-rhodopsin in disc membranes could have a relatively homogeneous lifetime of 12.5 nanoseconds. As in detergent-solubilized rhodopsin, the long lifetime of the dansyl fluorescence in disc membranes indicates that the dansyl group is in a relatively hydrophobic environment.

Unlike dansyl cadaverine-rhodopsin in detergent solution, membrane-bound dansyl cadaverine-rhodopsin does not change its fluorescence emission maxima upon bleaching. The dansyl emission spectra is shown in Fig. 31. In this case, there is probably little change in environment of the dansyl group upon bleaching, and the lifetimes before and after bleaching can be used to calculate an efficiency of energy transfer. This value was determined to be 32%. The transfer efficiency was used to estimate a distance

between the dansyl cadaverine group and the 11-cis retinal group according to Förster's theory of dipole-dipole interaction. The dansyl emission anisotropy starts at 0.2, indicating the probe has mobility and that the orientation factor, K^2, may be approximated as 2/3. Relevant parameters are shown in Table XI, and the distance is estimated to be about 60 Å.

The mobility of the probe on rhodopsin in disc membranes was also examined (Fig. 32). In contrast to the case in detergent, the dansyl group on membrane-bound rhodopsin showed restricted mobility as determined by the decay of anisotropy. Furthermore, little difference was noted upon bleaching the rhodopsin. The differing results could mean that the mobility seen in 1% Ammonyx LO is secondary to a detergent-produced change in protein conformation despite the evidence for preservation of structure by other spectroscopic techniques. On the other hand, this difference could result because the probe might be in a different environment in the membrane than it is in the purified protein. For example, the dansyl group in the membrane may extent into an immobilized ring of lipid surrounding the rhodopsin, while in detergent solution, it was located in a hydrophobic pocket of the protein.

C. Discussion

The evidence presented in this chapter showed that transglutaminase catalyzes the insertion of 1.0 ± 0.1 exogenous amines into rhodopsin. It does not prove that only one site of the rhodopsin molecule is labeled. The known specificity of the enzyme suggests that the group(s) modified must be a glutamine; this fact was not directly demonstrated. The reciprocal inhibition of rates between enzymatic cleavage and transglutaminase-catalyzed labeling suggests that these two actions occur in the same region of the polypeptide chain. However, these observations could also be explained by protein conformational changes occurring over a distance. The strongest evidence for locating the labeling site(s) and the cleavage site(s) in the same region of rhodopsin is the observation that subtilisin initially partitions some of ^{3}H-putrescine label on FS1 and some on FS2 followed by excision of the label from both fragments without appreciable change in the electrophoretic mobility of either fragment. The simplest interpretation of these data is that subtilisin cleaves rhodopsin at two or more closely spaced sites located on different sides of the site(s) labeled by transglutaminase. The initial proteolytic event can place the label on either fragment, and a second proteolytic event removes it. In the experiments with dansyl cadaverine-rhodopsin, the label after the initial event was almost exclusively bound to FS1; possibly the bulkier dansyl group provides some steric protection of the cleavage site between the transglutaminase site and the larger fragment. The other cleavage site is therefore attacked first. Similar results were seen with papain cleavage of transglutaminase-catalyzed labeled-rhodopsins (Table X).

If the above interpretation is correct, then the results of the fluorescence experiments are exceedingly interesting. The 60 Å distance between the dansyl group and the 11-cis retinal, as estimated by the change in energy transfer, confirms the observations made in detergent solutions that rhodopsin is probably elongated. From the work of Wu and Stryer (1972), the distance between site A and the 11-cis retinal group was also determined to be long, i.e. > 75 Å . If the dansyl cadaverine probe is on the edge of F2, and since both site A and the 11-cis retinal group are on F2, there must be two long distances on this small, approximately 10,000 dalton polypeptide domain. This suggests that the dansyl probe site and site A are probably close to each other. Preliminary measurements of energy transfer between AENS as an energy donor and transglutaminase-inserted ethyl orange cadaverine as an energy acceptor on double-labeled rhodopsins in 1% Ammonyx LO suggest distances on the order of 30 Å separating these sites, confirming the prediction.

CHAPTER VI

SUMMARY AND PROSPECTS

The data presented in this dissertation lead to a new model for the structure of rhodopsin. Thermolysin splits rhodopsin into two membrane-bound fragments. These two fragments have amino acid compositions that are strikingly similar to each other and to that of opsin. The larger fragment, called F1, contains the blocked amino terminus of opsin, the insertion site of galactose following modification by galactosyl transferase, the specific binding site for Con A, and all of the amino sugars of opsin. The smaller fragment, called F2, contains the 11-*cis* retinal binding site and the accessible sulfhydryl group alkylated by IAENS. F1 and F2 have estimated molecular weights of 25,000 ± 3,000 and 10,000 ± 2,000 respectively; these fragments account for most of the amino acid residues of whole rhodopsin. Both fragments behave as integral membrane proteins, i.e., neither can be solubilized from the membrane in aqueous solutions and both remain insoluble following disruption of the lipid bilayer structure. Other enzymes, such as subtilisin and papain, also cleave rhodopsin into similar pairs of fragments. These data are not consistent with the idea that rhodopsin has a hydrophobic core and a hydrophilic periphery, as previously suggested by other workers (Trayhurn *et al.*, 1974a, 1974b; Saari, 1974; van Breugel *et al.*, 1975). My model of rhodopsin has two membrane-bound domains of similar hydrophobicity.

Cleaved rhodopsin retains its 500 nm absorbance band. It is also regenerable to the same extent as whole rhodopsin by adding exogenous 11-*cis* retinal. Three methods of purification, deoxycholate differential extrac-

tion, hydroxylapatite chromatography, and Con A-agarose affinity chromatography, were used to isolate the 500 nm absorbing species. In all three cases, cleaved rhodopsin contained both F1 and F2. Thermolysin-cleaved rhodopsin has 90% as many amino acid residues as does whole rhodopsin. In the new model of rhodopsin, the two membrane-bound domains interact with each other by non-covalent forces. When the covalent, polypeptide bridge between the two domains is severed by proteolysis, the non-covalent interactions are of sufficient strength to keep the cleaved rhodopsin complex together in detergent solution.

The most exciting aspects of this new model are its dynamic features. Light dissociates enzymatically-cleaved rhodopsin in detergent solution into F1 and F2. This process may represent an experimentally accessible expression of a change in the non-covalent interactions between the two domains of the molecule. The region of polypeptide chain which connects F1 and F2 is highly susceptible to proteolysis by several proteases of differing specificity. This connecting region could be a molecular hinge, about which motions between the two domains of rhodopsin take place.

Guinea pig liver transglutaminase catalyzed the labeling of rhodopsin with exogenous amines. This enzyme inserted spectroscopic probes into the region of rhodopsin that is sensitive to proteases. The efficiency of energy transfer between a dansyl cadaverine label and the 11-cis retinal group in disc membranes was used to estimate a distance of over 60 Å separating these two sites. Thus, rhodopsin appears to be elongated in disc membranes. The dansyl probe is located near the amino terminus of F2. The 11-cis retinal chromophore is also bound to this small fragment. Therefore, F2, a 10,000 dalton length of polypeptide,must be at least 60 Å long. The

^{14}C-galactose insertion site is located on F1. If the galactosyl transferase can be used to insert a spectroscopic probe, an interesting triangulation experiment could be done to measure distances between the carbohydrate group(s) on F1, the transglutaminase labeling site in the putative hinge region, and the retinal group on F2.

What are some of the implications of this work for future research? The division of rhodopsin into F1 and F2 can be used to help locate the position of spectroscopic probes within the rhodopsin molecule. The photodissociation of F2 from F1 bound to a Con A-agarose column makes feasible large scale separation of the two fragments which could be used to order smaller peptides for the eventual sequencing of rhodopsin. Finally, the insertion of spectroscopic probes into the putative hinge region of the molecule may permit measurements of conformational changes in some of the metastable photolytic intermediates. This line of research may provide the link between the structure of rhodopsin and its function as a receptor protein.

TABLE I

PROPERTIES OF BOVINE RHODOPSIN

I. MOLECULAR WEIGHT

Method	Determination	Reference
Quantitative amino acid analysis	39,100 ± 900	Daemen et al., 1972
	38,000 ± 2,000	Heitzmann, 1972
Ultracentrifuge (sedimentation equilibrium)	35,000 ± 2,000	Lewis et al., 1974
Neutron scattering	38,000 ± 2,000	Yeager, 1976

II. EXTINCTION COEFFICIENT ϵ_{500}

Method	Determination	Reference
Retinal oxime absorbance	40,600	Wald & Brown, 1953
	42,000	Shields, 1970
	41,900	Bridges, 1970
Thiobarbituric acid retinal assay	40,000	Futterman & Saslaw, 1961
	42,000	Schichi et al., 1969
	43,000	Daemen et al., 1970
	41,000 ± 1,200	Zorn & Futterman, 1971
	40,000 ± 1,000	Heitzmann, 1972
	41,000 ± 1,000	Rotmans et al., 1972b

TABLE II

REPRESENTATIVE DETERGENTS USED WITH RHODOPSIN

Detergent	Class	Structure	500 nm	Regeneration	Miscellaneous
Digitonin	Steroid	Neutral Steroid, Carbohydrate	Preserved	Preserved	Expensive; low solubility, low purity; not suitable for chromatography
Cholate	Steroid	Acidic Steroid	Preserved	Preserved	Compatible with ammonium sulfate; not suitable for chromatography
Emulphogene	Non-Ionic	Tridecyl-$(O\text{-}CH_2CH_2)_{11}$-OH	Preserved	Lost	Low U.V. absorbance; compatible with chromatography
Triton X-100	Non-Ionic	Octylphenyl-$(O\text{-}CH_2CH_2)_{9.5}$-OH	Preserved	Lost	High U.V. absorbance; compatible with chromatography
Alkyl Glucosides	Non-Ionic	Alkane + Glucose	Preserved	Lost	Recently introduced
Tween 80	Non-Ionic	Mono-Oleyl Sorbitan $(O\text{-}CH_2CH_2)_{20}$-OH	Preserved	Partially Preserved	Limited value in chromatography
Ammonyx LO	Non-Ionic	12 and 14 Carbon Alkyl Dimethyl Amine Oxide	Preserved	Lost	Excellent for chromatography, spectroscopy; dialyzable
CTAB	Cationic	16 Carbon Alkyl Trimethyl Ammonium Bromide	Preserved	Lost	Not suitable for hydroxylapatite chromatography
DTAB	Cationic	12 Carbon Alkyl Trimethyl Ammonium Bromide	Preserved at 4° C	Lost	Dialyzable; compatible with hydroxylapatite chromatography
SDS	Anionic	12 Carbon Alkyl Sulfate	Lost	Lost	Excellent for electrophoresis

TABLE III

AMINO ACID COMPOSITION OF BOVINE RHODOPSIN (MOLE %)

Investigators	Shields et al. (1967)	Heller (1968)	Schichi et al. (1969)	Saari (1974)	Trayhurn et al. (1974b)	Plantner & Kean (1976)
Method of Purification	Gel filtration (digitonin)	Gel filtration(CTAB)	Calcium phosphate chromatography	Hydroxylapatite chromatography	Preparative SDS-gel electrophoresis	Con A-agarose affinity chromatography
RESIDUE						
Lys	4.4	4.3	3.1	3.5	3.9	3.9
His	2.2	1.7	1.6	1.9	1.4	1.9
Arg	3.7	2.6	2.4	2.2	2.3	2.6
Asp	7.1	6.5	7.4	5.8	6.1	6.9
Thr	7.9	7.4	9.8	6.7	7.2	8.3
Ser	5.6	5.2	5.9	3.8	4.3	5.9
Glu	8.2	9.1	9.8	10.7	8.7	9.0
Pro	5.2	5.7	6.3	7.4	6.7	6.0
Gly	7.9	7.0	8.2	7.0	7.4	7.5
Ala	8.2	8.7	9.8	8.6	9.2	8.7
Cys	2.2	2.2	2.4	---	---	2.2
Val	8.2	8.7	5.9	8.0	8.7	8.1
Met	3.1	3.5	3.1	4.2	4.3	3.3
Ile	5.2	5.7	3.9	5.1	5.6	4.8
Leu	8.2	8.7	7.8	9.3	8.5	8.0
Tyr	7.5	4.8	3.9	5.1	5.3	4.3
Phe	3.7	8.3	8.6	10.0	9.5	8.5
Trp	---	---	---	---	0.9	---
NAG		2-3				5-6
MANNOSE		3				7-9

TABLE IV

INDICATIONS OF CONFORMATIONAL CHANGE UPON BLEACHING

Observation	In strong detergent	In digitonin	In disc membranes
(1) Change in visible absorption	+	+	+
(2) Uptake of proton in Meta I → Meta II	+	+	+
(3) Appearance of $NaBH_4$ accessibility to retinyl-lysyl Schiff's base	+	+	+
(4) Appearance of newly titratable sulfhydryl groups	+	+	-
(5) Change in protein CD spectrum	+	-	-
(6) Change in spin-label probe mobility	+	-	-
(7) Loss of regenerability	+	-	-
(8) Change in accessibility to lectins			-
(9) Change in accessibility to proteases			-
(10) Change in disc membrane birefringence			+
(11) Change in disc membrane x-ray scattering			+
(12) Change in disc membrane freeze-fracture image			+

TABLE V

PROPERTIES OF PROTEOLYTIC ENZYMES USED TO DIGEST DISC MEMBRANES

Enzyme	Source	Type	Inhibitor	M.W.	Specificity
Papain	Carica papaya	Cysteine	Iodoacetamide	23,406	broad; Phe containing regions
Thermolysin	B. thermoproteolyticus	Zn^{++}, Ca^{++} requiring	EDTA	37,500	amino side of hydrophobic residues
Subtilisin	B. subtilis (Carlsberg)	Serine	DFP; Phenyl methyl sulfonyl fluoride	27,400	broad
α-Chymotrypsin	bovine pancreas	Serine	DFP; Phenyl methyl sulfonyl fluoride	25,000	carboxyl side of aromatic and bulky hydrophobic residues
Pronase	Streptomyces griseus	Mixture	Trichloroacetic acid	---	broad

*Source - The Enzymes, Boyer, P.D. (ed.)

TABLE VI

PROTEOLYSIS OF RHODOPSIN -

SUMMARY OF REPORTED RESULTS

Investigator	Enzyme(s)	Conclusions
Trayhurn et al.,1974	Papain	(1) Proteolysis liberates 1/3 of molecule, leaving a resistant "core" (2) Core contains carbohydrate and 11-cis retinal (3) Proteolysis preserves 500 nm absorbance and regeneration
Saari, 1974	Thermolysin, Subtilisin, and α-Chymotrypsin	(1) All three enzymes reduce rhodopsin to a 29,000 dalton fragment which is subsequently degraded further to 20,000-25,000 dalton fragments (2) All of these fragments contain carbohydrate and 11-cis retinal (3) A fragment of 18,000 daltons sometimes is seen (4) Proteolysis preserves 500 nm absorbance and regeneration
Van Breugel et al., 1975	Pronase, α-Chymotrypsin, and Subtilisin	(1) All enzymes attack rhodopsin, but few amino acid residues are liberated from the membrane (2) Borohydride reduces the retinal chromophore on to a fragment of 29,000 daltons and one of 15,000 daltons (3) The 29,000 dalton fragment bears carbohydrate

TABLE VII

AMINO ACID COMPOSITIONS OF OPSIN, F1 AND F2 (MOLE %)

(EXCLUDING CYS AND TRP)

Amino acid	Opsin	F1	F2
LYS	3.6	3.1	5.4
HIS	1.5	1.4	1.4
ARG	2.7	2.8	2.4
ASP	7.4	7.9	8.3
THR	7.6	6.9	9.0
SER	5.3	5.6	5.3
GLU	9.8	9.1	8.9
PRO	6.4	5.9	5.6
GLY	8.3	9.6	8.1
ALA	8.6	7.6	9.4
VAL	7.5	7.3	6.8
MET	3.3	3.5	4.0
ILE	4.7	4.2	5.6
LEU	9.0	10.8	6.3
TYR	5.2	5.5	5.1
PHE	8.8	8.8	8.5

TABLE VIII

SUMMARY OF DIFFERENCES BETWEEN F1 AND F2

Characteristic	F1	F2
Estimated molecular weight	25,000 ± 3,000	10,000 ± 2,000
Relative number of moles of amino acids in hydrolyzed gel bands	2.5 ± .5	1
N-retinyl fluorescence	Absent	Present
AENS fluorescence (relative intensity)	1	4
Galactose transferase-introduced ^{14}C-galactose radioactivity	Present	Absent
Periodate-created reactive groups	Present	Present
Specific binding site for Concanavalin A	Present	Absent
Mole % Lysine	3.1	5.4
Mole % Leucine	10.8	6.3

TABLE IX

COMPARISON OF FRAGMENTS PRODUCED BY PROTEOLYSIS OF RHODOPSIN

Enzyme	Fragment	SDS-gel M.W. (Weber & Osborn, 1969)	N-retinyl fluorescence	AENS fluorescence (relative intensity)	^{14}C galactose	Con A binding site
Thermolysin	F1	30,000	Absent	Weak	Present	Present
Subtilisin	FS1	30,000	Absent	Weak	Present	Present
Papain	FP1	30,000	Absent	Weak	Present	Present
Chymotrypsin	FC1	30,000	Absent	Weak	Present	Present
Chymotrypsin	FC1[1]	23,000	Absent	Weak	Present	Present
Thermolysin	F2	18,000	Present	Strong	Absent	Absent
Subtilisin	FS2	18,000	Present	Strong	Absent	Absent
Papain	FS2	18,000	Present	Strong	Absent	Absent
Chymotrypsin	FC2	18,000	Present	Strong	Absent	Absent

TABLE X

ACTION OF PROTEASES ON TRANSGLUTAMINASE-MODIFIED RHODOPSINS

Protease	Dansyl Cadaverine-Rhodopsin	Putrescine-Rhodopsin
Thermolysin	no proteolysis	no proteolysis
Subtilisin	early: dansyl fluorescence on FS1	early: putrescine radioactivity on FS1 and FS2
	late: dansyl fluorescence excised	late: putrescine radioactivity excised
Papain	early: dansyl fluorescence on opsin, FP1	early: putrescine radioactivity on opsin, FP1, FP2
	late: dansyl fluorescence excised	late: putrescine radioactivity excised

TABLE XI

FLUORESCENCE CHARACTERISTICS OF

DANSYL CADAVERINE-RHODOPSIN

Property	In 1% Ammonyx LO	In Disc Membranes
Dansyl lifetime in dark (τ_D)	10.1 nsec	12.5 nsec (corrected for partial bleaching)
Dansyl lifetime bleached (τ_B)	16.4 nsec	18.5 nsec
Correlation time, dark (decay of anisotropy)	5 nsec	~ 100 nsec
Correlation time, bleached (decay of anisotropy)	5 nsec; 75 nsec	~ 100 nsec
Efficiency of transfer		.32
Dansyl fluorescence emission maximum		525 nm
11-cis retinal absorption maximum		498 nm
Spectral overlap integral (J)		1.79667×10^{-13}
Dansyl fluorescence quantum yield (Q)		1.0
Distance at 50% transfer efficiency (R_o calc)		55 Å
Estimated distance: dansyl → 11-cis retinal		62 Å

Fig. 1. Sequence of photolytic intermediates following the absorption of a photon by rhodopsin.

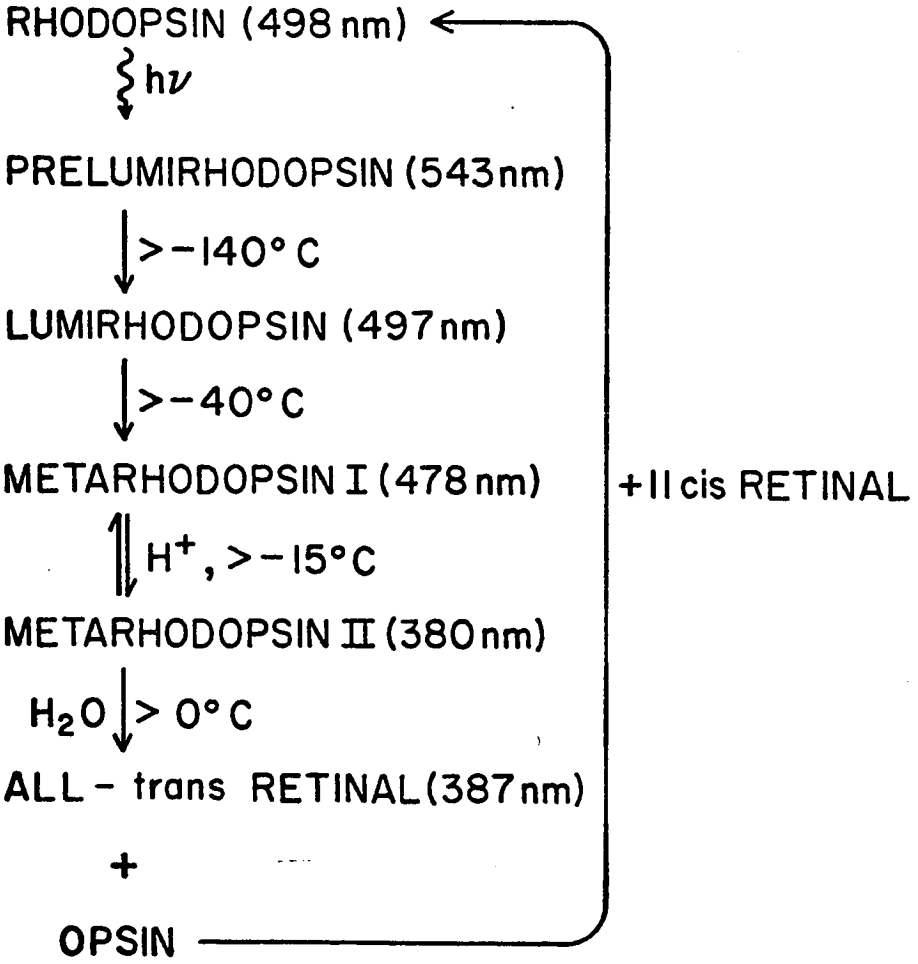


Fig. 2. Time course of the proteolysis of bovine retinal disc membranes by thermolysin as analyzed by electrophoresis on SDS-acrylamide gels. The gels were stained with Coomassie blue. Gels A through D represent 0 min, 10 min, 30 min and 3 hr, respectively of thermolysin treatment under conditions described under Methods. The predominant band at 0 min, which is opsin, was replaced by two bands having higher electrophoretic mobilities, called F1 and F2.

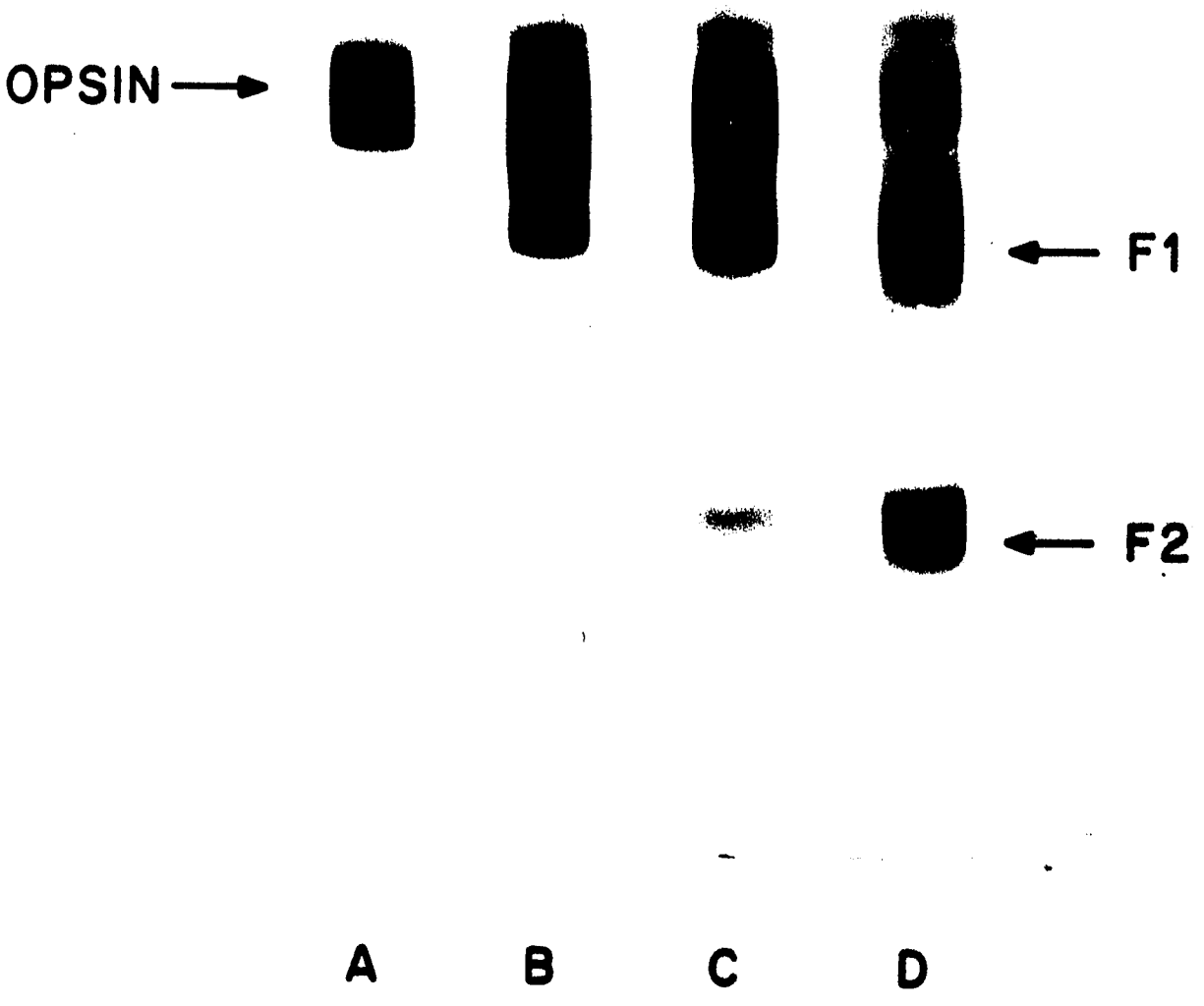


Fig. 3. Rhodopsin in 0.5% Triton X-100 solution purified by Con A-agarose affinity chromatography (A) before and (B) after 3 hrs of thermolysin proteolysis as analyzed by electrophoresis on SDS-acrylamide gels. The gels were stained with Coomassie blue. The opsin band was replaced by two bands having similar electrophoretic mobilities as F1 and F2, the pair of fragments produced by thermolysin proteolysis of disc membranes. The presence of Triton X-100 induces some aggregation of the opsin and opsin fragments.

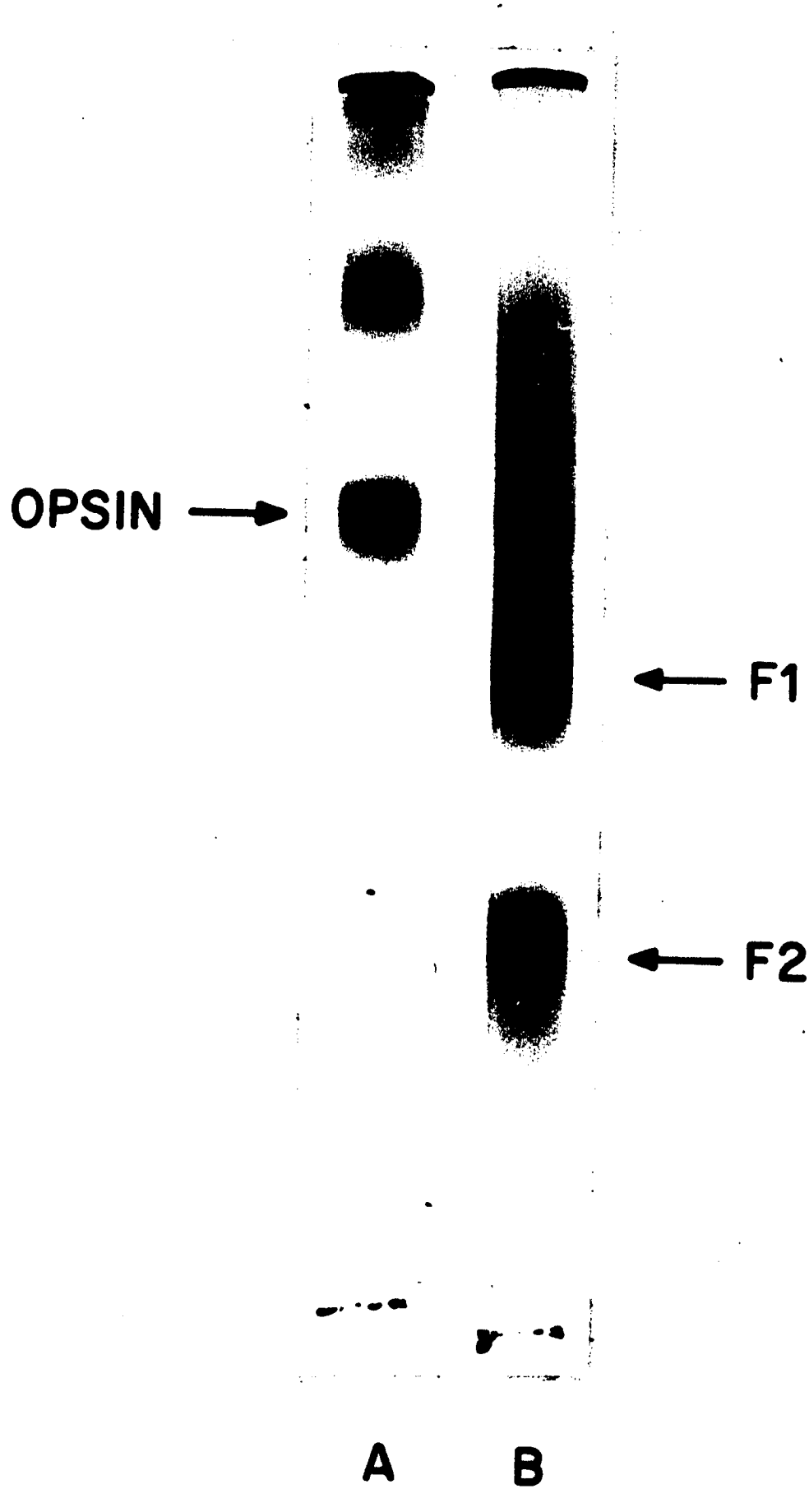


Fig. 4. (A) The absorbance spectra in the region of 400 nm to 600 nm wavelength of thermolysin-cleaved rhodopsin in 1.4% CTAB solution before and after bleaching. Thermolysin-cleaved rhodopsin retains the 500 nm absorbance band. (B) The absorbance spectra of thermolysin-cleaved rhodopsin in 1.4% CTAB solution following bleaching and subsequent regeneration in disc membranes of the 500 nm absorbance band by addition of 11-cis retinal. Thermolysin cleaved-rhodopsin is regenerable in disc membranes to the same extent as is untreated rhodopsin. The per cent regeneration is calculated as indicated under Methods by comparing the change in 500 nm absorbance produced by bleaching before and after regeneration.

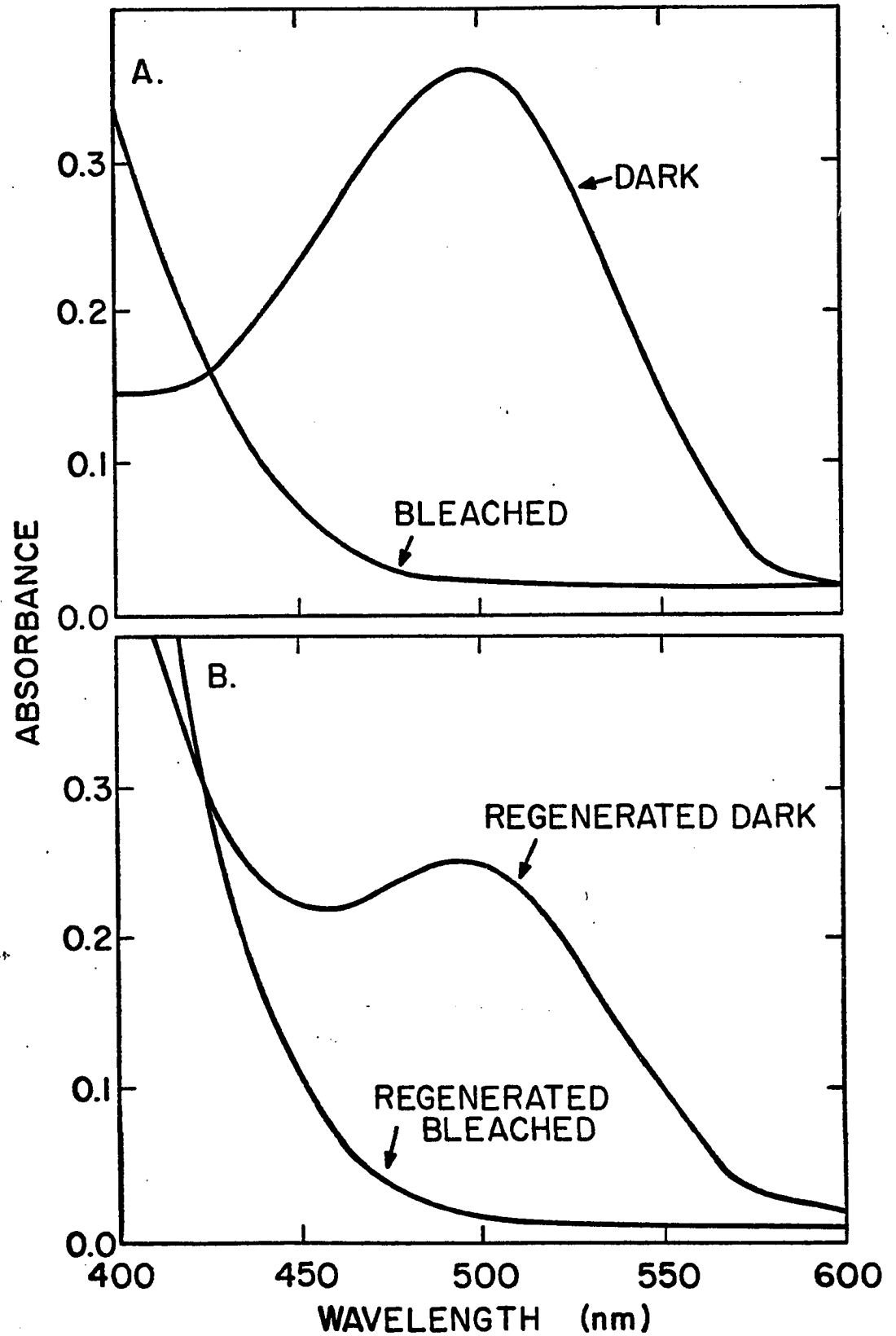


Fig. 5. Fluorescence scans of N-retinyl fluorescence on SDS-acrylamide gels of disc membranes (A) before and (B) after 6 hrs proteolysis by thermolysin. The excitation wavelength was 333 nm and the emission was viewed through a 3-72 Corning glass filter. The position of most of the N-retinyl fluorescence shifted from opsin to F2 following enzymatic cleavage. The N-retinyl fluorescence was produced by sodium borohydride reduction of rhodopsin in 2.5% SDS solutions as described under Methods. A small amount of fluorescence may be seen near the electrophoretic front, suggesting the formation of retinol or N-retinyl phosphatidyl ethanolamine.

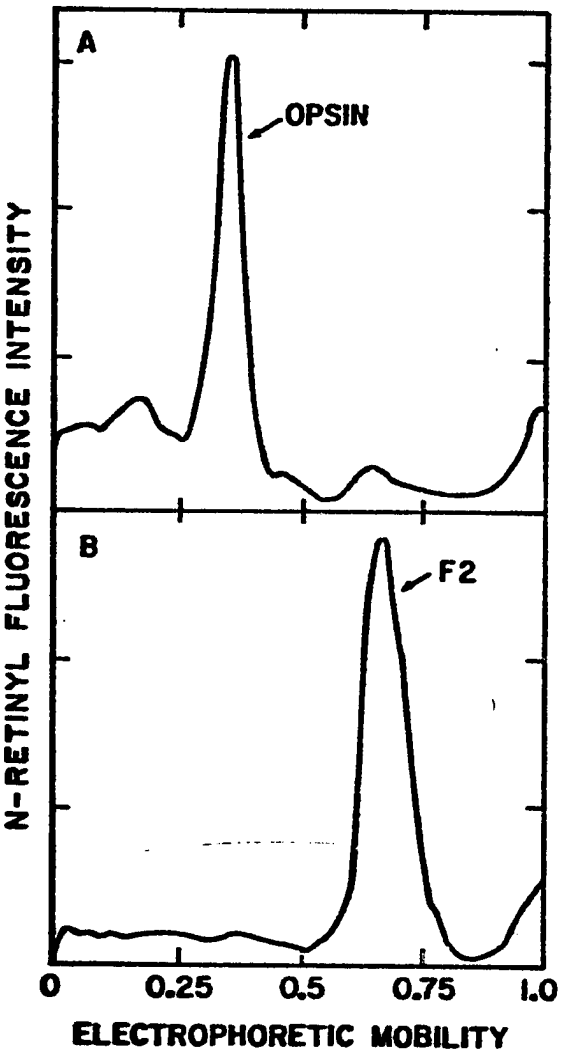


Fig. 6. Fluorescence scans of AENS fluorescence on SDS-acrylamide gels of AENS labeled disc membranes (A) before and (B) after 3 hr. of proteolysis by thermolysin. The excitation wavelength was 360 nm and the emission was viewed through a 3-72 Corning glass filter. The position of 80% of the AENS fluorescence shifted from opsin to that of F2 following enzymatic cleavage. About 20% of the fluorescence appeares at the positon of F1.

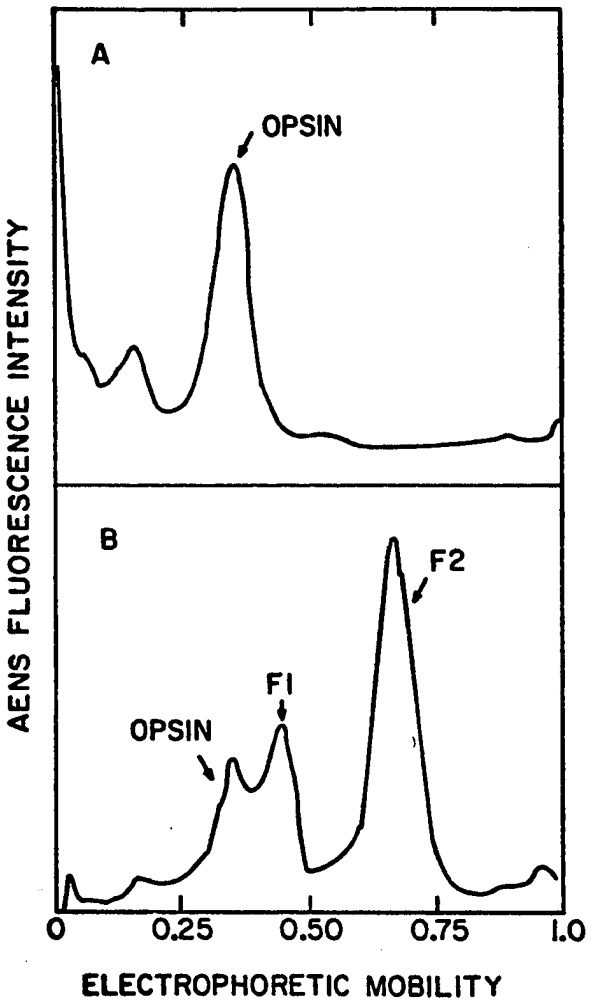


Fig. 7. Radioactive counting profile of SDS-acrylamide gels of ^{14}C galactose-labeled disc membranes (A) before and (B) after 6 hrs of proteolysis by thermolysin. Each gel was frozen and sliced into 5 mm pieces and prepared for liquid scintillation counting as described under Methods. The position of the ^{14}C-radioactivity shifted from opsin to that of F1 following enzymatic cleavage.

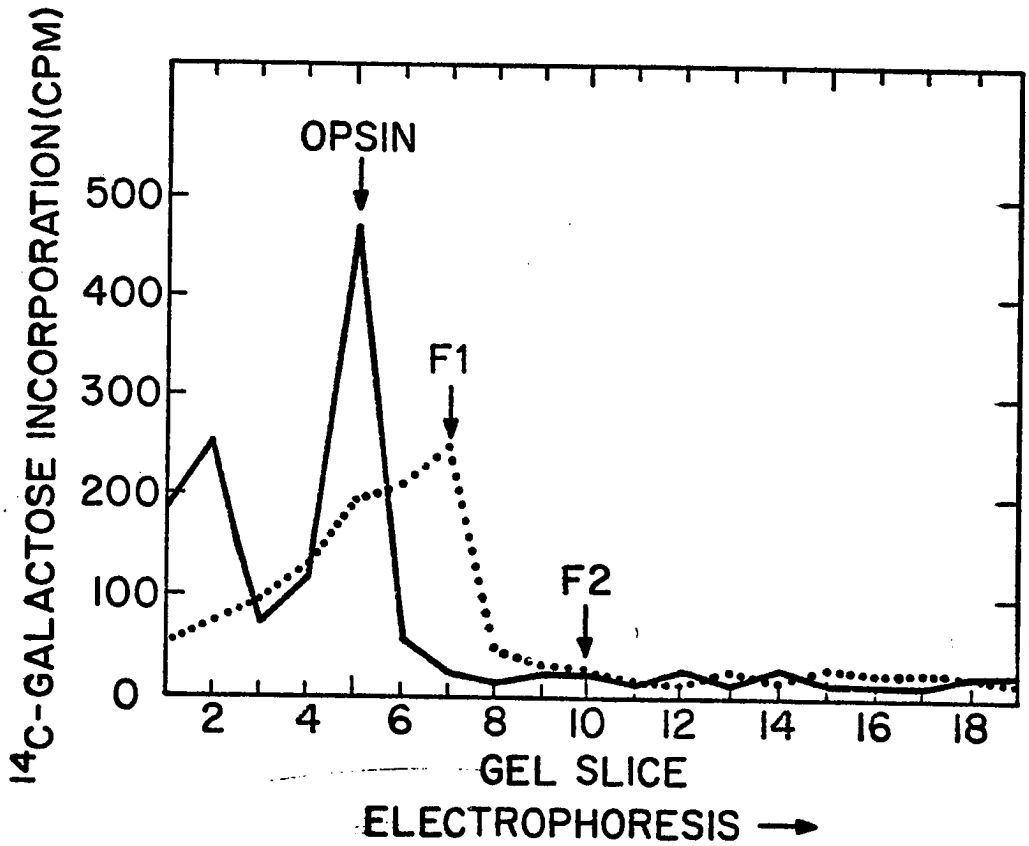


Fig. 8. Radioactive counting profile of SDS-acrylamide gels of ^{3}H-sodium borohydride reduced disc membranes, which had been periodate-oxidized prior to reduction (A) before and (B) after 6 hr of proteolysis by thermolysin. Each gel was frozen and sliced into 5 mm pieces and prepared for liquid scintillation counting as described under Methods. The position of the ^{3}H radioactivity shifts from opsin to that of F1 and F2 following enzymatic cleavage.

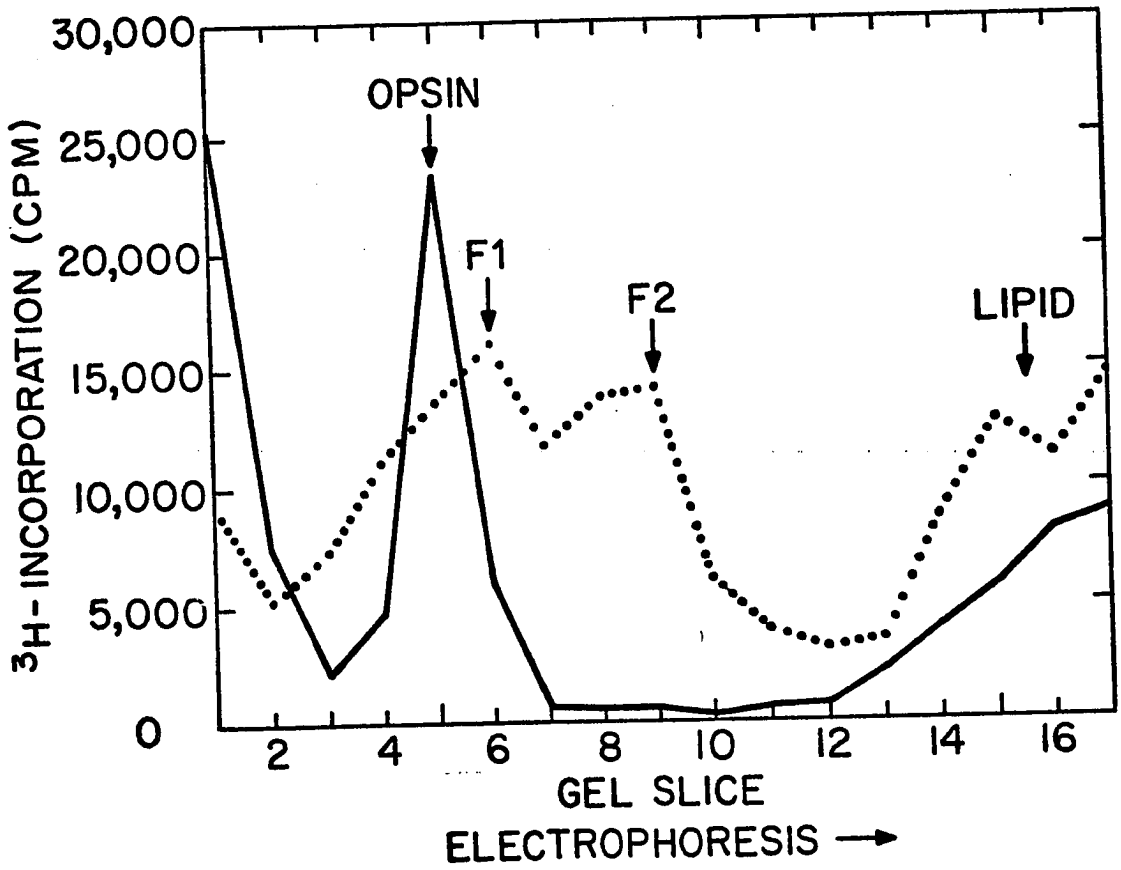


Fig. 9. Comparison of the polypeptides extracted differentially with increasing concentrations of deoxycholate from untreated and from thermolysin-cleaved disc membranes as analyzed by SDS-acrylamide gel electrophoresis. The gels were stained with Coomassie blue. Gels A through D represent the polypeptides sequentially extracted from untreated disc membranes with 0.1%, 0.15%, 0.2% and 0.4% deoxycholate, respectively. Gels E through H represent the polypeptides extracted from thermolysin-cleaved disc membranes with the same sequence of deoxycholate concentrations. Most of the 500 nm absorbing species in thermolysin-cleaved disc membranes was solubilized by 0.15% and 0.2% deoxycholate solution (gels F and G); these two fractions contain both F1 and F2 in equal proportion.

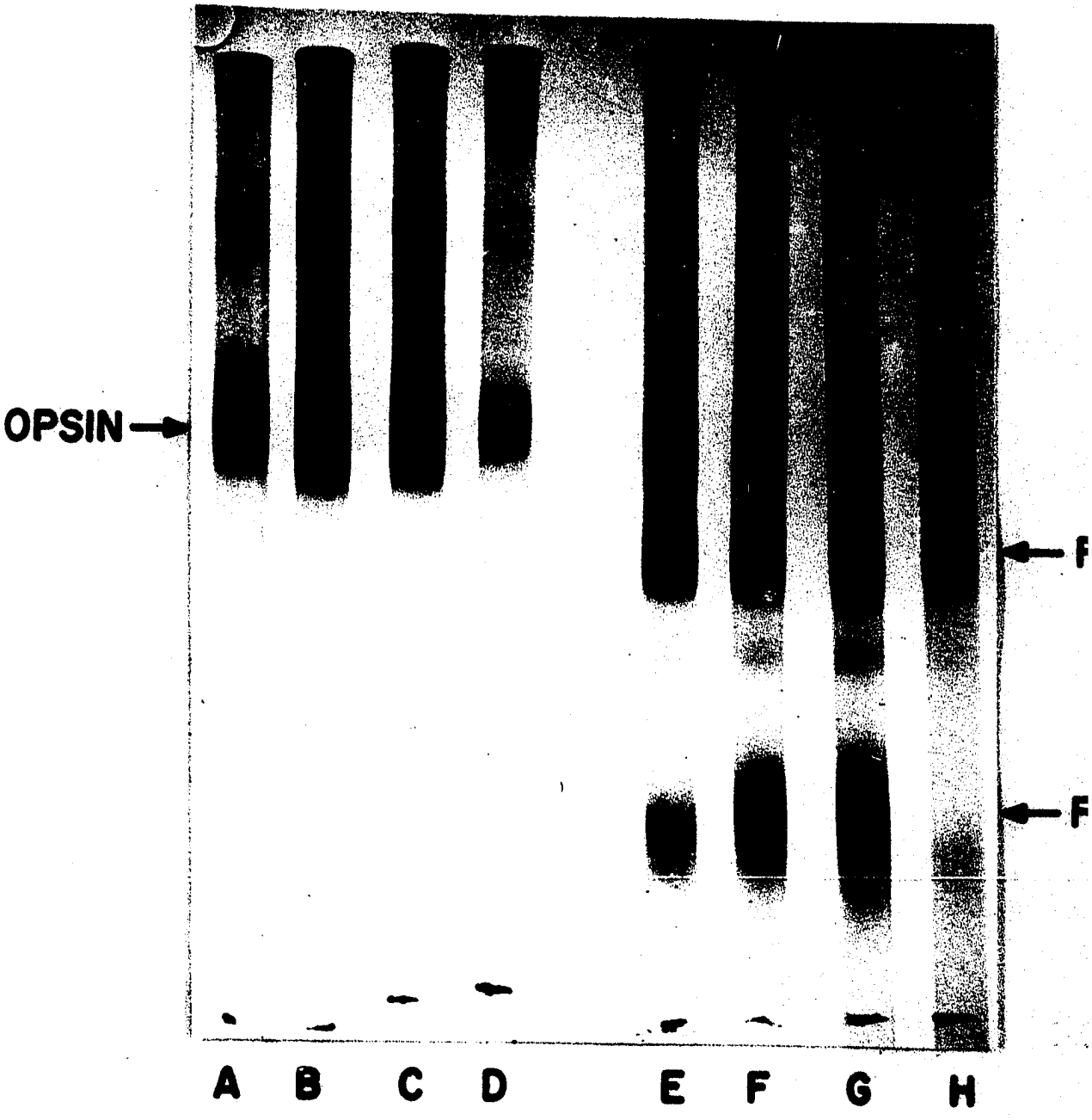


Fig. 10. (A) Whole and (B) thermolysin-cleaved rhodopsin after purification by Con A-agarose affinity chromatography as analyzed by SDS-acrylamide gel electrophoresis. The gels were stained with Coomassie blue. Cleaved-rhodopsin contains both F1 and F2.

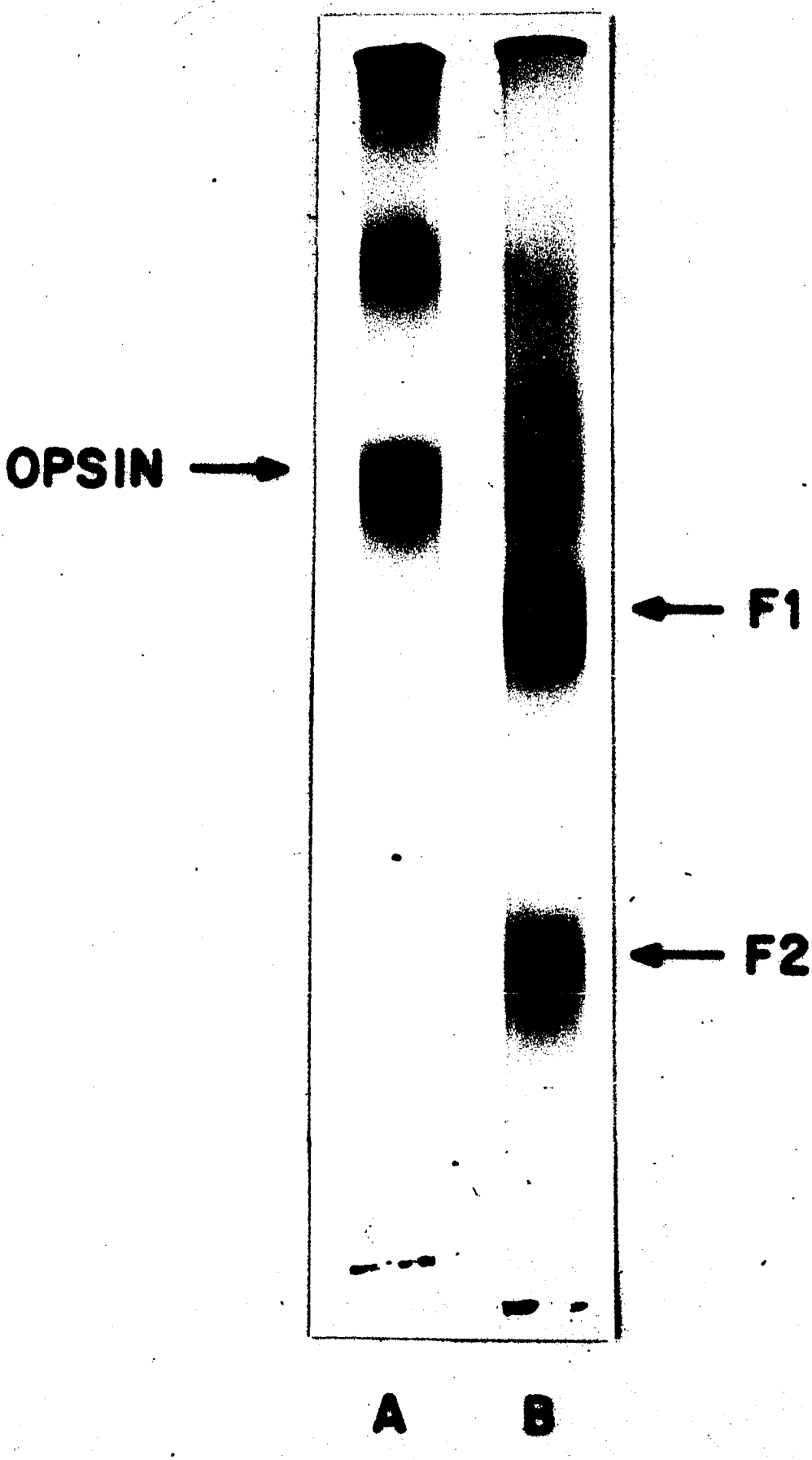
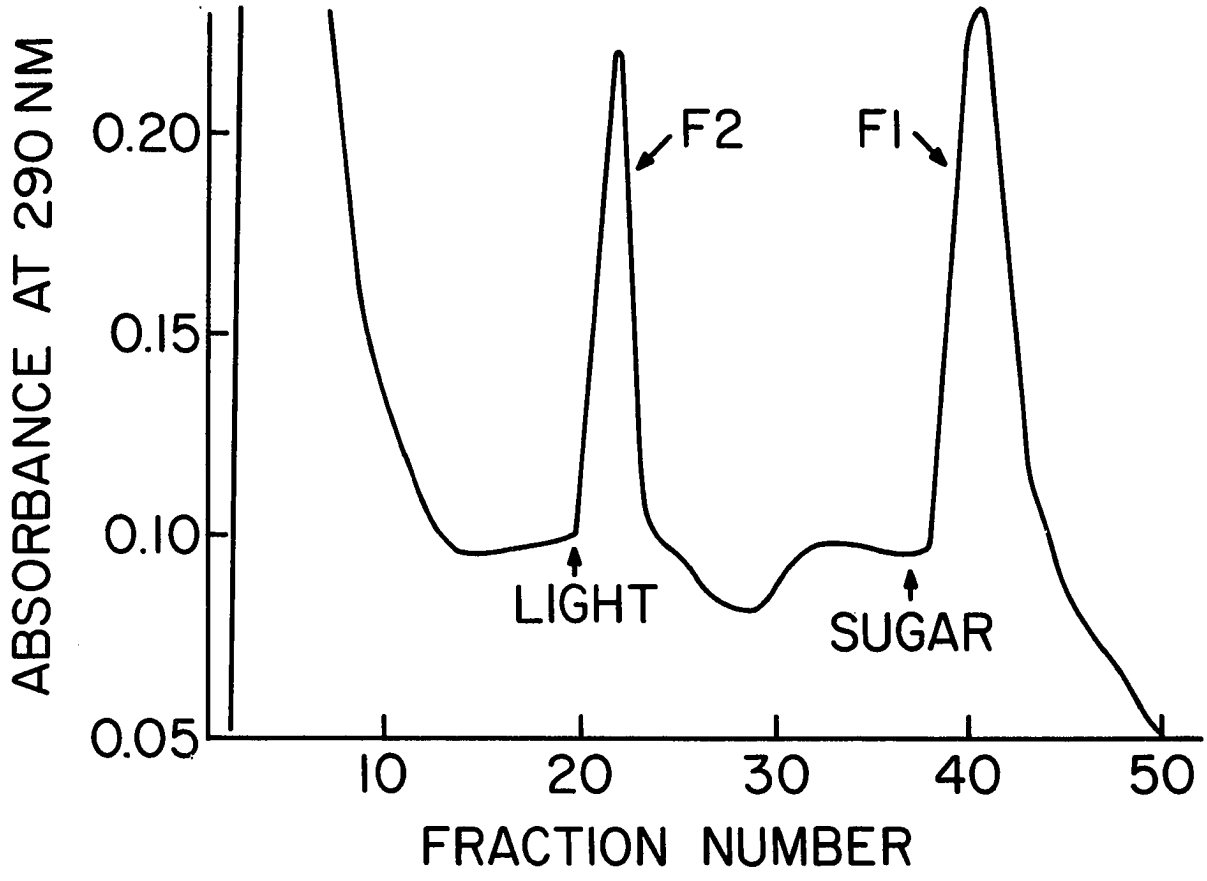


Fig. 11. (A) Elution profile of thermolysin-cleaved rhodopsin from a Con A-agarose column. The absorbance of the effluent was measured at 290 nm to maximize the absorbance of tryptophan to that of Triton X-100. Disc membranes were subjected to proteolysis by thermolysin, solubilized by Triton X-100 and loaded in the dark on a Con A-agarose column. The column was washed until the absorbance at 290 nm reached a plateau value. Exposure of the column to light led to the elution of 290 nm absorbing material. A second absorbance peak was observed following the addition of 0.1 M α-methyl mannoside. The presence of 2 mM dithiothreitol did not alter the elution pattern. (B) SDS-acrylamide gels stained with Coomassie blue show that these two peaks are F2 (top) and F1 (bottom) respectively. A small amount of undigested opsin is evident in the bottom gel.

A



B

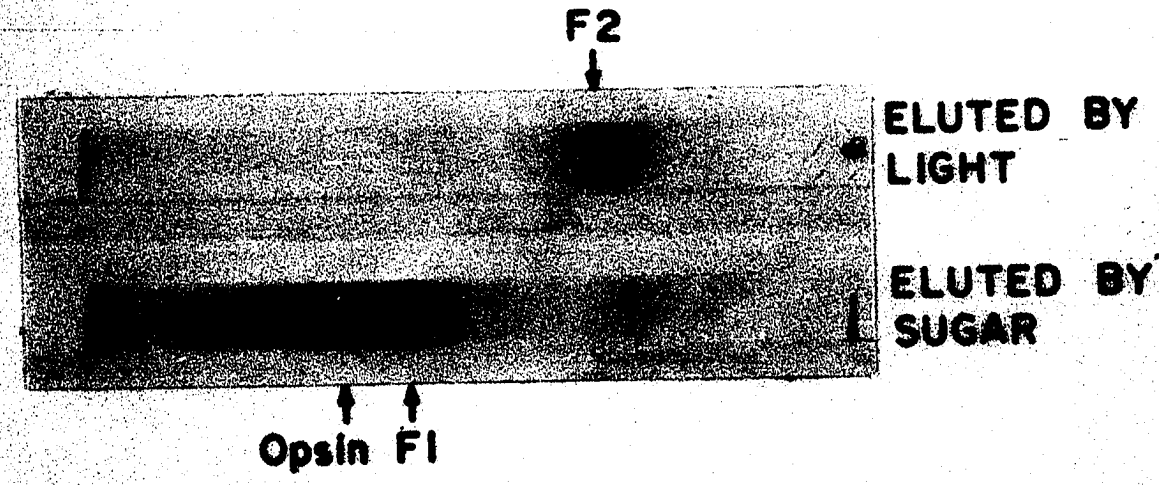


Fig. 12. Time course of the proteolysis of bovine retinal disc membranes by papain as analyzed by electrophoresis on SDS-acrylamide gels. The gels were stained with Coomassie blue. Gels A through E represent 0 hr, 1 hr, 2 hr, 4 hr and 8 hr respectively of papain treatment under conditons described under Methods. The predominant band at 0 hr, which is opsin, was replaced by two bands having higher electrophoretic mobilities, called FP1 and FP2. This pair of fragments has similar electrophoretic mobilities as the pair produced by thermolysin treatment of disc membranes, called F1 and F2.

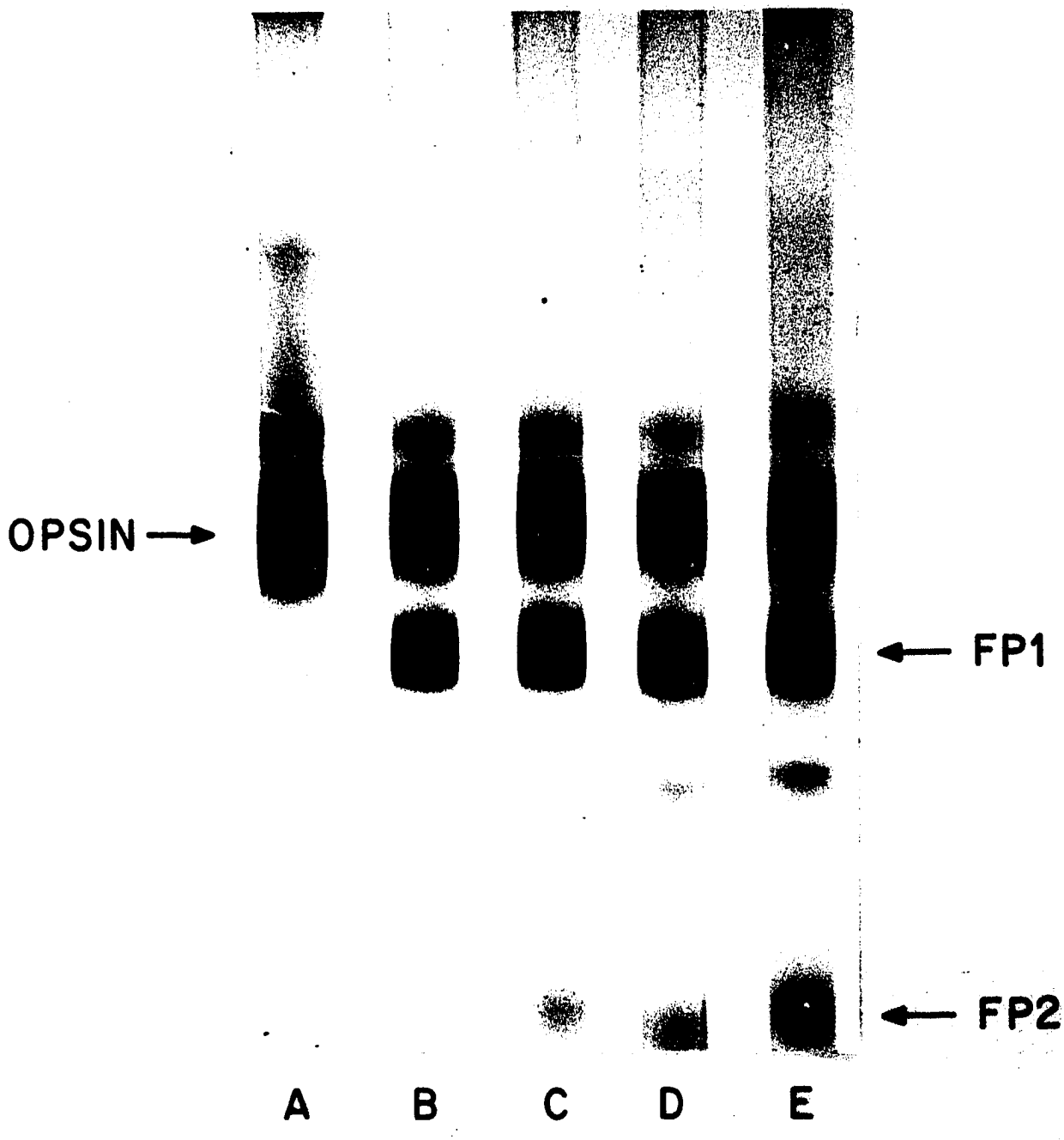


Fig. 13. Fluorescence scans of N-retinyl fluorescence on SDS-acrylamide gels of disc membranes (A) before proteolysis; (B) after 12 hr proteolysis by thermolysin; (C) after 12 hr proteolysis by papain; and (D) after 12 hr proteolysis by subtilisin. The excitation wavelength was 333 nm and the emission was viewed through a 3-72 Corning glass filter. The positon of the N-retinyl fluorescence after proteolysis with thermolysin, papain or subtilisin shifted to that of F2, FP2, or FS2 respectively. No N-retinyl fluorescence was seen the positions of F1, FP1 or FS1.

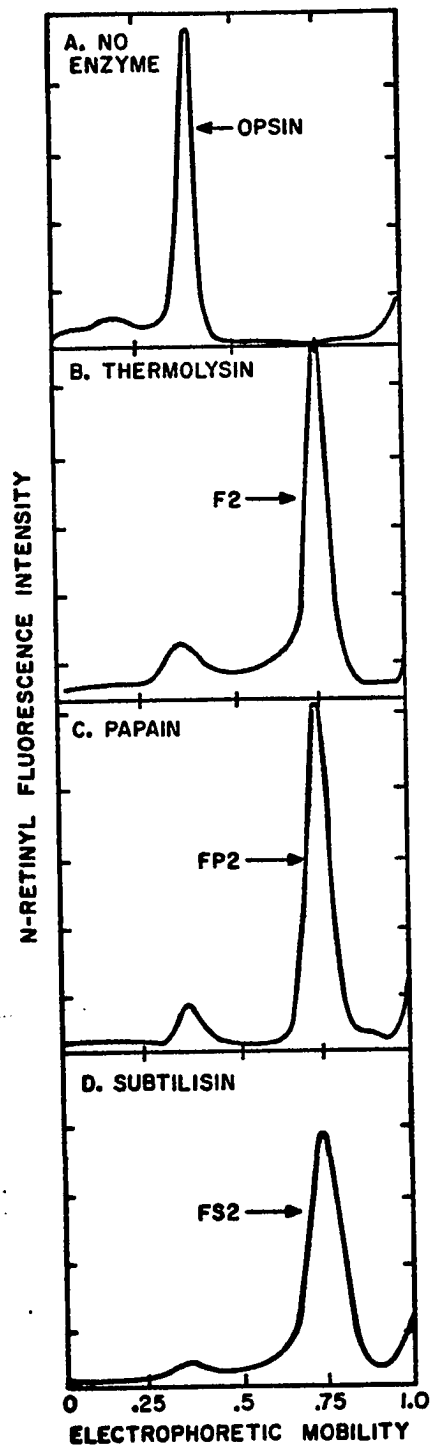


Fig. 14. SDS-acrylamide gels were used to analyze the polypeptides in the eluents (top) released by light and (bottom) released by 0.1 M α-methyl-mannoside from a Con A-agarose column loaded in the dark with papain-cleaved disc membranes in Triton X-100 solution. The gels stained with Coomassie blue show that light eluted FP2 while sugar caused the elution of FP1 and undigested opsin. These results are similar to those obtained using thermolysin-cleaved disc membranes.

Fig. 15. Time course of the proteolysis of bovine retinal disc membranes by subtilisin as analyzed by electrophoresis on SDS-acrylamide gels. The gels were stained with Coomassie blue. Gels A through F represent 15 min, 30 min, 1 h4, 2 hr, 5 hr and 24 hr respectively of subtilisin treatment under conditions described under Methods. The predominant band at 15 min, which is opsin, was replaced by two bands having higher electrophoretic mobilities, called FS1 and FS2. This pair of fragments has similar electrophoretic mobilities as the pair produced by thermolysin treatment of disc membranes called FL and F2.

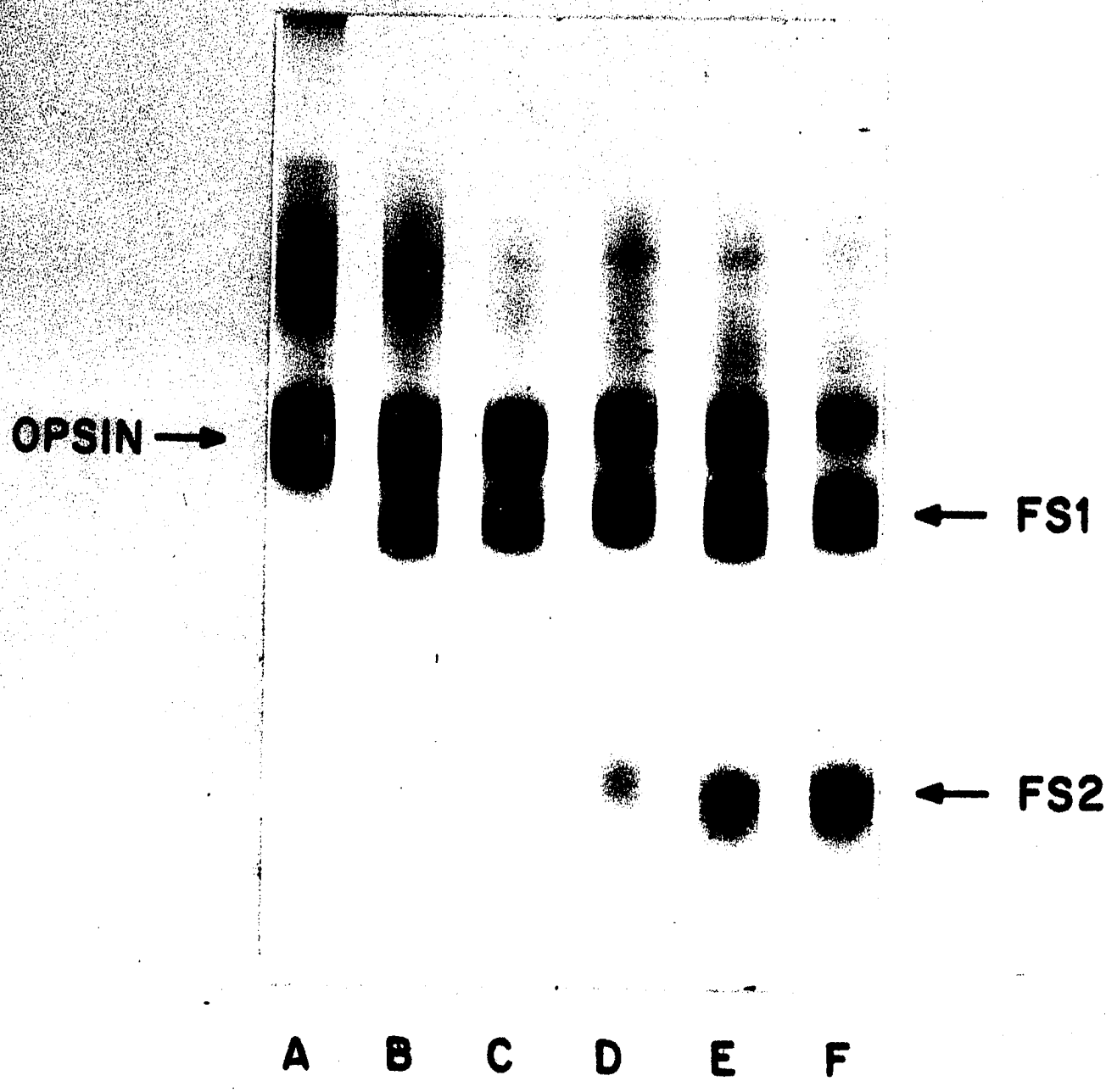


Fig. 16. SDS-acrylamide gels were used to analyze the polypeptides in the eluents (top) released by light and (bottom) released by 0.1 M α-methyl-mannoside from a Con A-agarose column loaded in the dark with subtilisin-cleaved disc membranes in Triton X-100 solution. The gels, stained with Coomassie blue, show that light eluted FS2 while sugar caused the elution of FS1 and undigested opsin. These results are similar to those obtained using thermolysin-cleaved disc membranes.

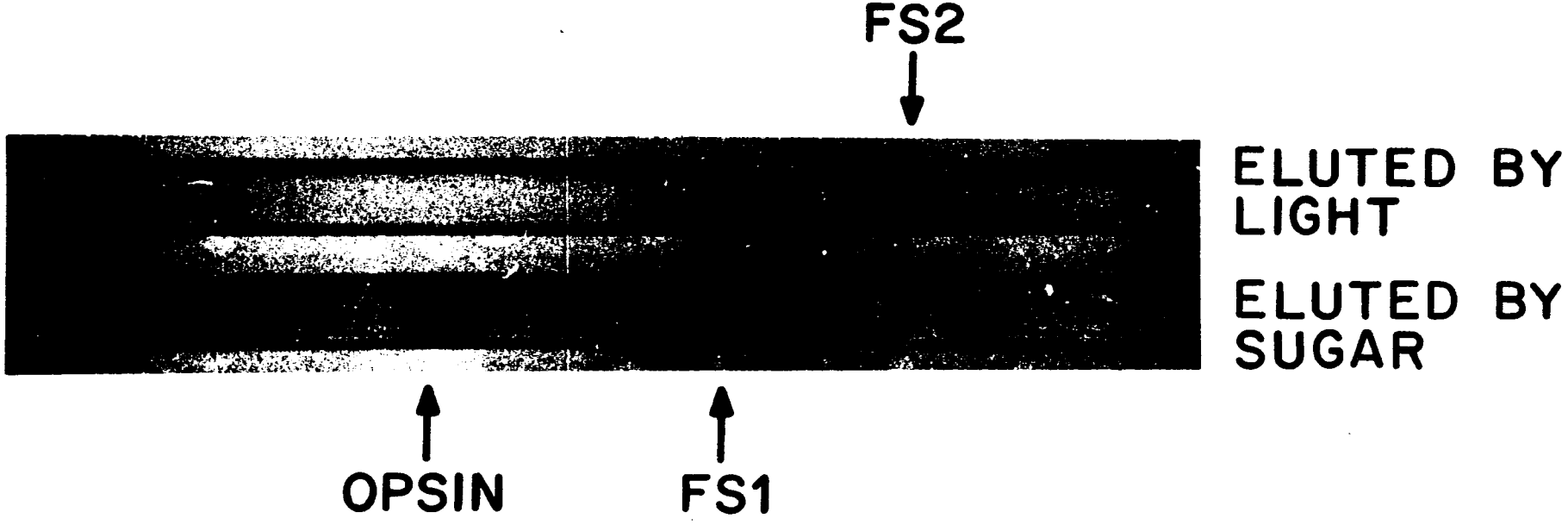


Fig. 17. Time course of the proteolysis of bovine retinal disc membranes by chymotrypsin as analyzed by electrophoresis on SDS-acrylamide gels. The gels were stained with Coomassie blue. Gels A through E represent 1 hr, 3 hr, 8 hr and 24 hr respectively of chymotrypsin treatment under conditions described in Methods. The predominant band at 0 hr, which is opsin, was partially replaced by one heavily staining band and two lightly staining bands having higher electrophoretic mobility. In the order of increasing mobility, these have been named FC1, FC1' and FC2.

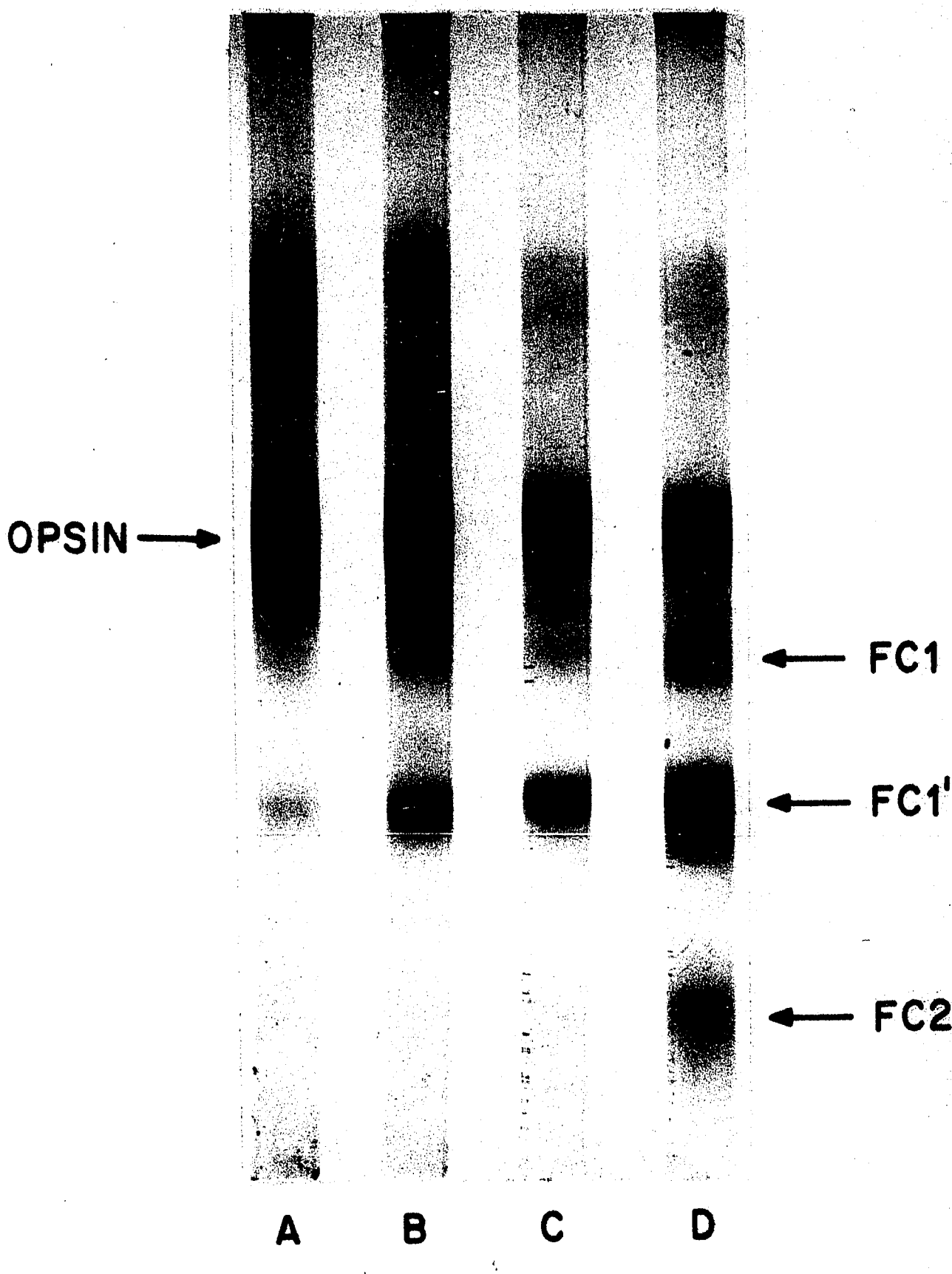


Fig. 18. Fluorescence scans of N-retinyl fluorescence on SDS-acrylamide gels of disc membranes cleaved by (A) subtilisin and (B) chymotrypsin. The excitation wavelength was 333 nm and the emission was viewed through a 3-72 Corning glass filter. The position of the N-retinyl fluorescence after proteolysis with subtilisin or chymotrypsin was that of FS2 or FC2, respectively. No N-retinyl fluorescence was seen at the positions of FS1, FC1 or FC1'.

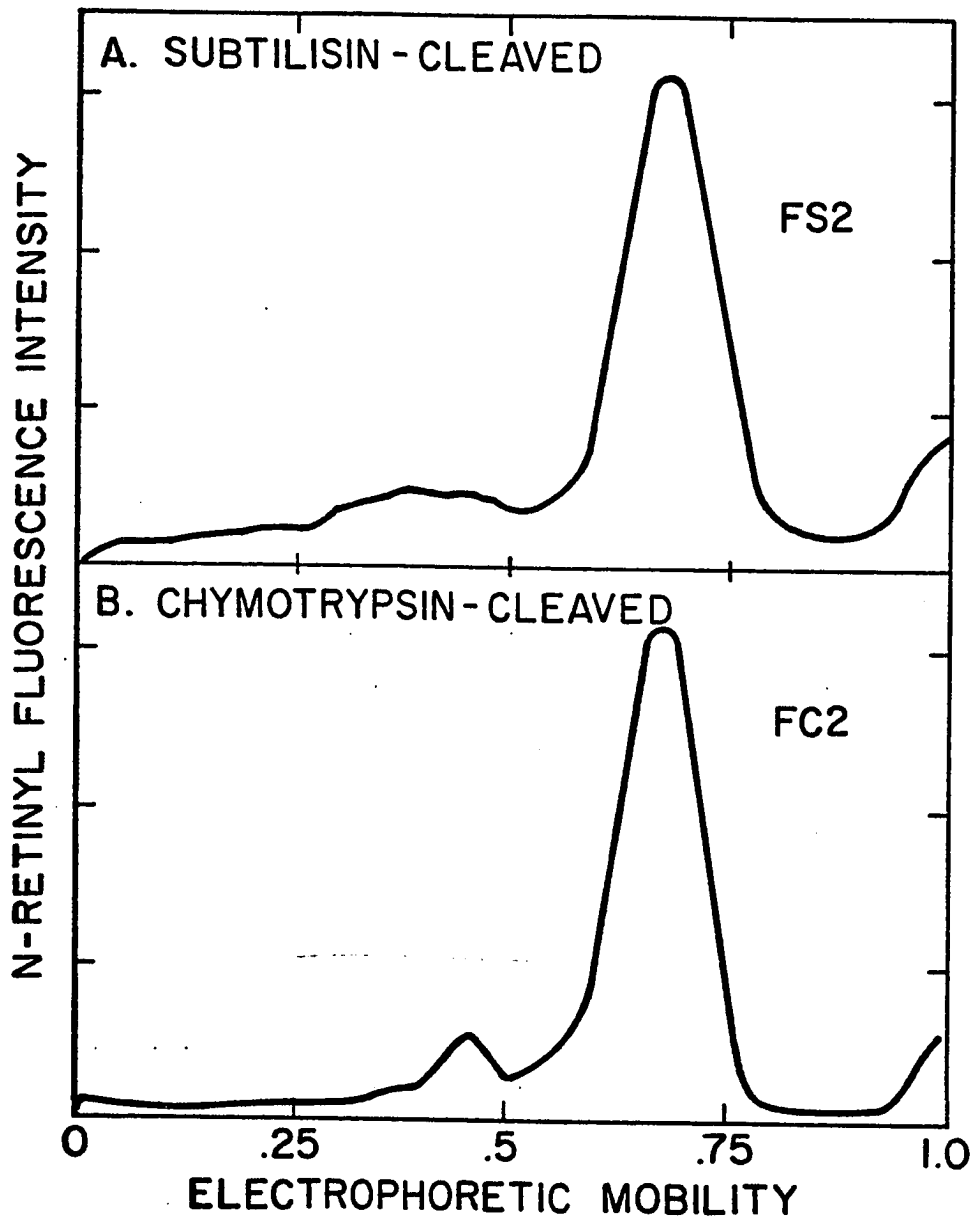


Fig. 19. SDS-acrylamide gels were used to analyze the polypeptides in the eluents (top) released by light and (bottom) released by 0.1 M α-methyl-mannoside from a Con A-agarose column loaded in the dark with chymotrypsin-cleaved disc membranes in Triton X-100 solution. The gels, stained with Coomassie blue, show that light eluted FC2 while sugar caused the elution of FC1 and FC1'.

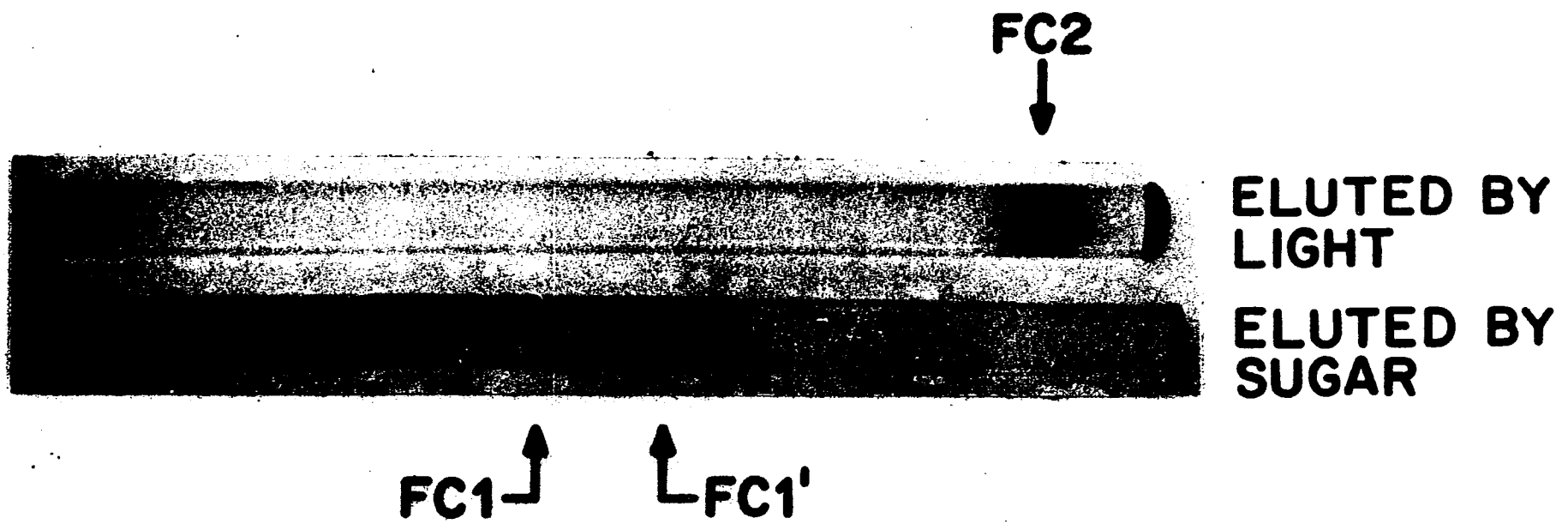


Fig. 20. Diagram illustrating the reaction catalyzed by guinea pig liver transglutaminase. The glutamine residue must be in a peptide but the primary amine may be free or peptide bound.

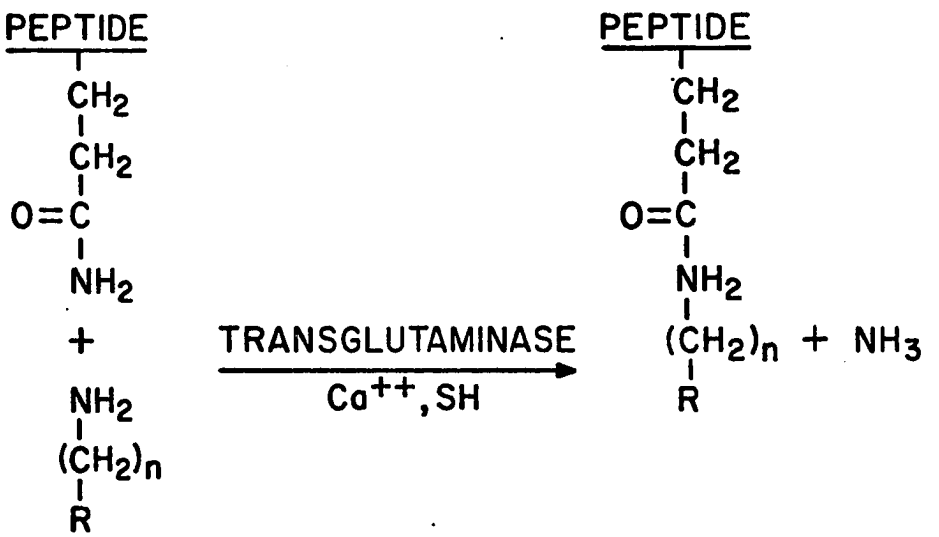
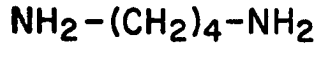


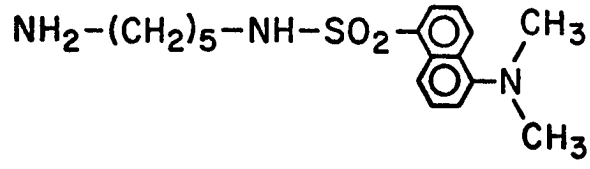
Fig. 21. A list of the primary amine substrates incorporated into rhodopsin by the action of transglutaminase. In general, the amine must be of the form $NH_2-(CH_2)_n-R$.

SUBSTRATES

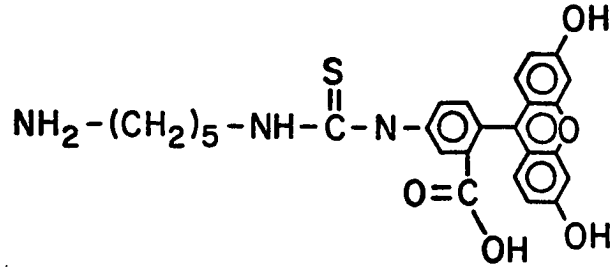
PUTRESCINE



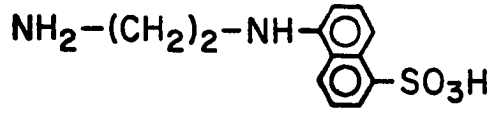
DANSYL CADAVERINE



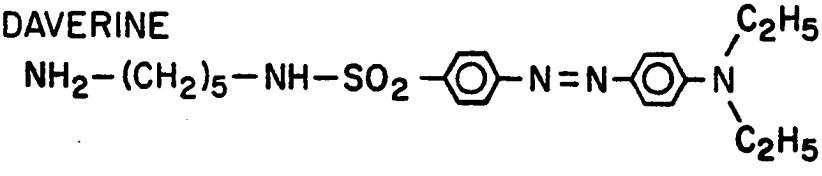
FLUORESCEIN CADAVERINE



N-(AMINOETHYL)-5-NAPHTHYLAMINE-1-SULFONIC ACID



ETHYL ORANGE CADAVERINE



GLYCINE METHYL ESTER

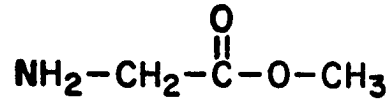


Fig. 22. SDS-acrylamide gels show the transglutaminase-catalyzed labeling of rhodopsin in retinal disc membranes. (A) and (B) are a photograph of the Coomassie blue staining pattern and an autoradiograph of the tritium radioactivity, respectively, of the same gel following electrophoresis of disc membranes which were transglutaminase-labeled for 8 hr with ^{3}H-putrescine. (C) is a photograph of an SDS-gel illuminated with ultraviolet light to exhibit dansyl fluorescence following electrophoresis of disc membranes which were transglutaminase-labeled for 8 hr with dansyl cadaverine. Tranglutaminase treatment of disc membranes causes the formation of opsin aggregates which cannot be dissociated by SDS, perhaps through the formation of glutamyl-lysyl interchain isopeptide bonds. Opsin, opsin aggregation products and transglutaminase are all labeled by putrescine or dansyl cadaverine in a transglutaminase-catalyzed reaction.

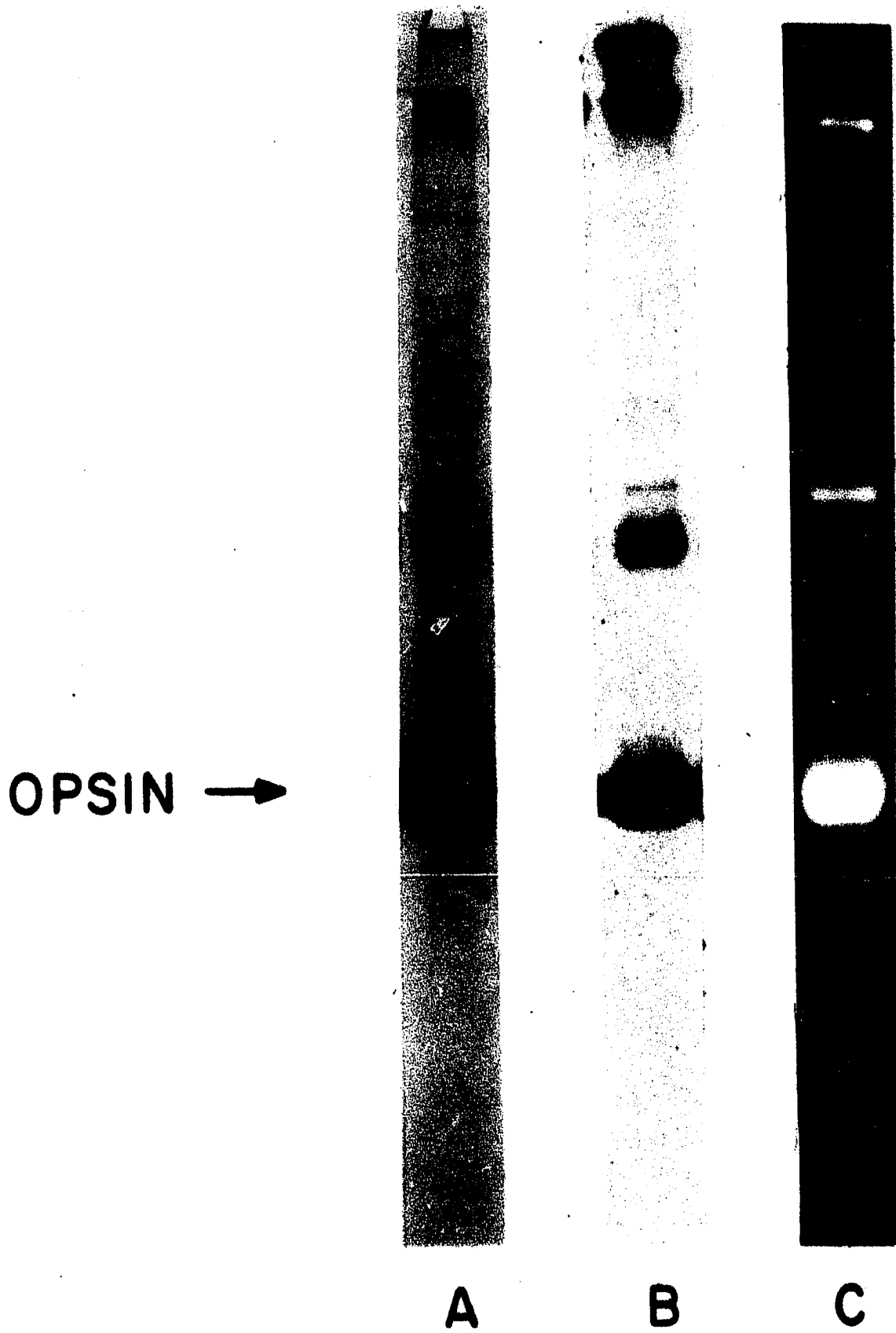


Fig. 23. Time course of the transglutaminase-catalyzed labeling of rhodopsin in disc membranes with ^{3}H putrescine (A) in the dark, and (B) after bleaching. The labeling of rhodopsin saturates after 4 hr. Opsin is labeled equally as well as unbleached rhodopsin.

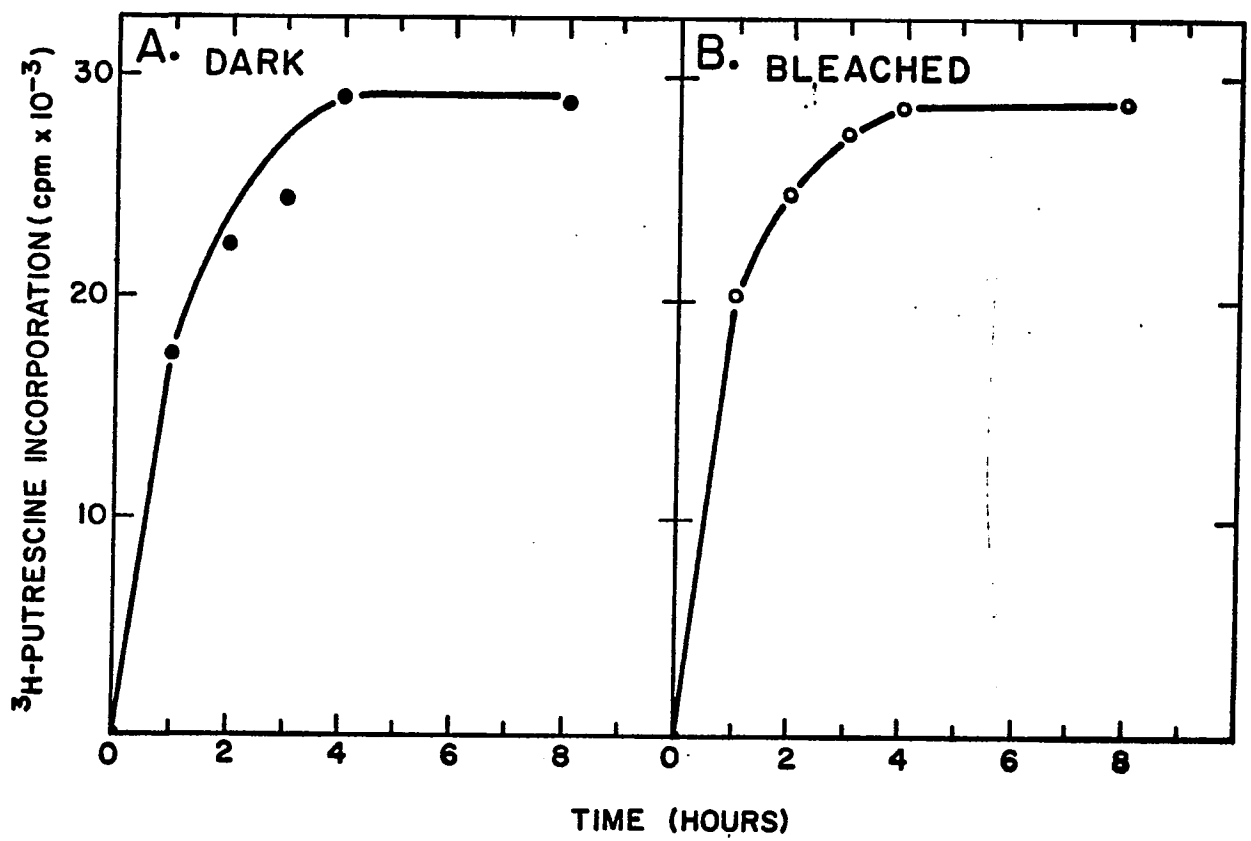


Fig. 24. Elution profile of 500 nm absorbance and ^{14}C radioactivity of disc membranes following 8 hr of transglutaminase-catalyzed ^{14}C-putrescine labeling from a hydroxylapatite column. The membranes were solubilized in Ammonyx LO, loaded on the column in the dark and washed with 35 mM sodium phosphate buffer until the level of radioactivity reached a plateau. Rhodopsin was eluted with 150 mM sodium phosphate buffer, and the stoichiometry of the peak fractions was calculated by comparing the ratio of the radioactivity to the 500 nm absorbance. The putrescine specific activity was 19.5 mCi/mmole; the extinction coefficient of rhodopsin was taken as 40,000. 1.0 ± 0.1 mole of putrescine was incorporated per mole of rhodopsin.

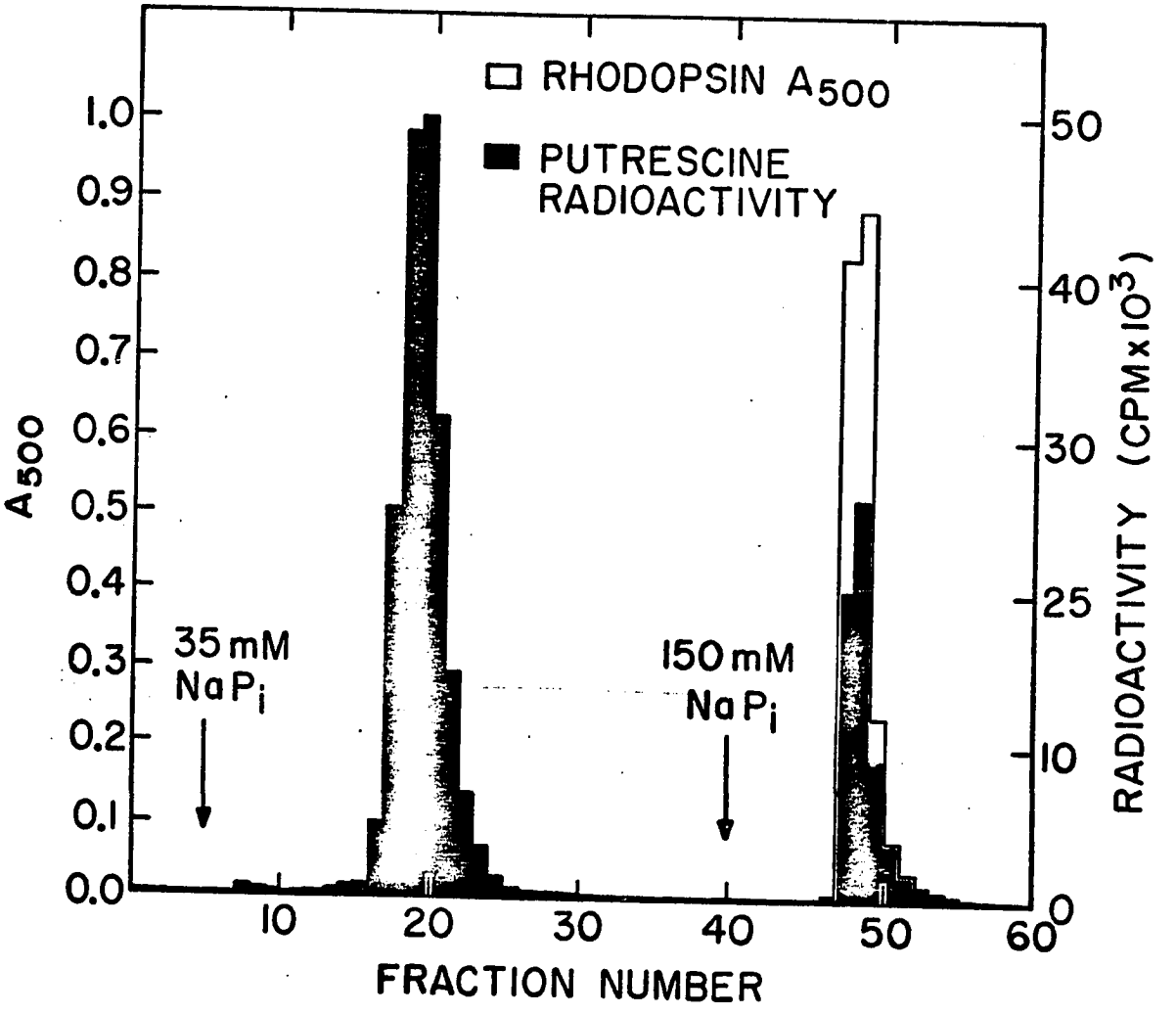


Fig. 25. Fluorescence scans of dansyl fluorescence on SDS-gels following electrophoresis of disc membranes which were transglutaminase-labeled with dansyl cadaverine for 8 hr. The gels were excited with light of 360 nm and emission was viewed through a 3-73 Corning glass filter. A sample of membranes was divided into two tubes. One half of the membranes were pre-labeled with putrescine for 3 hrs in a transglutaminase-catalyze reaction; the other half were incubated with putrescine in the absence of transglutaminase as a control. The membranes which were prelabeled incorporate less than 10% of the amount of dansyl fluorescence as do the control membranes. Thus, putrescine and dansyl cadaverine compete for the same site.

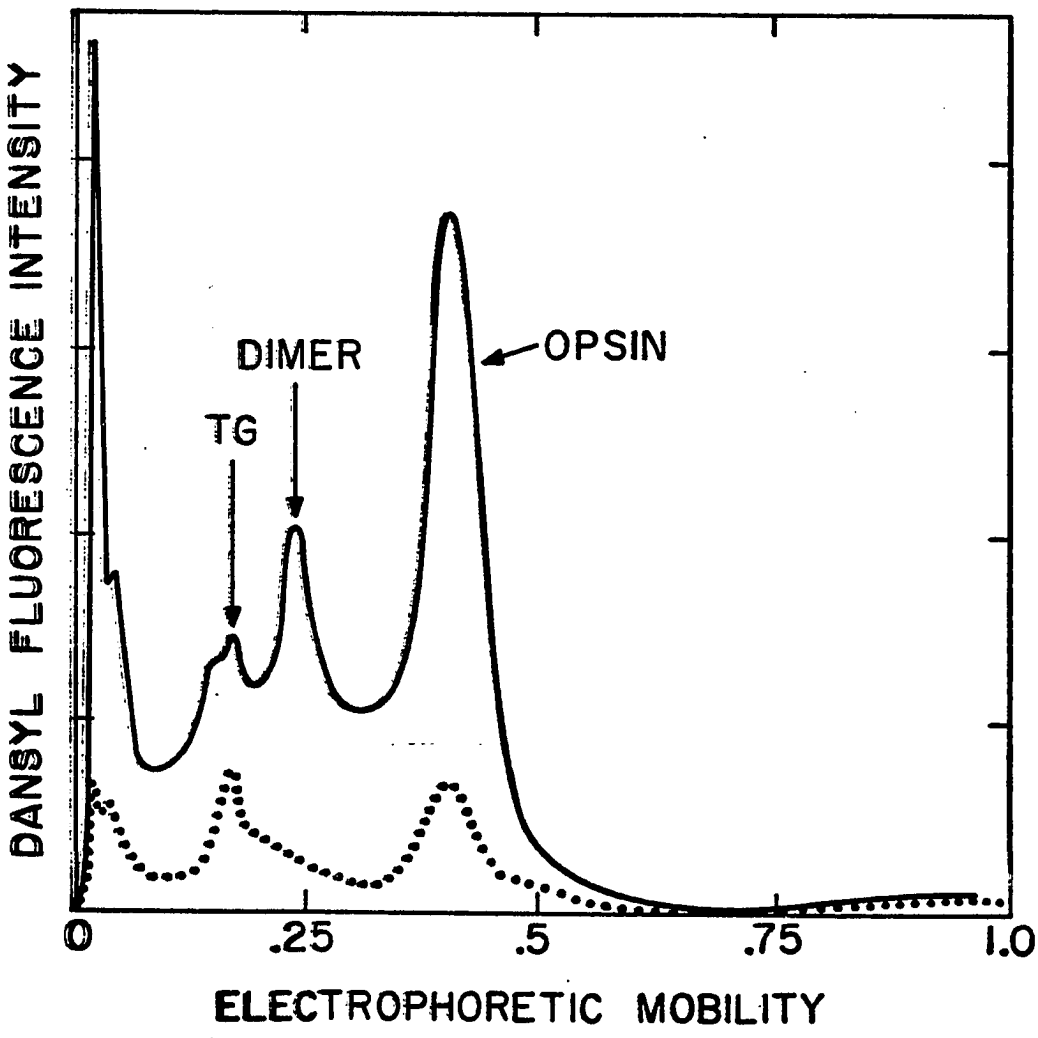


Fig. 26. Photograph (left) and autoradiograph (right) of an SDS-slab gel following electrophoresis of disc membranes which were transglutaminase-labeled with ^{3}H putrescine and subsequently subjected to proteolysis by subtilisin. Gels A through F represent 0 min, 15 min, 30 min, 1 hr, 2 hr and 3 hr of subtilisin treatment, respectively. The gel was stained with Coomassie blue. Immediately following proteolysis, the radioactivity may be associated with either FS1 or FS2, suggesting that the putrescine is located between at least two closely spaced cleavage sites. After longer times of proteolysis, the radioactivity is excised from the fragments. A comparison with the Coomassie staining pattern shows that there has not been an appreciable concomitant change in the electrophoretic mobility of either fragment.

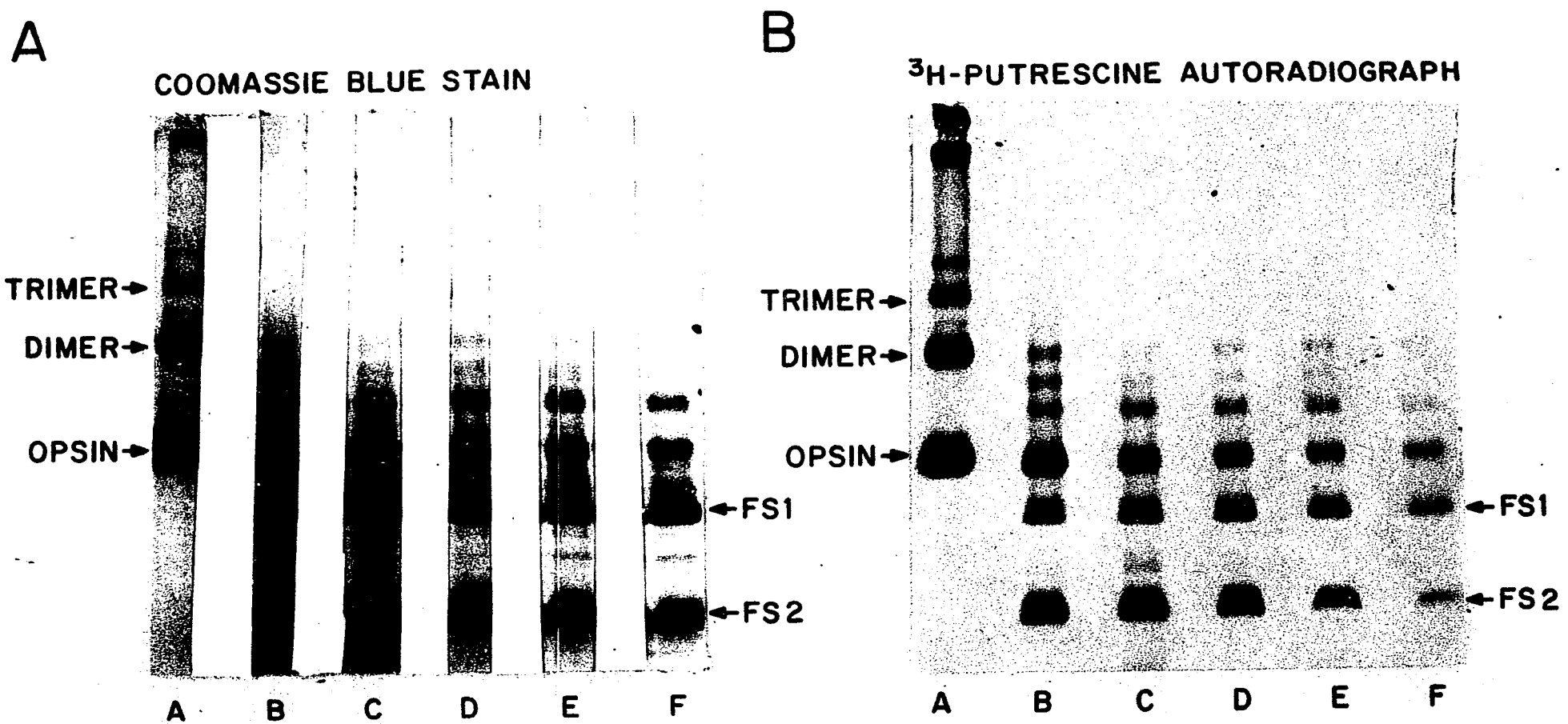


Fig. 27. Fluorescence gel scans of dansyl fluorescence exhibited by disc membranes on SDS-gels following electrophoresis. The membranes were transglutaminase-labeled with dansyl cadaverine and subjected to (A) 0 hr, (B) 3 hr and (C) 24 hr of proteolysis by subtilisin. The gels were excited with light at 360 nm and emission was viewed through a 3-73 Corning glass filter. The position of the fluorescence shifts from that of opsin and its aggregates to that of FS1 after 3 hrs, and disappears almost entirely from the membrane after 24 hrs. Coomassie blue staining of the gels revealed that dansyl cadaverine was excised from FS1 without an appreciable concomitant change in the electrophoretic mobility of the fragment.

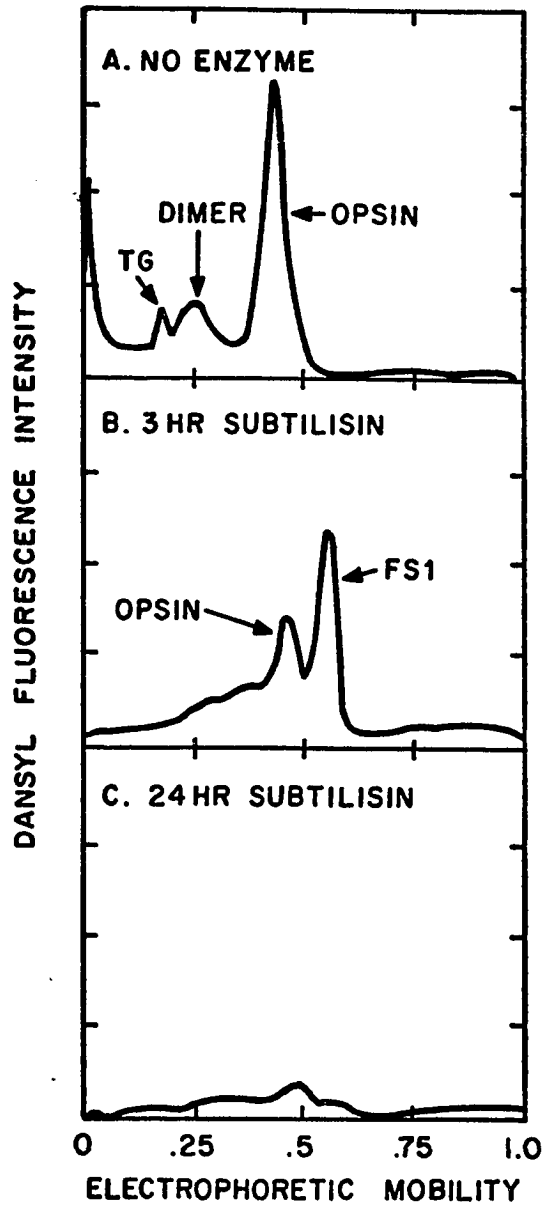


Fig. 28. Nanosecond decay curves of dansyl emission from dansyl cadaverine-rhodopsin, purified by hydroxylapatite chromatography, in 1% Ammonyx LO solution. The decay curves of samples kept dark and after bleaching are compared. The average dansyl fluorescence lifetimes computed from these data are 10.1 nsecs in rhodopsin and 16.4 nsecs in opsin.

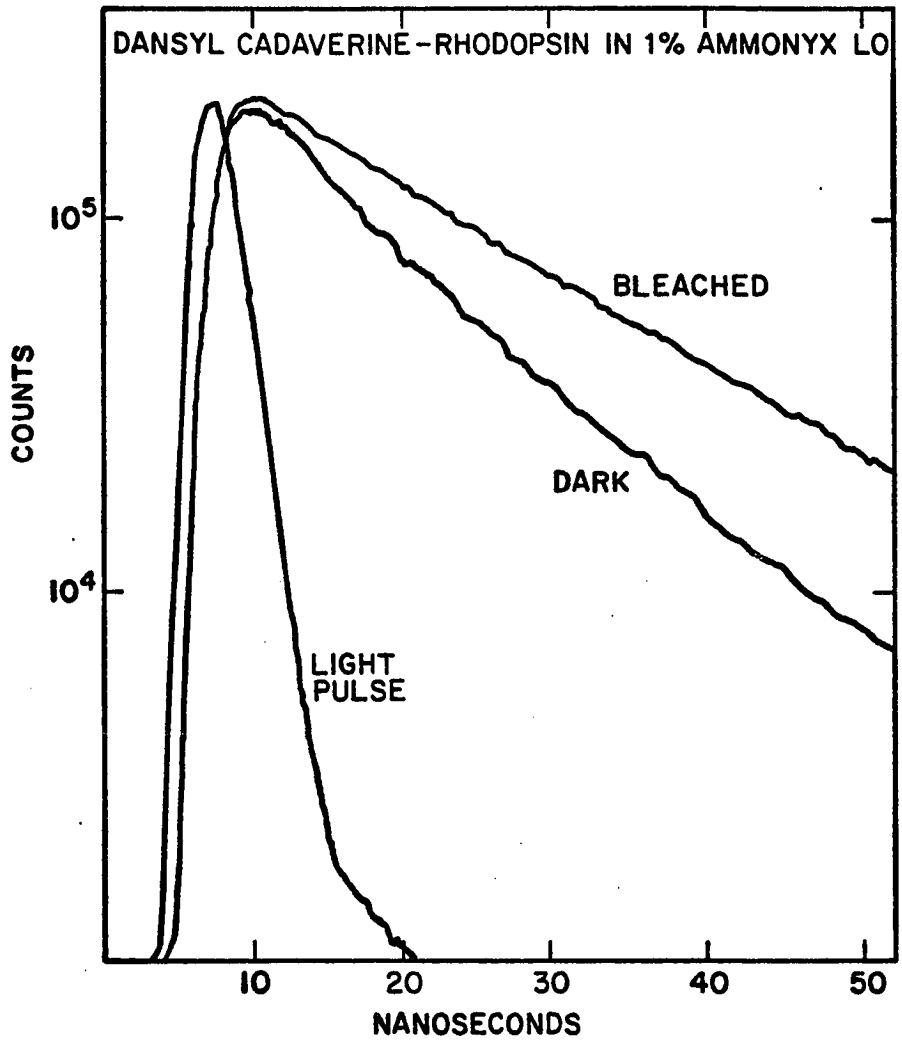


Fig. 29. A comparison of the nanosecond decay of dansyl emission anisotropy from dansyl cadaverine-rhodopsin, in 1% Ammonyx LO solution (A) before and (B) after bleaching of the rhodopsin. The sample was purified by hydroxylapatite chromatography. For a rigid sphere, the unbleached sample would have a rotational correlation time of approximately 5 nsec and the bleached sample has at least two rotational correlation times of approximately 5 and 75 nsec.

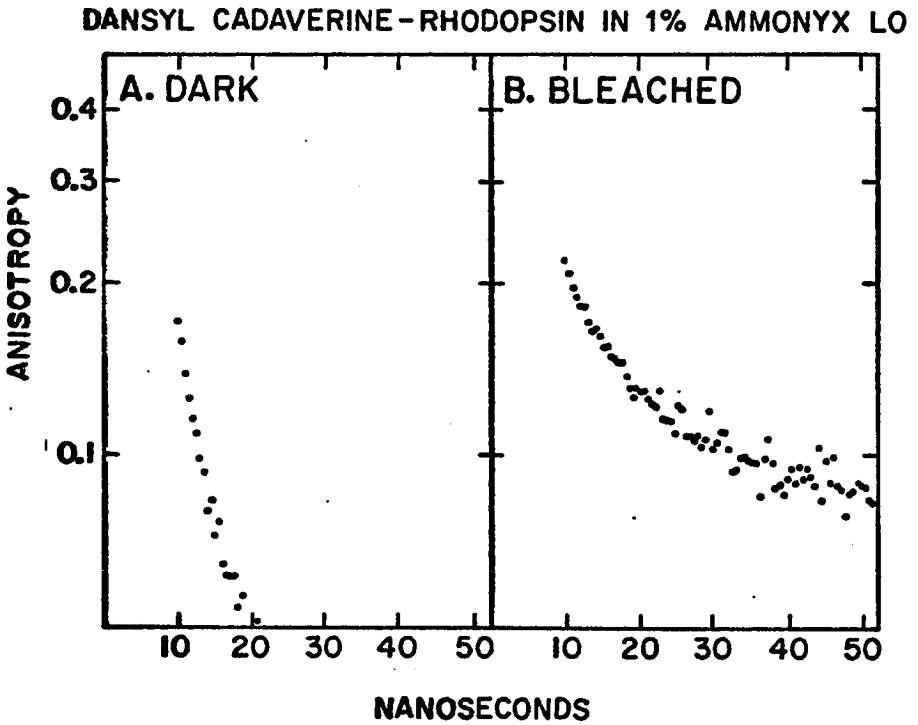


Fig. 30. Nanosecond decay curves of dansyl emission from dansyl cadaverine-rhodopsin in disc membranes. The decay curves of samples kept dark and after bleaching are compared. The average dansyl fluorescence lifetimes computed from these data are 13.7 nsec in rhodopsin and 18.5 nsec in opsin.

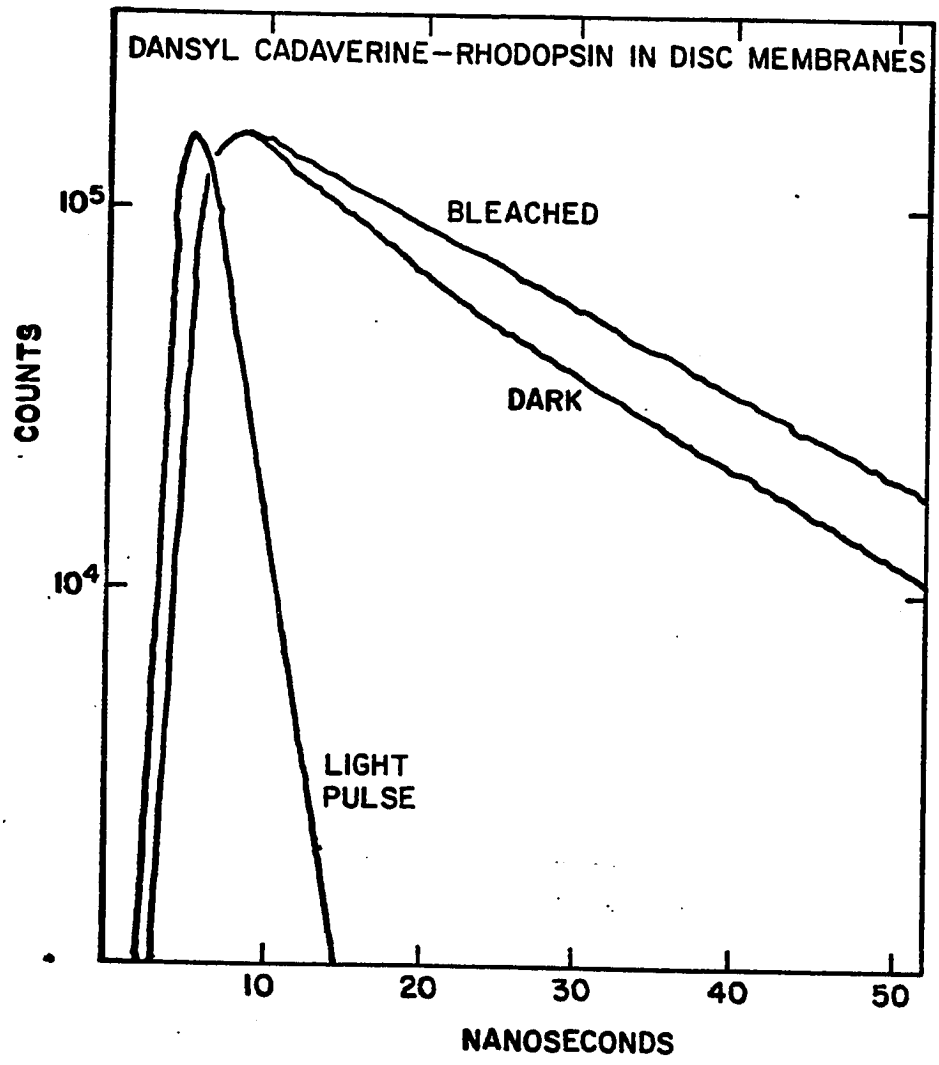


Fig. 31. A comparison of the dansyl fluorescence emission spectrum of dansyl cadaverine-rhodopsin and of the rhodopsin absorbance spectrum. The dansyl emission spectrum was recorded from sonicated, albumin-washed disc membranes and corrected for variability of the detector with wavelength. The absorbance spectrum was recorded from rhodopsin in 1% Ammonyx LO solution, purified by hydroxylapatite chromatography.

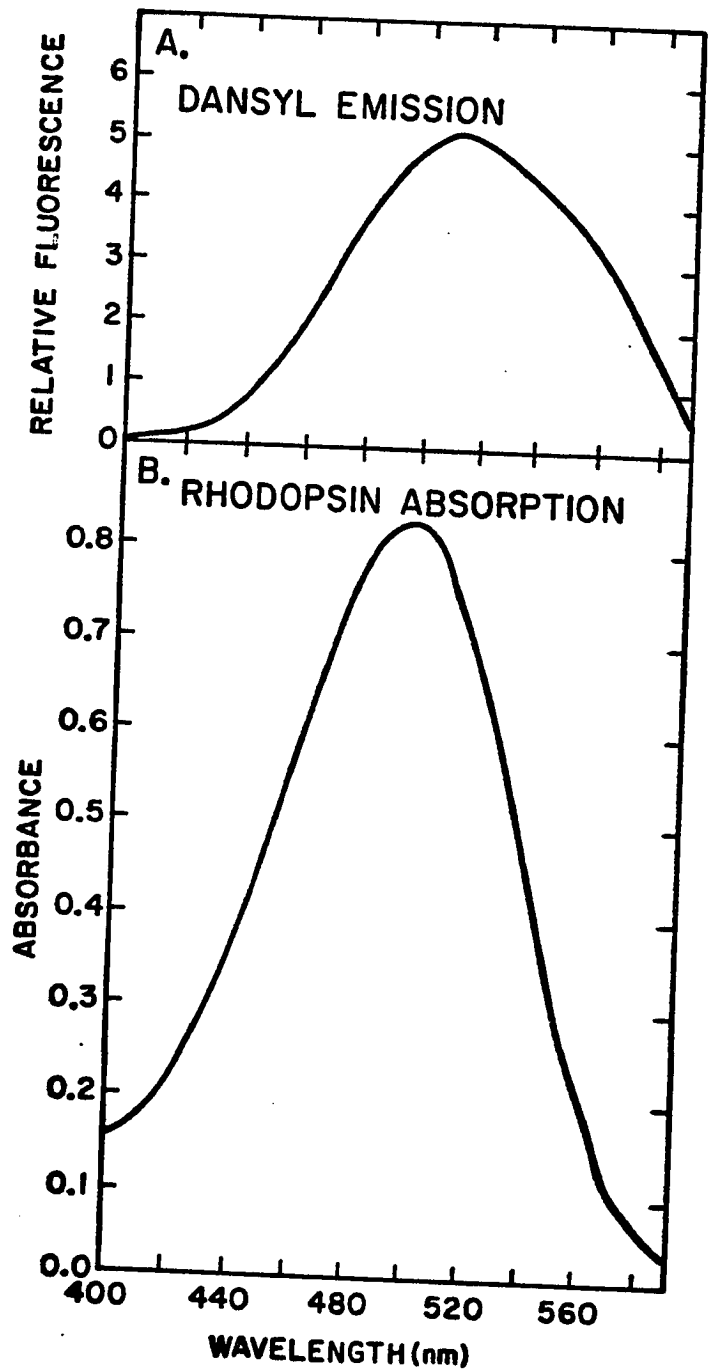


Fig. 32. A comparison of the nanosecond decay of dansyl emission anisotropy from dansyl cadaverine-rhodopsin in disc membranes (A) before and (B) after bleaching of the rhodopsin. For a rigid sphere, both samples would have rotational correlation times of approximately 100 nsec.

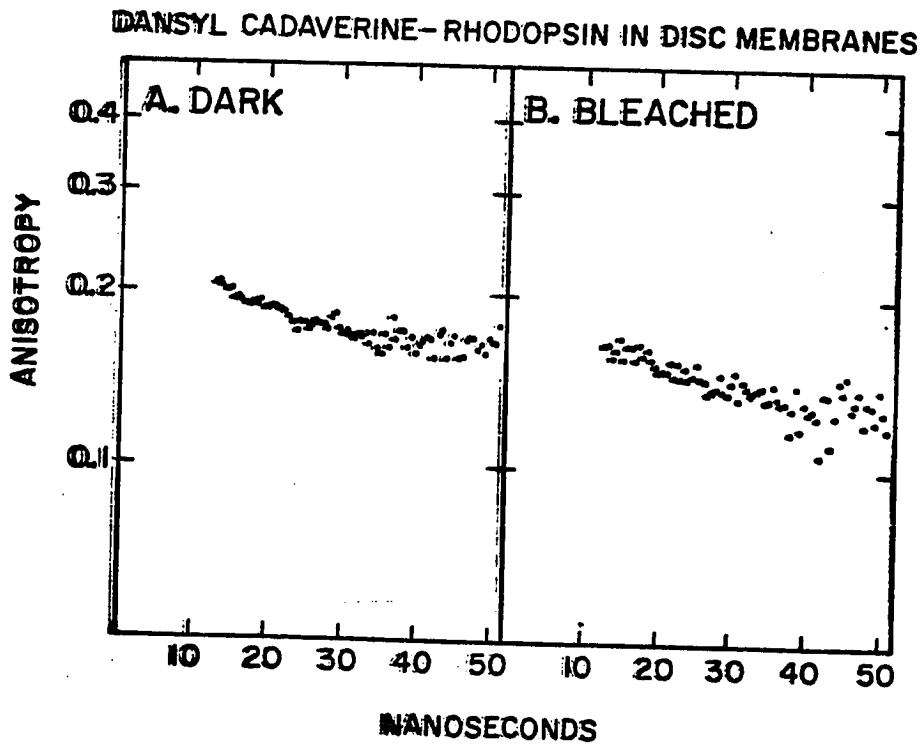


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Fig. 3. Rhodopsin in 0.5% Triton X-100 solution purified by Con A-agarose affinity chromatography (A) before and (B) after 3 hrs of thermolysin proteolysis as analyzed by electrophoresis on SDS-acrylamide gels. The gels were stained with Coomassie blue. The opsin band was replaced by two bands having similar electrophoretic mobilities as F1 and F2, the pair of fragments produced by thermolysin proteolysis of disc membranes. The presence of Triton X-100 induces some aggregation of the opsin and opsin fragments.