PHYSICAL-CHEMICAL STUDIES OF RHODOPSIN

by

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I hereby recommend that this dissertation prepared under my direction by Robert Anthony Henselman

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be accepted as fulfilling the dissertation requirement of the degree of Doctor of Philosophy

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SIGNED: Robert Anthony Hensleman

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ABSTRACT

Rhodopsin, the visual pigment protein, mediates dim light and black and white vision and functions as a transducer converting light energy into synaptic activity in the vertebrate retinal rod cell. In the work reported here two aspects of rhodopsin were studied. One portion of this work dealt with the partial characterization of a newly solubilized form of rhodopsin. The second portion of this work dealt with the establishment of a reaction mechanism to account for the recombination of rhodopsin from 11-*cis* retinal and the photolyzed visual pigment apoprotein opsin.

It was found that rhodopsin could be extracted from lyophilized rod outer segment membranes in high yields using solutions of sodium cholate at a concentration of 20 mg/ml in pH 7.0, 0.10 *M* potassium phosphate buffer. These initial rhodopsin extracts were partially purified by an ammonium sulfate fractionation procedure. The overall yield of the recombination reaction between photolyzed rhodopsin (opsin) and 11-*cis* retinal was dependent upon sodium cholate concentration, ranging from 90-100% recombination at 1-4 mg/ml to 5-10% at 20 mg/ml sodium cholate. The pH optimum for the maximum extent of recombination in rhodopsin solutions containing 2-3 mg/ml sodium cholate was between pH 7.0 and 7.4.

The kinetic aspects of the recombination reaction were studied both in solutions of sodium cholate solubilized rhodopsin and in suspensions of isolated rod outer segment membranes. The kinetic results obtained from both of the above studies suggested the recombination of rhodopsin from 11-cis retinal and either sodium cholate solubilized opsin or membrane bound opsin proceeded via a complex mechanism. The major features of the mechanism proposed to account for the recombination of rhodopsin included the formation of an intermediate addition compound between a lysine residue of opsin and 11-cis retinal. The dehydration of this intermediate addition compound was proposed to result in the formation of rhodopsin in which 11-cis retinal was covalently bonded to the protein opsin via a protonated aldimine linkage. This dehydration step was postulated to involve a group on opsin which acted as a general acid and facilitated the dehydration of the intermediate compound.

CHAPTER 1

INTRODUCTION

Rhodopsin, the visual pigment protein which mediates dim light vision (Abrahamson and Ostroy 1967) is located in the outer segment of the vertebrate retinal rod cell. The outer segment of the rod cell is the site of visual excitation where the absorption of light, signal amplification, and finally conversion into synaptic activity takes place (Daemen 1973). As rhodopsin accounts for some 80-85% of the protein in isolated rod outer segment membranes (Daemen, DeGrip, and Jansen 1972; Heitzmann 1972) it is not surprising that rhodopsin is of central importance in the visual process. Although the entire role which rhodopsin has in the visual process is unknown, it is quite clear that rhodopsin functions as the primary photoactive material in the rod outer segment (Abrahamson and Ostroy 1967; Hagins 1972).

The studies reported here are primarily concerned with the physical and chemical properties of rhodopsin and the characterization of the dark reaction between the photolyzed visual pigment, opsin, and 11-*cis* (or 9-*cis*) retinal which results in the recombination of rhodopsin (or isorhodopsin). In the following pages the literature on the physical and chemical properties of bovine rhodopsin and on

the recombination reaction is reviewed and the goals of this work are stated.

Physical and Chemical Properties of Rhodopsin

As indicated above rhodopsin is located in the outer segment of the vertebrate retinal rod cell. The isolation of rod outer segment membranes and of rhodopsin is greatly facilitated because of the structure of the rod cell. Morphologically this cell is divided into an inner and an outer segment connected by a small cilium (Daemen 1973). Homogenization of the retina results in the cleavage of the connecting cilium permitting the ready separation of the inner and outer segments by either differential or sucrose gradient centrifugation (Matthews et al. 1963; McConnell 1965; Papermaster and Dreyer 1974). The current literature indicates that the best preparations of bovine rod outer segments are obtained when the membranes are isolated from sucrose density gradients at a density of 1.12-1.13 g/ml (Papermaster and Dreyer 1974; McConnell 1965; DeGrip, Daemen, and Bonting 1972) or when the membranes are subjected to repeated floatations in 1.02 M sucrose solutions (Heller 1968; Zorn and Futterman 1971; Hong and Hubbell 1973). Analysis of these isolated rod outer segment membranes by a variety of workers indicates the membranes are composed of about 35-40% protein, 40-60% lipid, and 1-4% carbohydrate on a dry weight basis (Daemen 1973; Nielsen, Fleischer, and

McConnell 1970; Heller and Lawrence 1970). Rhodopsin is the major protein constituent of isolated rod outer segment membranes and accounts for 80-85% of the protein present in these membranes (Daemen et al. 1972; Heitzmann 1972; Papermaster and Dreyer 1974). Phospholipids account for 80-90% of the total lipids present in rod outer segments (Nielsen et al. 1970; Daemen 1973; Poincelot and Zull 1969; Shichi 1971). The phospholipid composition of these membranes has been determined by a number of workers and their reports demonstrated phosphatidylethanolamine and phosphatidylcholine each comprise about 40% of the total phospholipid present while phosphatidylserine and phosphatidylinositol account for approximately 12-13% and 2-6%, respectively.

Rhodopsin itself is insoluble in aqueous solutions but has been extracted from rod outer segment membranes in a solubilized form by the use of a variety of surfactants or detergents including digitonin (Tansley 1931; Wald and Brown 1952), cetyltrimethylammonium bromide (Bridges 1957; Heller 1968), Triton X-100 (Crescitelli 1967; Osborne, Sardet, and Helenius 1974), Ammonyx LO (Ebrey 1971), dodecyltrimethylammonium bromide (Hong and Hubbell 1973), Emulphogene BC 720 (Shichi et al. 1969), and Tween-80 (Zorn and Futterman 1973). Rhodopsin has been purified to homogenity with respect to protein composition in most of the above detergents to yield preparations which have an apparent molecular weight between

36,000 and 39,000 as determined by sodium dodecylsulfate disc gel electrophoresis (Osborne et al. 1974; Daemen et al. 1972; Heitzmann 1972; Papermaster and Dreyer 1974). This molecular weight is consistent with that determined by amino acid analysis which indicates rhodopsin has a molecular weight of about 39,000 per mole of retinal (Daemen et al. 1972).

In the context of the work reported here the most important chemical and physical properties of rhodopsin are those which influence the ability of photolyzed rhodopsin (opsin) to undergo the recombination reaction with 11-*cis* or 9-*cis* retinal. The reaction between opsin and 11-*cis* retinal to form rhodopsin specifically refers to the reaction represented by equation 1 and is termed the recombination reaction of rhodopsin.

$$\text{opsin} + \text{11-}\textit{cis}\text{ retinal} \rightleftharpoons \text{rhodopsin} \qquad (1)$$

The recombination reaction involves only the last step in the overall regeneration of rhodopsin. Regeneration refers to the sequence of reactions, *in vivo* or *in situ*, which take place after the photolysis of rhodopsin and ultimately lead to recombination. The exact pathway or sequence of reactions leading to the regeneration of rhodopsin is unknown. Several groups of workers have postulated different pathways for the regeneration of rhodopsin and these possible pathways are

illustrated in Figure 1. Shichi and Somers (1974) have postulated that the *in situ* or *in vivo* regeneration of rhodopsin may involve the formation of a complex between phosphatidylethanolamine and all-*trans* retinal. The action of light upon this complex promotes the photoisomerization of the retinal to the 11-*cis* form which can then react with opsin to form rhodopsin. Other postulated mechanisms of regeneration include both the enzymatic (Hubbard 1956; Amer and Akhtar 1972) and nonenzymatic conversion (Futterman and Rollins 1973) of all-*trans* retinal to 11-*cis* retinal.

The characterization of solubilized rhodopsin has been greatly hampered because of the lack of a functional assay for the active protein. Two properties of rhodopsin have been considered to be unique characteristics of this pigment. The first is the absorption spectrum of rhodopsin with maxima at 278, 340, and 500 nm (Shichi 1970) which is stable in the dark over a wide pH range. Upon exposing a rhodopsin solution to white light, the absorption maximum at 500 nm is lost and replaced by a new peak at approximately 380 nm. These spectral changes in rhodopsin are the result of the photo-induced isomerization of the 11-*cis* retinal chromophore to the all-*trans* form (Hubbard and Kropf 1958) and the release of all-*trans* retinal (Abrahamson and Ostroy 1967). The second unique characteristic of rhodopsin is its recombination capacity. When the apoprotein opsin (photolyzed rhodopsin) is incubated with exogenous 11-*cis* retinal

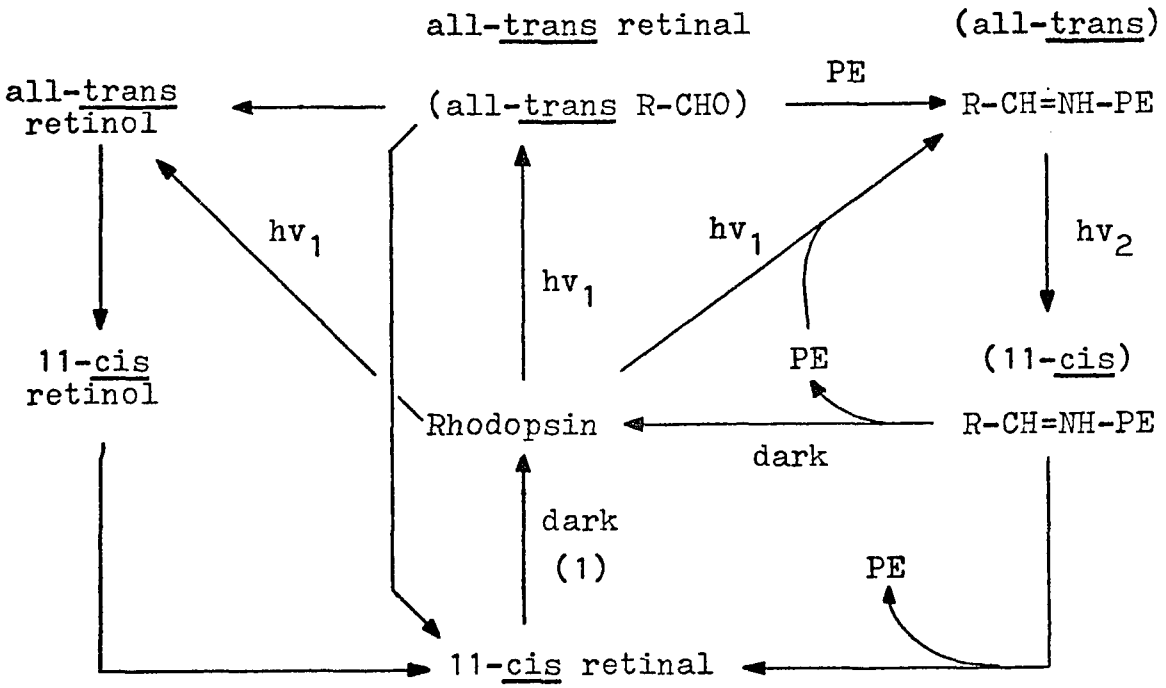


Figure 1. Possible Pathways for the Regeneration of Rhodopsin.

PE represents phosphatidylethanolamine and reaction 1 indicates the recombination reaction between opsin and free 11-cis retinal.

in the dark, the so-called recombination reaction takes place (equation 1) and a light sensitive pigment undistinguishable from native rhodopsin is formed. The recombination reaction is extremely sensitive to the environment of the apoprotein. This reaction has been reported to occur in high yields only in isolated rod outer segment membranes (DeGrip et al. 1972; Hong and Hubbell 1973), digitonin solutions (Wald and Brown 1956), and to a lesser extent in solutions of Tween-80 (Zorn and Futterman 1973).

The linkage between the apoprotein portion of rhodopsin and the chromophore, 11-cis retinal, has been the subject of a number of investigations. The suggestion that 11-cis retinal was bound to rhodopsin via a protonated aldimine linkage (Schiff base) was made as early as 1955 and was based mainly upon model compound studies (Morton and Pitt 1955). Positive evidence for this model came from the reports that digitonin solutions of rhodopsin could be reduced with sodium borohydride when illuminated (Bownds and Wald 1965) and the resulting solutions contained retinal which was covalently bound to a lysine residue of the protein (Bownds 1967).

The above experiments demonstrated that in bleached rhodopsin the chromophore could be reductively fixed to an ϵ-NH_2 group of a lysine residue but did not answer the question of what was the binding site in native and unbleached

rhodopsin. Recent experiments with $NaBH_3CN$ (sodium cyanoborohydride) indicated this reagent reacted with rhodopsin in the dark and reductively fixed the retinal group to an ϵ-NH_2 group of lysine (Fager, Sejnowski, and Abrahamson 1972). Raman spectral studies of intact bovine retinas also support the now generally accepted belief that opsin and 11-cis retinal are bound to each other through a protonated aldimine linkage (Rimai, Kilponen, and Gill 1970). The position of the retinal chromophore binding site in the protein is unknown since the amino acid sequence of rhodopsin has not yet been determined. Bownds (1967) isolated a small peptide from illuminated, sodium borohydride treated, and pronase digested rhodopsin which contained one alanine, one ϵ-N-retinyl-lysine, and two phenylalanine residues. Other than the above work by Bownds (1967) the literature is devoid of any reported attempts to sequence either the protein or peptides in the retinal binding site. The entire retinal polyene chain and the aldimine linkage are presumed to be buried in the interior of the rhodopsin protein. This hypothesis is supported by the fact that neither sodium borohydride or hydroxylamine will attack native dark rhodopsin. Both of the above reagents attack the aldimine linkage of model compounds in solution or rhodopsin after it has been exposed to light. The retinal binding site in rhodopsin is highly selective for the stereochemistry of the

retinal ligand. Only 11-cis or 9-cis stereoisomers of retinal will react with opsin to form rhodopsin or a rhodopsin analogue. The other stereoisomers of retinal, all-trans, 13-cis, or 9,13-dicis retinal do not form visual pigment analogues with opsin (Hubbard and Wald 1952).

The characterization of the recombination reaction of rhodopsin in isolated rod outer segment membranes has received only a limited amount of attention in the literature. A small number of workers have reported that bleached or photolyzed rod outer segment membranes have the capacity to form rhodopsin in high yield when incubated with exogenous 11-cis retinal in the dark (Hong and Hubbell 1973; DeGrip et al. 1972; Futterman and Rollins 1973). The pH optimum for the maximum extent of recombination was reported to be between pH's 6.0 and 7.5 (Futterman and Rollins 1973). This pH optimum is in disagreement with the earlier value of pH 5 determined by Amer and Akhtar (1972). The basis for this discrepancy is unclear but presumably is related to the procedures used to isolate the rod outer segment membranes. Amer and Akhtar (1972) isolated their membranes at a density of 1.20 g/ml while Futterman and Rollins (1973) employed sucrose solutions of density 1.13 g/ml in their isolation procedure.

Hong and Hubbell (1973) have shown that rhodopsin-lipid artificial membranes composed of rhodopsin and selected

lipids, egg phosphatidylcholine or digalactosyl diglyceride, were capable of carrying out the recombination reaction in the presence of exogenous 11-cis retinal. Hong and Hubbell (1973) concluded from these experiments that when rhodopsin was incorporated into artificial lipid bilayers there was no specific requirement for a particular polar head group on the lipid and in the case of phosphatidylcholine bilayers there was not a requirement for a particular type of hydrocarbon chain. It was pointed out by the authors (Hong and Hubbell 1973) that only the overall capacity of the rhodopsin to undergo recombination after photolysis was determined.

DeGrip, Van De Laar et al. (1973) have shown that in suspensions of isolated rod outer segment membranes two sulfhydryl groups per rhodopsin molecule can be modified with either 5,5'-dithiobis-(2-nitrobenzoic acid) or N-ethylmaleimide and these modifications did not significantly affect the overall recombination capacity of the membrane preparation. In another series of experiments DeGrip, Daemen, and Bonting (1973) modified 98% of the available amino groups in isolated rod outer segment membranes in darkness with methyl acetimidate and observed a 30% loss in both the 500 nm absorbance and in recombination capacity. When they used 1,3,5-trinitrobenzene-2-sulfonate to modify the amino groups in darkness they observed a complete loss of both the recombination capacity and the 500 nm absorbance (DeGrip, Daemen, and

Bonting 1973). From these experiments it was concluded that the introduction of the small positively charged acetimidine group had little effect on the structure of rhodopsin in rod outer segment membranes while the introduction of the large uncharged trinitrophenyl group seriously disrupted the rhodopsin structure.

A large portion of the peptide chain of rhodopsin apparently has no role in maintaining either the characteristic absorption spectrum or the recombination reaction of rhodopsin. Trayhurn, Mandel, and Virmaux (1974a) treated isolated rod outer segment membranes with papain and removed a large fragment from rhodopsin. The residue or so-called rhodopsin core retained the characteristic absorption spectrum of rhodopsin when it was solubilized with Emulphogene. Further experiments indicated the rhodopsin core had a molecular weight of approximately 25,000 and was capable of undergoing the recombination reaction with 11-cis retinal (Trayhurn, Mandel, and Virmaux 1974b).

With the exception of the single experiment by Hubbard (1958) on the kinetics of the recombination reaction in isolated rod outer segments there are no quantitative reports on this reaction. Unfortunately, Hubbard (1958) did not calculate the rate constant and only showed that at a 5-fold excess of 11-cis retinal the recombination reaction proceeded by pseudo first-order kinetics. Currently there

are no quantitative descriptions of the recombination reaction in isolated rod outer segment membranes.

When detergent solubilized solutions of rhodopsin are bleached the chromcphore is isomerized and hydrolytically cleaved from the protein portion of rhodopsin. Hubbard and Wald (1952) demonstrated that only 11-cis retinal was capable of forming a visual pigment indistinguishable from native rhodopsin when digitonin solutions of opsin were incubated with various stereoisomers of retinal. The pH optimum for the maximum extent of recombination in solutions of digitonin solubilized rhodopsin was found to be at pH 6.3-6.4 (Radding and Wald 1956). Zorn and Futterman (1973) demonstrated that Tween-80 solubilized rhodopsin was also capable of recombination when the bleached pigment was incubated with exogenous 11-cis retinal in the dark. The recombination reaction in Tween-80 had the same pH optimum, 6.3-6.4, as in digitonin but the maximum extent of recombination was only about 60% of that theoretically expected (Zorn and Futterman 1973). Preparations of rhodopsin which have been solubilized with Triton X-100 (Johnson and Williams 1970), cetyltrimethylammonium bromide (Heller 1968), Ammonyx LO (Ebrey 1971), Emulphogene BC 720 (Shichi et al. 1969), and dodecyltrimethylammonium bromide (Hong and Hubbell 1973) are all incapable of recombination with 11-cis retinal. The basis for this lack of recombination capacity is unclear but is

most probably due to the action of the surfactant itself upon bleached rhodopsin. Rhodopsin which has been previously purified in dodecyltrimethylammonium bromide and then exchanged into a digitonin solution is fully capable of carrying out the recombination reaction (Hong and Hubbell 1973). In a similar manner digitonin solutions of rhodopsin lose their recombination capacity in the presence of 0.1% Emulphogene BC 720 (Shichi 1971) or 0.28% cetyltrimethylammonium bromide (Plante and Rabinovitch 1972).

A considerable amount of work has been done on detergent solubilized rhodopsin in an attempt to determine and describe those properties of rhodopsin which are esstential in the recombination reaction. The pioneering studies of Wald and Brown (1952) on the recombination reaction utilizing digitonin solubilized rhodopsin demonstrated the requirement for a free sulfhydryl group. These studies were further extended by other workers (DeGrip, Van De Laar et al. 1973; Zorn 1974) who showed that two available sulfhydryl groups in the dark rhodopsin could be modified with either N-ethylmaleimide or 5,5'-dithiobis-(2-nitrobenzoic acid) without significantly decreasing the overall recombination capacity of the rhodopsin preparation. When bleached samples of digitonin solubilized rhodopsin were treated with 5,5'-dithiobis-(2-nitrobenzoic acid), four sulfhydryl groups were modified which resulted in a 70% decrease in the overall recombination

capacity (DeGrip, Van De Laar et al. 1973). Zorn (1974) treated bleached digitonin solutions of rhodopsin with N-ethylmaleimide and modified approximately 3.2 sulfhydryl groups which resulted in only a 9-10% decrease in the overall recombination capacity of the preparation.

The question of the role, if any, that phospholipids have in the recombination reaction of solubilized rhodopsin has received a great deal of attention by a number of workers. Zorn and Futterman (1971) observed that the removal of phospholipids from Triton X-100 solubilized rhodopsin resulted in a loss of the recombination capacity of aqueous suspensions of the photolyzed protein when the phospholipid to rhodopsin ratio was decreased below 30 to 1 on a molar basis. The recombination capacity of the rhodopsin suspension was recovered if phosphatidylethanolamine was added back to the protein in a final molar ratio of 30 to 1 or greater (Zorn and Futterman 1971). In this study the phospholipids were removed by extracting the dried rhodopsin-Triton X-100 material with toluene and then measuring the recombination capacity of the residue as an aqueous suspension. There appears to be some difficulties with the interpretation of these results since the complete removal of Triton X-100 from the material used for regeneration was not demonstrated. This is an extremely important point because Triton X-100 is known to inhibit the recombination reaction (Johnson and

Williams 1970). Hong and Hubbell (1973) have shown that rhodopsin solubilized with dodecyltrimethylammonium bromide and chromatographed on a column of hydroxylapatite resulted in a preparation which contained less than 0.8 mole of organic phosphate per mole of rhodopsin. When this essentially phospholipid free sample of rhodopsin was exchanged into digitonin solutions Hong and Hubbell (1973) observed that the photolyzed protein was fully capable of recombination when incubated in the dark with exogenous 11-cis retinal for 5-12 hours.

A limited amount of work has been done on the kinetics of the in vivo recombination of rhodopsin. Wald and Brown (1956) demonstrated that digitonin solutions of opsin and 11-cis retinal react to form rhodopsin and this reaction followed second-order kinetics with a rate constant of 43 M^{-1} sec^{-1} at 23 °C and pH 6.4. Plante and Rabinovitch (1972) also studied the reaction in digitonin solutions and a calculation from their data yields an apparent second-order rate constant of about 380 M^{-1} sec^{-1} at pH 6.8. Other than the above reports little is known about the kinetics of detergent solubilized rhodopsin recombination and there are no literature reports on the dependence of these kinetics on pH, ionic strength, surfactant concentration, surfactant type or purity of the rhodopsin preparation with respect to other proteins or to phospholipids.

The current state of knowledge with respect to the *in vivo* recombination reaction of rhodopsin can be summarized in a few sentences. Opsin, as present in isolated rod outer segment membranes (DeGrip et al. 1972; Futterman and Rollins 1973; Hong and Hubbell 1973) or in solution with digitonin (Hubbard and Wald 1952) or Tween-80 (Zorn and Futterman 1973), is capable of reacting with 11-*cis* retinal and forming rhodopsin. The pH optimum for the maximum extent of recombination in isolated rod outer segments is between pH 6.0 and 7.5 (Futterman and Rollins 1973) while in digitonin or Tween-80 solutions the pH optimum is 6.3-6.4 (Radding and Wald 1956; Zorn and Futterman 1973). At least two sulfhydryl groups of rhodopsin can be modified in the dark with either N-ethylmaleimide or 5,5'-dithiobis-(2-nitrobenzoic acid) without seriously affecting the recombination capacity (DeGrip, Van De Laar et al. 1973; Zorn 1974). The recombination of rhodopsin from opsin and exogenous 11-*cis* retinal, at least with respect to the overall yield, does not appear to have an absolute dependence upon phospholipids (Hong and Hubbell 1973). The kinetic aspects of the recombination reaction in either isolated rod outer segment membranes or in detergent solutions have not been extensively studied.

Purpose Statement

The goal of this research was to establish a plausible mechanism to account for the *in vivo* recombination

reaction of the visual pigment rhodopsin. Mechanism, in the context of this work, was taken to mean a description of the reaction between 11-*cis* (9-*cis*) retinal and the apoprotein opsin which results in the formation of rhodopsin (isorhodopsin). This description includes the determination of the conditions with respect to surfactants, pH, and associated phospholipids which allow the recombination reaction to proceed. The kinetic aspects of recombination have been studied where possible with respect to the above conditions and these results have been used in formulating a reaction mechanism to describe the recombination reaction of rhodopsin. Circular dichroism studies on rhodopsin were carried out in the near ultraviolet spectral region and were utilized as a probe to the secondary and tertiary structure of rhodopsin. The availability of certain amino acid residues to chemical modification under different conditions was assessed. These results were used to supplement the circular dichroism studies in determining conformation differences between active and inactive forms of rhodopsin.

The completion of the studies outlined above led to the following results which are esstential in describing the overall mechanism of rhodopsin recombination.

1. A qualitative description of the binding of 11-*cis* retinal to the apoprotein opsin in both detergent solubilized and membrane bound forms.

2. The generation of a reaction mechanism which accounts for the recombination of rhodopsin in quantitative chemical terms.
3. A preliminary description of some of the differences in the structure of active and inactive forms of rhodopsin as indicated by circular dichroism spectroscopy and sulfhydryl group availability.

A portion of the work reported here has already been described in an earlier publication (Henselman and Cusanovich 1974).

CHAPTER 2

MATERIALS AND METHODS

Materials

Bovine retinas, dark adapted and frozen, were obtained from the George Hormel Company, Austin, Minnesota and from American Stores Packing Company, Lincoln, Nebraska. The term "dark adapted" indicates the excised eyes were stored in the dark before the retinas were dissected and frozen. This process insures that the rhodopsin content of the retinas will be at a maximum level. No differences were noted in the final rhodopsin samples prepared from either source. Digitonin was purchased from Sigma Chemical Company and was used without further purification. Cholic acid was also obtained from Sigma and was purified by charcoal treatment and 2-3 recrystallizations from hot absolute ethanol. The acid was converted to the sodium salt by titration to pH 10 with sodium hydroxide and was then lyophilized to dryness. Enzyme grade ammonium sulfate was obtained from Nutritional Biochemicals Corporation. All-*trans* and 9-*cis* retinal as well as 5,5'-dithiobis-(2-nitrobenzoic acid) was obtained from Sigma and used without further purification. Crystalline 11-*cis* retinal was a generous gift of Hoffman-La Roche Inc., Nutley, New Jersey and was used as received.

All other chemicals used in this work were of reagent grade quality.

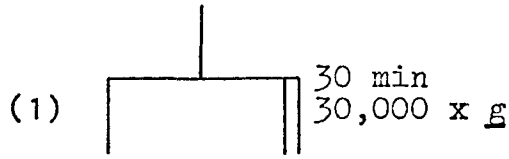
Methods

Preparation of Rod Outer Segment Membranes and Extractions of Rhodopsin

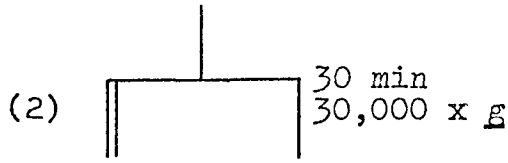
All procedures involving rhodopsin and its extraction were done in total darkness or under dim red light (Kodak Wratten Series 1A Filter) at 4 °C unless otherwise stated. Two different methods were employed for the preparation of rod outer segment membranes during the course of this work. Method A was used to prepare rod outer segments that were subsequently extracted with digitonin. These digitonin extracts were used in the early experiments on the recombination of isorhodopsin from 9-*cis* retinal and opsin. Method A is basically a floatation procedure involving 40% sucrose similar to that described by Shichi et al. (1969) and is fully detailed in Figure 2. Rhodopsin was extracted from the wet rod outer segments (Method A) by homogenization with 67 mM potassium phosphate buffer, pH 6.5, containing 2% digitonin. The homogenate was allowed to incubate at 4 °C for 1-4 hours and was then centrifuged for 45 minutes at 40,000 x *g*. The clear red supernatant containing rhodopsin was carefully removed and the pellet was discarded.

The second procedure used for the preparation of rod outer segment membranes involved the repeated floatation of

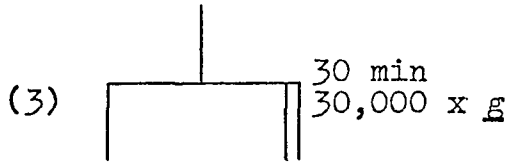
50 Thawing retinas were ground to a slurry in a cold mortar and the total volume adjusted to 100-ml with buffer A.



The pellet was taken up in 30-ml of 40% sucrose, homogenized and was then layered under an equal volume of buffer A.



The material at the interface plus the upper buffer layer was removed and diluted with 1 volume buffer A.



The pellet was taken up in 30-ml of 40% sucrose and gently homogenized. The floatation and pelleting procedure (steps 2-3) was repeated one additional time.

The final pellet was washed twice with buffer A by centrifugation at 30,000 x g for 15 minutes. The isolated rod outer segments were then extracted with 2% digitonin in buffer A as indicated in the Methods section.

Figure 2. Isolation of Rod Outer Segment Membranes--Method A.

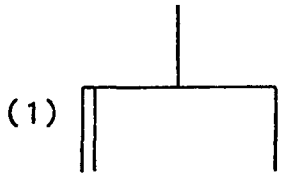
Buffer A was 67 mM potassium phosphate buffer at pH 6.5.

the retinal homogenate in 1.02 M sucrose. Rod outer segment membranes isolated by this procedure (Method B) were used for all the studies involving the recombination reaction of rhodopsin (opsin + 11-cis retinal) in sodium cholate solutions and in suspensions of the isolated membranes. Method B is essentially the same procedure as described by Hong and Hubbell (1973) and is illustrated in Figure 3. Rhodopsin was extracted from lyophilized rod outer segments (Method B) by homogenization with 0.10 M potassium phosphate buffer, pH 7.0, containing 20 mg/ml sodium cholate and in some cases 1 mM dithioerythritol. The homogenate was incubated at 4 °C for 1 hour and then centrifuged for 45 minutes at 40,000 x g. The clear red supernatant containing rhodopsin was removed and the pellet discarded. In those cases where rod outer segment membrane suspensions were used the lyophilized membranes were gently homogenized in the presence of pH 7.0, 0.10 M potassium phosphate buffer containing 1 mM dithioerythritol and then adjusted to the desired concentration.

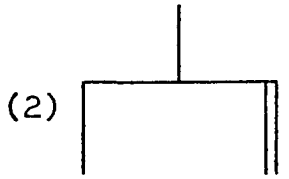
Recombination of Rhodopsin or Isorhodopsin

Recombination experiments were conducted on both digitonin and sodium cholate solubilized rhodopsin as well as on suspensions of rod outer segment membranes. The various retinal isomers were added to the reaction mixtures as concentrated solutions in either absolute ethanol or in 1,4-dioxane. The volumes of the added ligand solutions

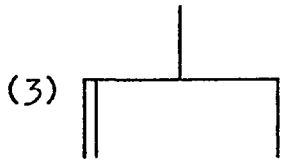
100 Thawing retinas were ground to a slurry in a cold mortar and the total volume adjusted to 100-ml with 1.38 M sucrose in buffer A.



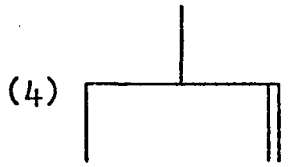
The supernatant was collected and diluted with one volume of buffer A.



The pellet was taken up in 40-ml of 1.02 M sucrose in buffer A and vigorously homogenized in a glass-Teflon homogenizer.



The supernatant with floating material was collected and diluted with one volume of buffer A.



The pellet was taken up in 1.02 M sucrose in buffer A and homogenized. The floatation and pelleting procedure (steps 3-4) was repeated two more times.

The final pellet was washed three times with distilled water by sedimenting the rod outer segments each time using a 15 minute, 40,000 x g centriguation. The isolated rod outer segment membranes were then lyophilized to dryness.

Figure 3. Isolation of Rod Outer Segment Membranes--Method B.

Buffer A was 67 mM potassium phosphate buffer at pH 6.5 and all centrifugations were carried out at 4 C.

never exceeded 1% of the total reaction mixture volumes. Retinal concentrations were determined from the known extinction coefficients of the particular stereoisomers in ethanol which are shown in Table 1 (Morton 1972). Two types of recombination experiments were carried out on each form of rhodopsin, equilibrium and kinetic studies, and the methods which were employed in each type of experiment are discussed below.

The equilibrium studies on the recombination reaction included all experiments in which only the extent of recombination was measured. The effects of pH, surfactant type, surfactant concentration, and chemical modifications upon the extent of rhodopsin (isorhodopsin) recombination were examined in this work. These equilibrium type experiments were carried out by the following general method. The absorption spectrum of each sample was recorded before and after bleaching with white light on either a Cary 11 or a Cary 118 recording spectrophotometer. The recombination of each sample was then initiated by the addition of exogenous 9-cis or 11-cis retinal and each reaction mixture was incubated in the dark at room temperature for at least 1 hour. After the incubation period the reaction was terminated by the addition of hydroxylamine (final concentration of 20 mM) and the absorption spectrum of each sample was again recorded before and after a second bleaching. The

Table 1. Spectral Properties and Extinction Coefficients[a] of Retinal Stereoisomers in Ethanol.

Stereoisomer	λ max	ϵ max (M^{-1} cm^{-1})
all-*trans* retinal	381	43,400
9-*cis* retinal	373	36,100
11-*cis* retinal	376.5	24,900

a. The data in Table 1 were taken from Morton (1972).

extent or percent of recombination observed in each sample was determined by the relationship given in equation 2 where ΔA and $\Delta A'$ were the observed differences in absorbance before and after bleaching in the presence of hydroxylamine.

$$\text{Percent Recombination} = \frac{\Delta A' \text{ (recombined)}}{\Delta A \text{ (native)}} \times 100 \qquad (2)$$

In experiments on the overall recombination of isorhodopsin (9-cis retinal + opsin) $\Delta A'$ was measured at 487 nm and in experiments on the recombination of rhodopsin (11-cis retinal + opsin) $\Delta A'$ was determined at 500 nm. In both of the above experiments ΔA was determined at 500 nm. In those studies which involved suspensions of isolated rod outer segment membranes all spectra were recorded as difference spectra between dark and bleached membrane preparations. The exact experimental conditions used in these experiments with regard to buffers, pH, rhodopsin concentrations, retinal concentrations, and surfactants are given in the results section.

Kinetic studies on the recombination reaction were carried out generally as described above except that immediately after the addition of the exogenous 9-cis or 11-cis retinal the increase in absorbance was monitored as a function of time. Absorbance increases were measured at 500 nm (rhodopsin and isorhodopsin recombination) or at 487 nm (isorhodopsin recombination) with either a single beam

spectrophotometer constructed in this laboratory or with a Cary 118 recording spectrophotometer. In the case of isorhodopsin recombination there was no difference in the calculated rate constants whether the absorbancy increases were monitored at 487 nm or 500 nm. Temperatures were maintained at the indicated values with thermostated cell holders and were measured in the spectrophotometer cells with a YSI Model 42SC Tele-Thermometer (Yellow Springs Instrument Co., Yellow Springs, Ohio). Further experimental details of the conditions employed in the kinetic analysis of the recombination reaction are given in the results section.

Sulfhydryl Group Reactivity of Rhodopsin

The sulfhydryl group reactivity of rhodopsin was determined with 5,5'-dithiobis-(2-nitrobenzoic acid) basically as described by Ellman (1959) and the number of reactive sulfhydryl groups was estimated using an extinction coefficient of 13,600 $\underline{M}^{-1}$ cm^{-1} at 412 nm. Control experiments using cysteine as the reactive sulfhydryl compound indicated the extinction coefficient at 412 nm was not significantly affected (less than a 2% decrease) by carrying out the assay at pH 7.0 in the presence of 20 mg/ml sodium cholate. Reaction mixtures were 1 ml in volume and contained pH 7.0, 0.10 $\underline{M}$ potassium phosphate buffer and were 5-6 $\underline{\mu M}$ in rhodopsin. 5,5'-Dithiobis-(2-nitrobenzoic acid) was added to the reaction mixtures either in the dark or after 10

minutes bleaching with white light as indicated in the results section.

Ammonium Sulfate Fractionation of Sodium Cholate Solubilized Rhodopsin

Solutions of rhodopsin in pH 7.0, 0.10 M potassium phosphate buffer containing 20 mg/ml sodium cholate and 1 mM dithioerythritol or mercaptoethanol were brought to 25% saturation by the addition of solid ammonium sulfate. Following incubation at 4 °C for 10 minutes, the mixture was centrifuged for 20 minutes at 10,000 x g. The supernatant solution was removed, brought to 30% saturation in ammonium sulfate, and was then recentrifuged as above. The supernatant solution was again collected and brought to 36% saturation in ammonium sulfate and after incubation at 4 °C for 10 minutes was centrifuged for 20 minutes at 10,000 x g. The supernatant solution was poured off and the pellet containing rhodopsin was immediately resolubilized in pH 7.0, 0.10 M potassium phosphate buffer containing 20 mg/ml sodium cholate and 1 mM dithioerythritol or mercaptoethanol. In those cases where a second ammonium sulfate fractionation was employed the above procedure was repeated immediately. Residual ammonium sulfate was removed from the fractionated rhodopsin solutions by either gel filtration on Sephadex G-25 or dialysis.

Miscellaneous

Concentrations of digitonin solubilized rhodopsin were calculated using a molar extinction coefficient of 40,600 $\underline{M}^{-1}$ cm^{-1} at 498 nm for rhodopsin solutions in pH 6.5, 67 mM potassium phosphate buffer containing 2% digitonin (Wald and Brown 1952). The rhodopsin concentration in isolated rod outer segment membranes was estimated using an extinction coefficient of 40,000 $\underline{M}^{-1}$ cm^{-1} and the observed difference in absorbance at 500 nm upon bleaching in the presence of 20 mM hydroxylamine. Concentrations of sodium cholate solubilized rhodopsin were calculated using a molar extinction coefficient at 498 nm of 41,400 $\underline{M}^{-1}$ cm^{-1} for rhodopsin solutions in pH 7.0, 0.10 M potassium phosphate buffer containing 20 mg/ml sodium cholate. This extinction coefficient was determined using the thiobarbituric acid assay for retinal (Futterman and Saslaw 1961) and the observed decrease in absorbance at 498 nm of the rhodopsin solution upon bleaching with white light in the presence of 20 mM hydroxylamine.

Circular dichroism spectra of digitonin and sodium cholate solubilized rhodopsin were recorded on a Cary 60 spectropolarimeter equipped with a Cary 6001 CD attachment in one cm pathlength cells at 30 °C. The circular dichroism data are reported in terms of the molecular ellipticity (equation 3).

$$[\theta]_\lambda = 100\ \theta_\lambda / [\underline{M}] \qquad (3)$$

In equation 3 $[\theta]_\lambda$ is the molecular ellipticity (deg-cm^2/decimole), θ_λ is the measured ellipticity in degrees, and $[\underline{M}]$ is the molar concentration of rhodopsin.

The quantitation of the amount of organic phosphorus associated with solubilized rhodopsin was carried out by the following method. Aliquots of the rhodopsin solutions were quantitated with respect to the amount of rhodopsin using the extinction coefficients given above and were then transferred to dialysis bags. The samples were dialyzed against 3-5 changes of distilled water (1000 volumes) for 49-72 hours at 4 °C. After dialysis each sample was removed and the dialysis bag was thoroughly rinsed with distilled water to insure complete removal of the precipitated material. The recovered material was then evaporated to dryness on a warm water bath (40 °C) with the aid of a stream of air. Phosphate analysis on this dried material, after perchloric acid digestion, was then carried out by the method of Bartlett (1959) for samples containing up to 0.3 µmoles of phosphorus or by the method of Fiske and SubbaRow (1925) for samples containing between 0.3 and 1.5 µmoles of phosphorus. In both procedures monobasic potassium phosphate (KH_2PO_4) served as the standard. The above analytical method measures all organic phosphate which is nondialyzable including that from phospholipids (Folch, Lees, and Stanley 1957) and from any

phosphorylated proteins which may be present. This method, as pointed out by Hong and Hubbell (1973), therefore yields an estimate of the upper limit of the phospholipid content of the rhodopsin solutions.

Sodium cholate concentrations were determined by either the use of the appropriate dilution factors or by the method of Boyd, Eastwood, and MacLean (1966). In this method (Boyd et al. 1966) samples containing 5-100 µg of sodium cholate were acidified by the addition of 5 ml of 70% sulfuric acid. After 5 minutes incubation at room temperature 1 ml of aqueous furfural (0.25%) was added to each sample and after color development for 60 minutes the absorbance of the chromogen was measured at 510 nm. Recrystallized sodium cholate (Materials Section) served as the standard and the furfural was redistilled under a nitrogen atmosphere prior to its use.

CHAPTER 3

RESULTS

The results of this work are presented in this chapter and cover the work done on digitonin and sodium cholate solubilized rhodopsin as well as the results with rhodopsin as present in isolated rod outer segment membranes.

Digitonin Solubilized Rhodopsin

All of the rhodopsin used in experiments reported in this section was extracted from rod outer segment membranes prepared using the procedures described in Method A of the Methods section. Rhodopsin was extracted from these membranes with 2% digitonin in 67 mM potassium phosphate buffer at pH 6.5 as described in the Methods section.

Spectral Properties and Purity

Figure 4 presents a typical absorption spectrum of rhodopsin solubilized in 2% digitonin containing pH 6.5, 67 mM potassium phosphate buffer and 20 mM hydroxylamine. The solid line of Figure 4 represents the absorption spectrum of rhodopsin in the dark while the broken line illustrates the spectrum of the same material after bleaching for 10 minutes with white light. These crude digitonin extracts of rhodopsin typically had A_{280}/A_{500} ratios between 3.3 and 4.5.

Figure 4. UV-Visible Absorption Spectrum of Digitonin Solubilized Rhodopsin.

The solid line represents the spectrum recorded in the dark and the broken line represents the spectrum recorded after bleaching with white light for 10 minutes. The rhodopsin sample was in 67 mM potassium phosphate buffer, pH 6.5, containing 2% digitonin and 20 mM hydroxylamine. The spectra are presented on a normalized scale where the absorbance at 280 nm was set equal to one absorbance unit.

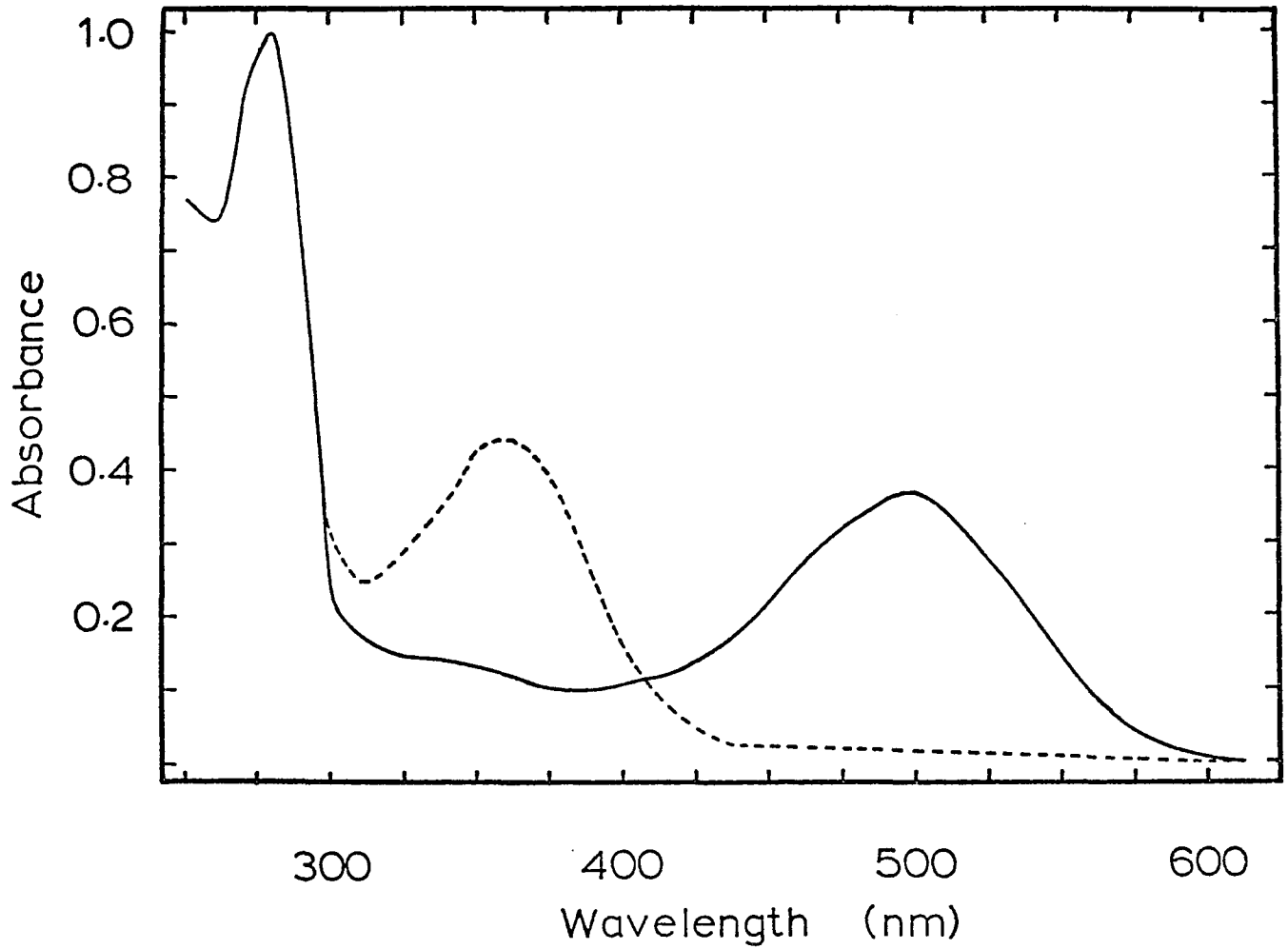


Figure 4. UV-Visible Absorption Spectrum of Digitonin Solubilized Rhodopsin.

Rhodopsin which has been purified to homogeneity generally has an A_{280}/A_{500} ratio between 1.6 and 1.8 (Shichi et al. 1969; Hong and Hubbell 1973) and therefore the crude rhodopsin preparations used in these studies are not considered to be homogenous.

Figure 5 (broken line) illustrates the circular dichroism spectrum of digitonin solubilized rhodopsin in the region from 250 to 400 nm. In this experiment, rhodopsin (15 μM) was solubilized in 67 mM potassium phosphate buffer, pH 6.5, containing 2% digitonin and had an A_{280}/A_{500} ratio of 2.8. The circular dichroism peaks at 338 nm and 280-285 nm were nearly completely lost upon bleaching the sample with white light (solid line, Figure 5). The band at 338 nm (the so-called cis peak) has been assigned to the chromophore, 11-cis retinal, and results from its induced asymmetry when it is bound to the apoprotein opsin (Waggoner and Stryer 1971). The band(s) at 280-285 nm have not been previously studied in rhodopsin but most probably are due to an induced asymmetry in typrosine and/or tryptophan residues.

Equilibrium Properties of Isorhodopsin Recombination

In this work the overall extent of recombination of either rhodopsin or isorhodopsin is considered to be an equilibrium property of the recombination reaction.

Figure 5. Circular Dichroism Spectrum of Digitonin Solubilized Rhodopsin.

The rhodopsin sample (15 μM) was in 67 mM potassium phosphate buffer, pH 6.5, containing 2% digitonin. The circular dichroism spectrum of rhodopsin is shown by the broken line (-----) and the spectrum of the bleached material, opsin, is represented by the solid line (——).

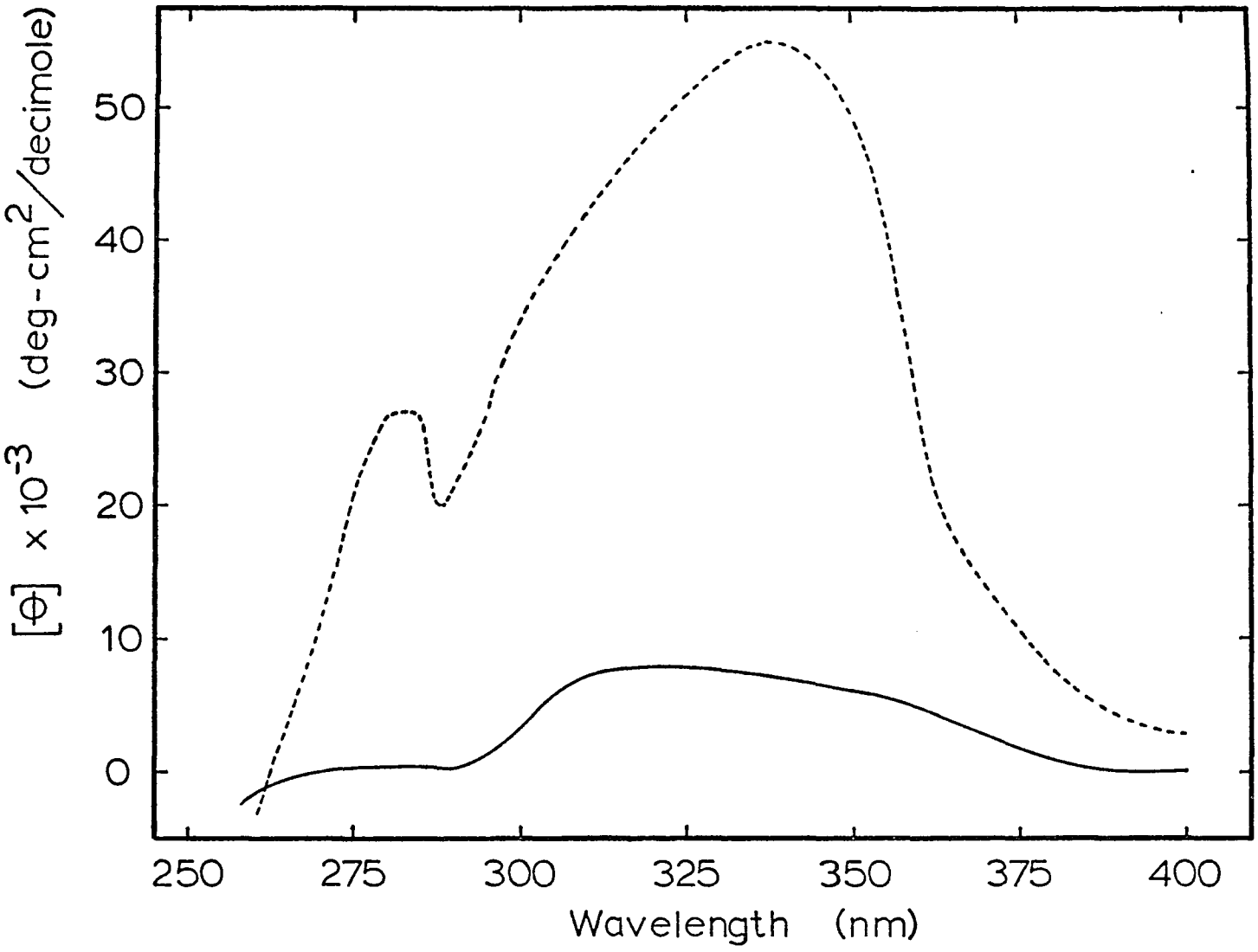


Figure 5. Circular Dichroism Spectrum of Digitonin Solubilized Rhodopsin.

The results of the experimental determination of the pH optimum for the maximum extent of isorhodopsin recombination in digitonin solutions are presented in Figure 6. These experiments were carried out using rhodopsin (8-8.5 μM) solubilized in a 2% digitonin solution containing 0.05 M each of sodium phosphate, sodium citrate, and sodium borate at the indicated pH values. The concentration of 9-cis retinal used in these experiments was approximately 35-36 μM. The rhodopsin samples used in these experiments were the crude digitonin extracts, were not subjected to any further purification, and the A_{280}/A_{500} ratios for these samples varied from 3.3 to 4.5. Figure 6 clearly illustrates the overall extent of isorhodopsin recombination (at equilibrium) under the conditions employed was optimal at pH 6.5. This pH optimum is very close to the reported pH optimum of 6.3-6.4 for the maximum extent of rhodopsin recombination in either digitonin (Radding and Wald 1956) or Tween-80 solutions (Zorn and Futterman 1973).

Kinetic Properties of Isorhodopsin Recombination

Since the proceeding experiments dealt only with the equilibrium properties of isorhodopsin recombination, it was of interest to determine if the dynamic or kinetic properties of this reaction paralleled the equilibrium properties or if they were different. From the data in Figure 6 it was concluded that the reaction was best carried out at pH 6.5

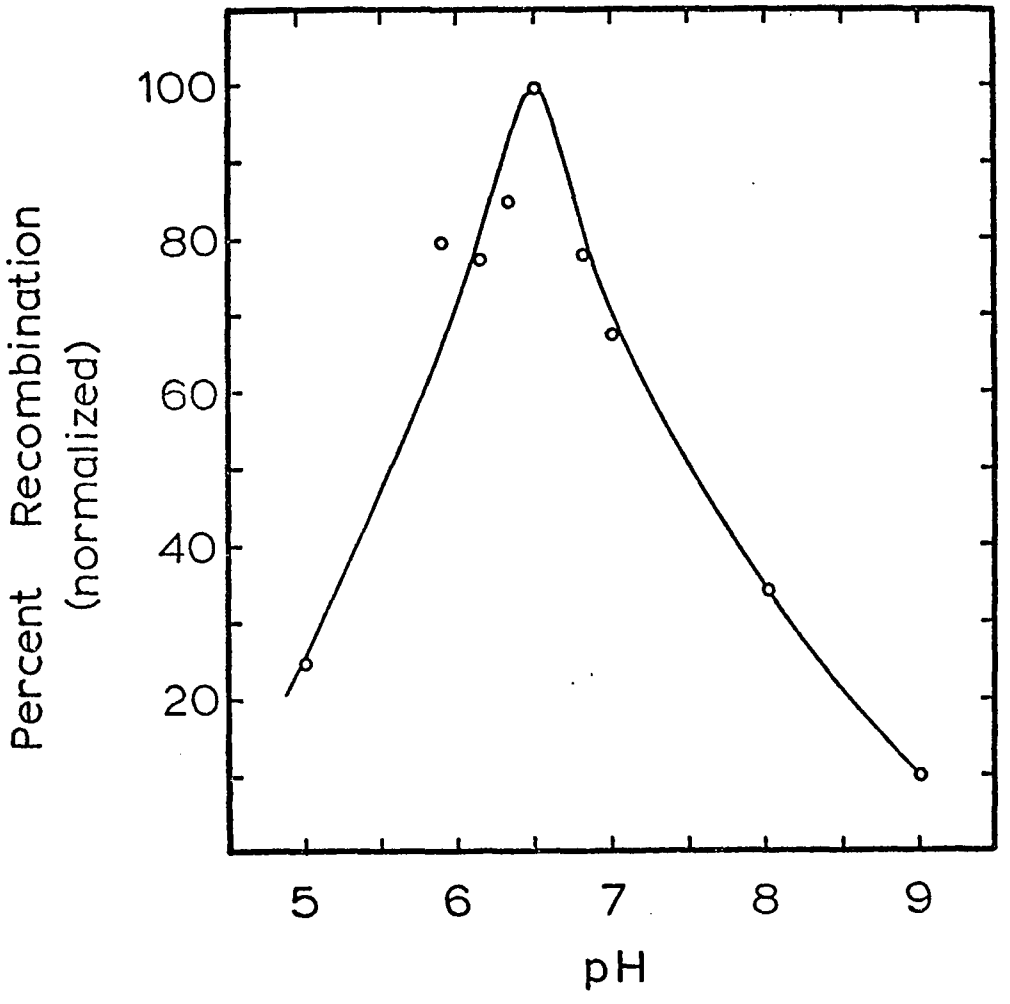


Figure 6. The Effect of pH Upon the Extent of Isorhodopsin Recombination.

The experimental conditions were as noted in the text.

since the greatest extent of recombination was obtained at this pH.

The kinetic properties of isorhodopsin recombination were initially studied and analyzed assuming the simplest possible mechanism for the reaction which is shown in equation 4 where 9-*cis* represents the ligand 9-*cis* retinal.

$$9\text{-}\underline{cis} + \text{opsin} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{isorhodopsin} \qquad (4)$$

If the rate constant for the reverse reaction (k_{-1}) is small then the rate law for the forward reaction is given by equation 5 where dx/dt represents the rate of formation of isorhodopsin, (*cis*) and (opsin) are the molar concentrations of 9-*cis* retinal and opsin respectively, and k_1 is the second-order rate constant for the forward reaction.

$$dx/dt = k_1 (\underline{cis}) (\text{opsin}) \qquad (5)$$

Under the special condition where the concentration of 9-*cis* retinal is much larger than that of opsin, the concentration of 9-*cis* retinal does not change appreciably during the course of the reaction and can be treated as a constant. The rate of such a reaction is then given by equation 6 where k_{obs} is the observed first-order rate constant and is equal to k_1(cis).

$$dx/dt = k_{obs} (\text{opsin}) \qquad (6)$$

A reaction which obeys equation 6 is termed pseudo first-order because under these conditions the rate of the reaction follows first-order kinetics. The k_{obs} values are determined from a plot of the ln ΔA versus time for each kinetic run since equation 6 can be integrated to yield equation 7.

$$\ln \Delta A = k_{obs} t \qquad (7)$$

In equation 7 the term ΔA is the change in absorbance at 487 or 500 nm (final absorbance - absorbance at time t) which is directly proportional to the change in the molar concentration of the produce isorhodopsin.

A series of pseudo first-order kinetic experiments were performed using unpurified digitonin extracts of rhodopsin obtained from isolated rod outer segment membranes prepared by the procedure outlined in Method A. The results of these kinetic experiments are illustrated in Figure 7 and demonstrate that the recombination reaction followed pseudo first-order kinetics under the conditions employed since the ln ΔA versus time plots are linear. Experiments were performed in which ΔA was monitored at both 487 and 500 nm and no differences were noted in the final calculated values of k_{obs}.

The second-order rate constant for isorhodopsin recombination was determined from a plot of the k_{obs} values against the concentration of 9-cis retinal which is shown in

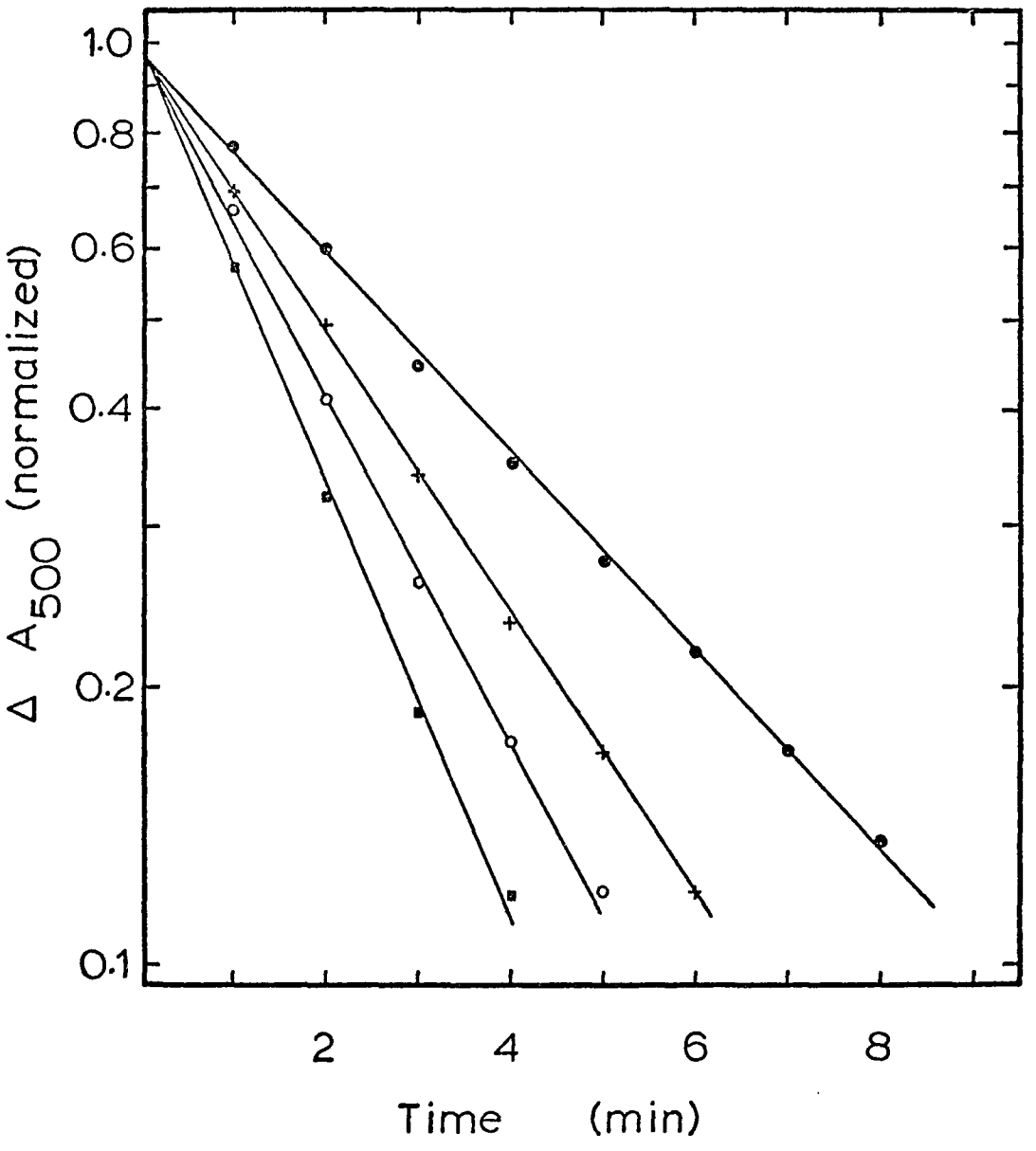


Figure 7. Pseudo First-Order Kinetic Plots for the Recombination of Isorhodopsin.

In these experiments the initial concentration of bleached rhodopsin was 8 uM in 67 mM potassium phosphate buffer, pH 6.5, containing 2% digitonin. The concentrations of 9-cis retinal were as follows: 22.4 uM (—●—); 33.6 uM (—+—); 44.7 uM (—○—); and 55.7 uM (—■—).

Figure 8. A least squares analysis of the data in Figure 8 yields a value of 142 ± 21 $\underline{M}^{-1}$ sec^{-1} for k_1 at 23 °C and pH 6.5. Inspection of Figure 8 reveals that the extrapolated least squares line does not pass through the origin. The intercept of this plot therefore yields an estimate of k_{-1} as approximately 6×10^{-4} sec^{-1} but because of the large degree of scatter in the experimental data the significance of this value of k_{-1} cannot be rigorously interpreted.

Since the data in Figure 8 showed a considerable degree of scatter it was of interest to determine if the experimental results were better represented by a different kinetic mechanism. The next more complicated kinetic mechanism involves the formation of an intermediate compound in the reaction pathway and equation 8 illustrates this mechanism.

$$9\text{-}\underline{cis} + \text{opsin} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{INT} \xrightarrow{k_2} \text{isorhodopsin} \qquad (8)$$

In equation 8 the ligand 9-cis retinal is represented by 9-cis and the intermediate compound in the reaction pathway is represented by INT. The breakdown of the intermediate compound (INT) into the product isorhodopsin was assumed to be essentially irreversible and characterized by the rate constant k_2. The observed rate of the reaction given in equation 8 at any 9-cis retinal concentration, assuming steady state kinetics, is then given by equation 9 where k_1 is the

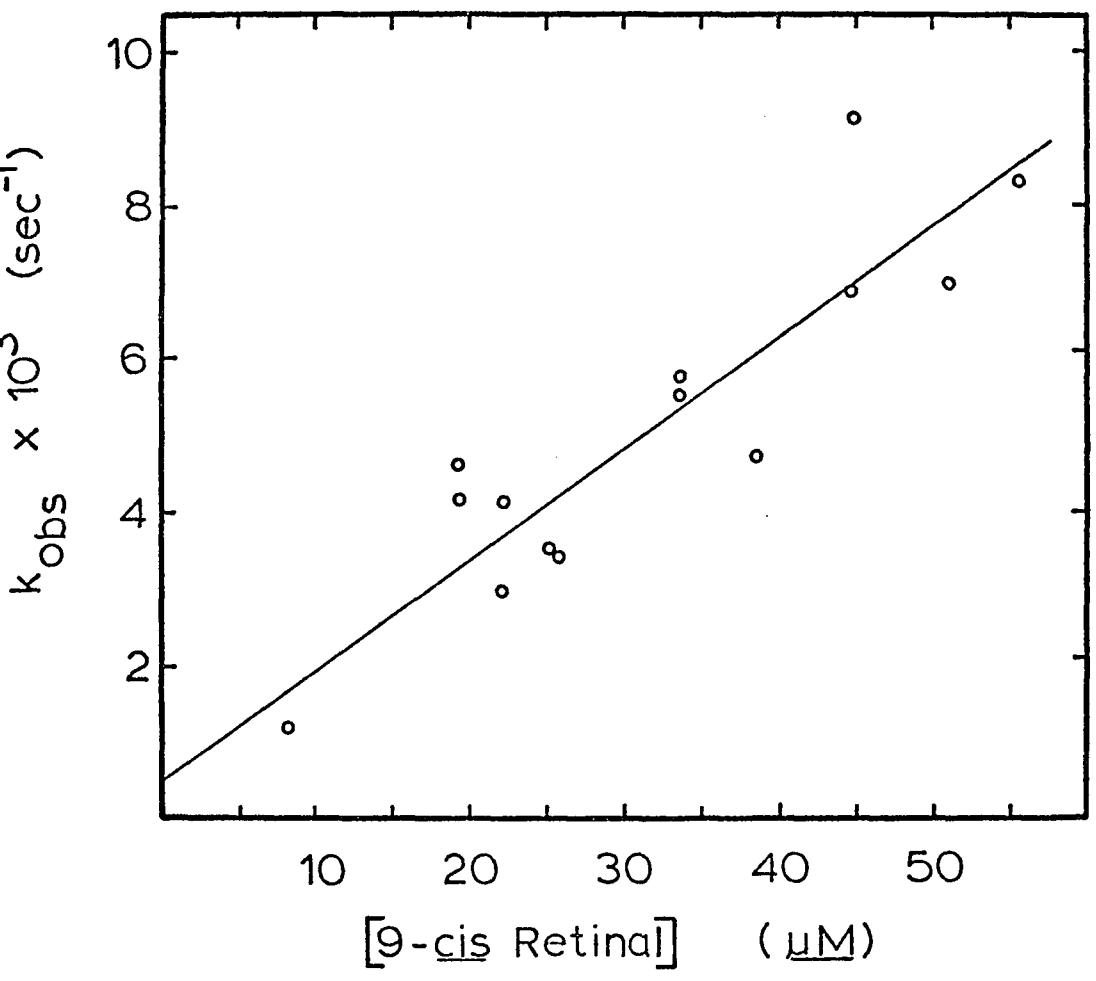


Figure 8. Second-Order Plot for the Recombination of Isorhodopsin.

The experimental conditions were the same as noted in Figure 7 and the reactions were carried out at 23 °C and pH 6.5

rate constant for the formation of the intermediate compound and k_{-1} is the rate constant for the breakdown of the intermediate compound to reactants.

$$k_{obs} = k_1 k_2 (\underline{cis}) / [k_{-1} + k_2 + k_1 (\underline{cis})] \qquad (9)$$

Equation 9 can be rearranged to yield equation 10 which can be graphically represented in a plot of $1/k_{obs}$ versus $1/(\underline{cis})$.

$$1/k_{obs} = [1/k_2] + [(k_{-1} + k_2)/k_1 k_2 (\underline{cis})] \qquad (10)$$

The intercept of such a plot yields the reciprocal of k_2 and the slope is equal to the quantity $(k_{-1} + k_2)/k_1$ divided by k_2. The reciprocal plot for the recombination of isorhodopsin is presented in Figure 9 and yields values of 0.5 sec^{-1} for k_2 and 2.62 x 10^{-4} M for the quantity $(k_{-1} + k_2)/k_1$ at pH 6.5 and 23 °C. Since the errors of the data presented in Figures 8 and 9 were so large it was not possible to determine which of the two kinetic mechanisms best described the recombination of isorhodopsin from digitonin solubilized opsin and 9-cis retinal.

pH Dependence. Since the studies on the effect of pH upon the extent of isorhodopsin recombination were only a measure of the overall equilibrium for isorhodopsin formation, it was of interest to determine if the kinetics of isorhodopsin recombination had a pH dependence similar to or different from that observed in the equilibrium measurements.

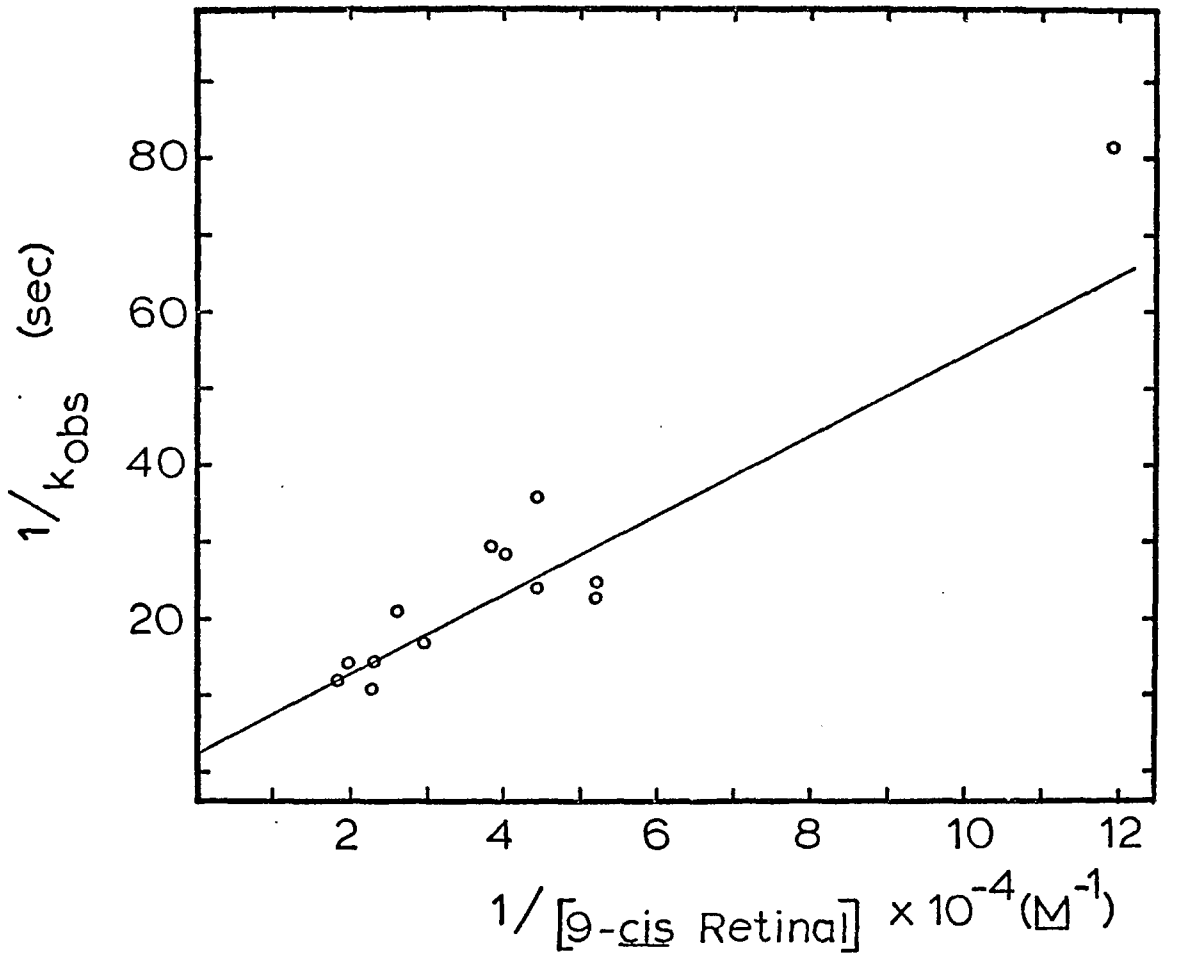


Figure 9. Reciprocal Plot of the Rate of Isorhodopsin Recombination.

The experimental data were the same as in Figure 8.

Figure 10 illustrates some typical pseudo first-order reaction plots for the recombination of isorhodopsin in 2% digitonin solutions at several pH values. In these experiments the initial rhodopsin concentration was approximately 6 μM (before bleaching) and the reactions were carried out in 67 mM potassium phosphate buffer at the indicated pH values. The concentration of 9-cis retinal used in these studies was 35-36 μM. The k_{obs} versus pH plot in Figure 11 partially demonstrates the pH dependence for the rate of isorhodopsin recombination in digitonin. Unfortunately, it was not possible to accurately measure the kinetics of recombination above pH 7 or below pH 5 because of the low recombination yields and hence small absorbance changes (0.05-0.15) at 500 nm. However enough data were observed to demonstrate that the pH optimum for the extent of recombination (Figure 6) was not the same as for the rate of recombination (Figure 11).

Effect of Temperature. The dependence of the rate constant for isorhodopsin recombination upon temperature was studied in order to evaluate the thermodynamic activation parameters for this reaction. Considerable difficulties were encountered in the temperature study and the data (not presented) had a large error associated with it. An apparent Arrhenius energy of activation, E_a, equal to approximately 27 kcal/mole was obtained for the recombination

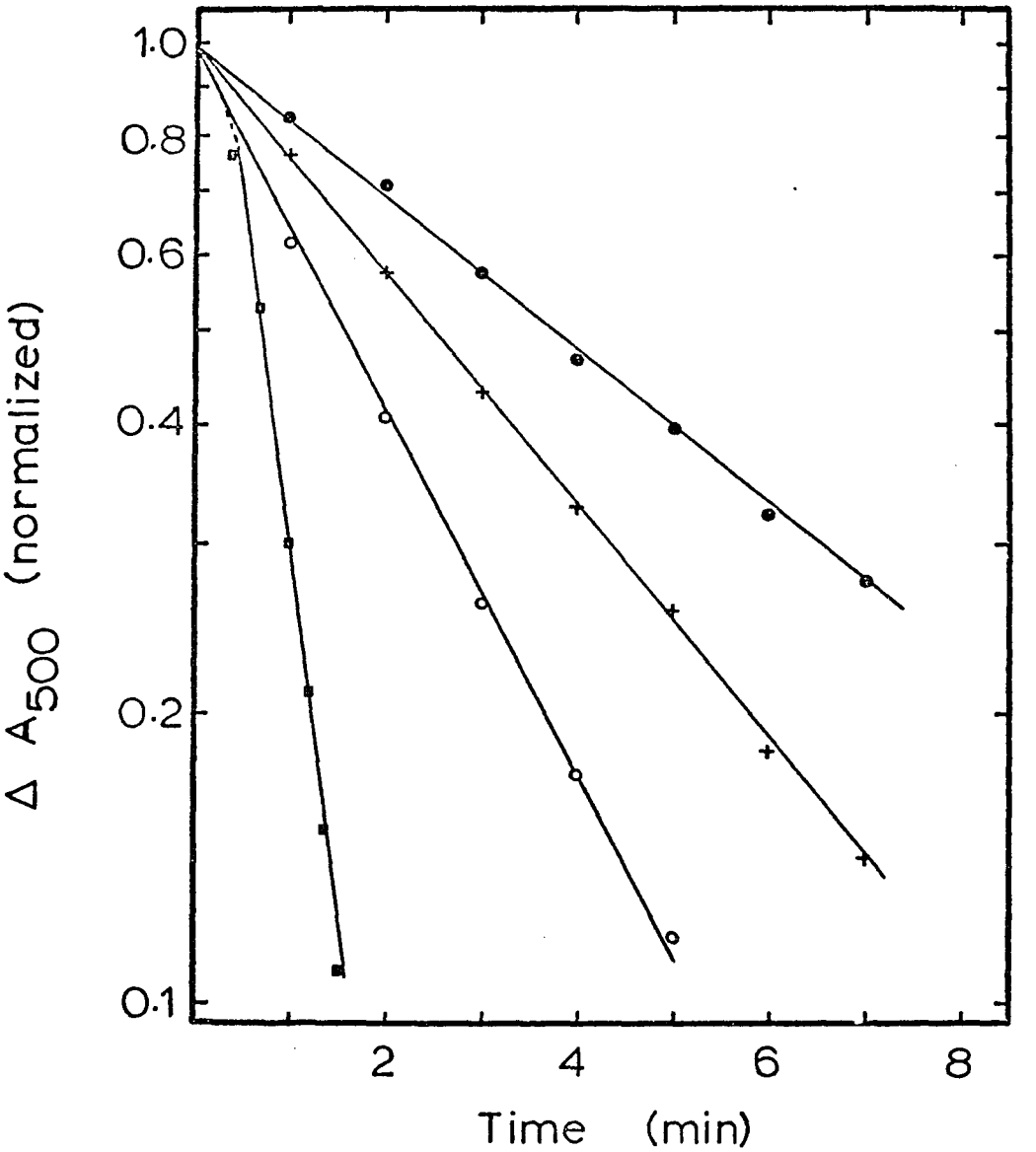


Figure 10. Effects of pH Upon the Pseudo First-Order Plots for the Recombination of Isorhodopsin.

pH 5.0 (—●—); pH 5.9 (—○—); pH 6.5 (—+—); pH 7.0 (—■—). In these experiments the opsin concentration was 6 μM and the concentration of exogenous 9-cis retinal was 35-36 μM. Each reaction mixture contained 67 mM potassium phosphate buffer and 2% digitonin.

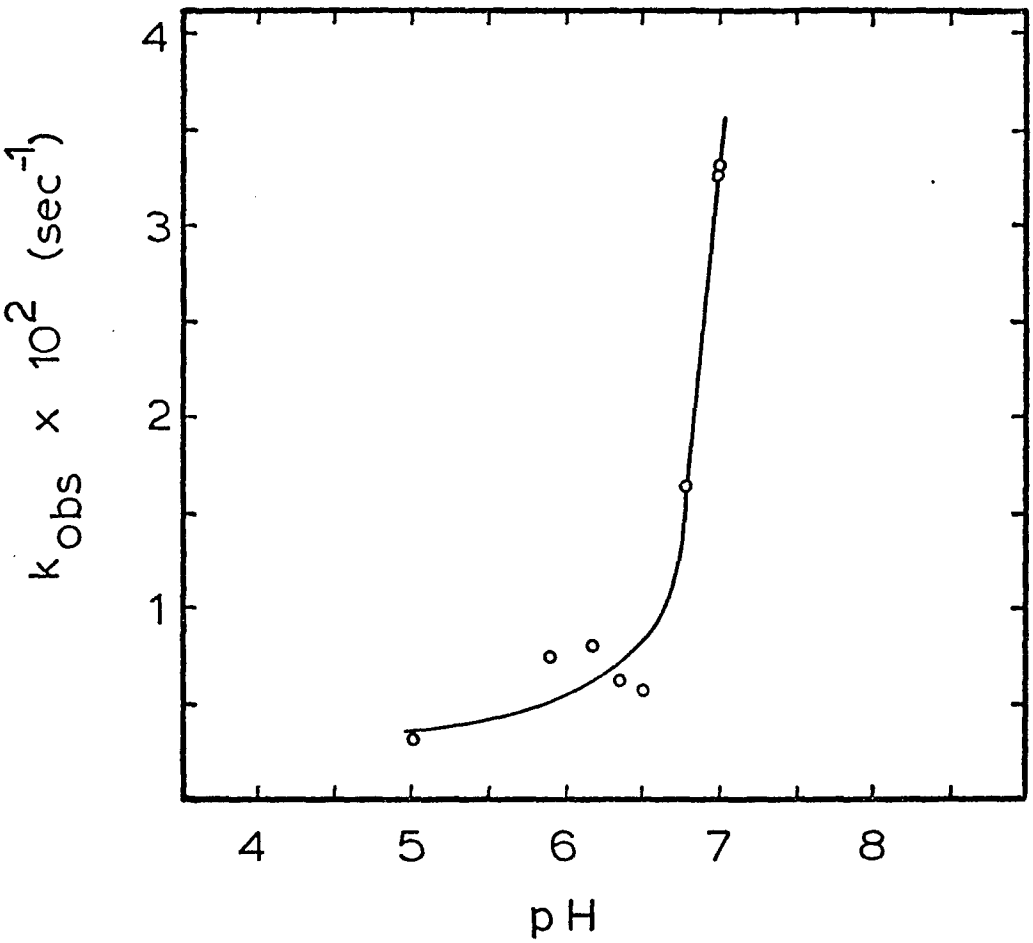


Figure 11. pH Dependence of the Rate of Isorhodopsin Recombination.

The experimental conditions were the same as indicated in Figure 10.

reaction. This value of E_a was obtained using second order rate constant (k_1) determined at different temperatures assuming the reaction proceeds via the mechanism shown in equation 4. This value of E_a was quite close to the value (24 kcal/mole) determined by Abrahamson and Ostroy (1967).

Summary. Two different mechanisms (equations 4 and 8) were evaluated through kinetic studies on the recombination of isorhodopsin in digitonin solutions. Because of the errors associated with these measurements it was not possible to decide which of the two above mechanisms better described the recombination reaction. One surprising result was the demonstration that the pH optimum for the extent of recombination (pH 6.5) was different than for the rate of recombination (pH $\geq$ 7). A summary of the results obtained for the recombination of isorhodopsin in digitonin solutions is presented in Table 2.

Sodium Cholate Solubilized Rhodopsin

Lyophilized rod outer segment membranes were prepared using the procedures described in Method B of the Methods section and were used as the basic starting material for all the studies involving sodium cholate solubilized rhodopsin.

Solubilization and Properties of Rhodopsin

Preliminary experiments were carried out in order to determine the optimum conditions, pH and surfactant

Table 2. Properties of Isorhodopsin Recombination.[a]

	Extent	Rate
Optimum pH for Recombination	6.5	$\geq$7.0
	k_1	k_{-1}
Rate Constants for[b] Recombination (Eq. 4)	142 ± 21 $\underline{M}^{-1}$ sec^{-1}	$6 \pm 3 \times 10^{-4}$ sec^{-1}
	k_2	$(k_{-1} + k_2) / k_1$
Rate Constants for[c] Recombination (Eq. 8)	0.5 sec^{-1}	2.62×10^{-4} $\underline{M}$

a. All kinetic experiments were at 23 °C, using digitonin (2%) solubilized rhodopsin in pH 6.5, 67 mM potassium phosphate buffer.

b. Rate data calculated according to the reaction mechanism shown in equation 4 from data obtained at pH 6.5.

c. Rate data calculated according to the reaction mechanism shown in equation 8 from data obtained at pH 6.5.

concentration, for the extraction of rhodopsin from the isolated and lyophilized rod outer segment membranes. It was found that the concentration of sodium cholate used for the extraction was critical since at concentrations of less than 15 mg/ml sodium cholate the extractions gave poor yields of rhodopsin and the resulting solutions were turbid and difficult to clarify. Altering the pH of the extracting solution, 6.5-7.6, did not significantly affect the amount of rhodopsin extracted but the A_{280}/A_{500} ratios of the resulting rhodopsin solutions increased with increasing pH (Figure 12). Using the above information it was decided that the best compromise involved extracting the lyophilized membranes with a 20 mg/ml sodium cholate solution in 0.10 M potassium phosphate buffer at pH 7.0. At pH values below 7.0 cholic acid tended to crystallize out of the solutions upon storage and at pH values greater than 7.0 the observed A_{280}/A_{500} ratio increased. Solutions of sodium cholate (20 mg/ml) in the presence of 50 mM Hepes buffer (N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid) were totally incapable of extracting or solubilizing rhodopsin from the lyophilized rod outer segment membranes.

The extraction of lyophilized rod outer segment membranes with pH 7.0, 0.10 M potassium phosphate buffer containing 20 mg/ml sodium cholate resulted in solubilizing 0.5-0.7 umoles of rhodopsin per 100 bovine retinas. These

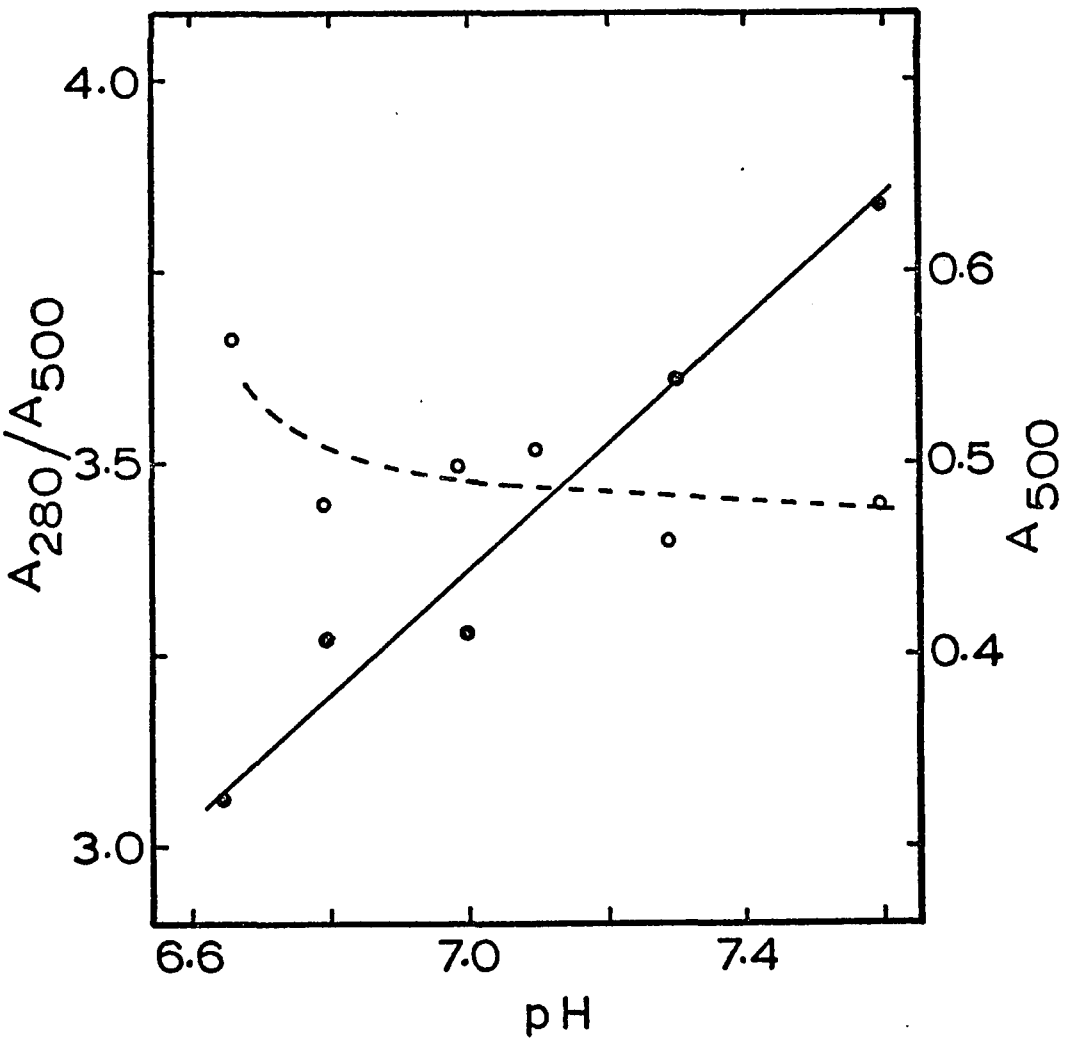


Figure 12. Effect of pH on the Extraction and Purity Ratio of Rhodopsin Using Sodium Cholate.

Equal amounts of lyophilized rod outer segment membranes were extracted with 0.10 M potassium phosphate buffer containing 20 mg/ml sodium cholate as described in the Methods section. A_{280}/A_{500} (—•—); A_{500} (- -o- -).

crude rhodopsin extracts had an average A_{280}/A_{500} ratio of 2.89 and varied from 2.0 to 3.8 (average and range of 15 separate preparations) and in most cases were also quite concentrated with the absorbance at 500 nm as high as 6. Figure 13 illustrates the UV-visible absorption spectrum of a typical rhodopsin preparation in 0.10 M potassium phosphate buffer, pH 7.0, containing 20 mg/ml sodium cholate and 20 mM hydroxylamine. The solid line in Figure 13 is the spectrum recorded in the dark and the broken line is the spectrum recorded after 10 minutes bleaching with white light. Figure 13 demonstrates an important point which is that sodium cholate solubilized rhodopsin is stable towards hydroxylamine in the dark and that the retinal-protein linkage is attacked by this reagent only after bleaching. In other experiments it was demonstrated that sodium borohydride behaves in a similar manner and these data indicate sodium cholate solubilized rhodopsin and other reported detergent solutions of rhodopsin have identical reactivities toward hydroxylamine or sodium borohydride.

The spectral properties and the observed A_{280}/A_{500} ratios of the initial extracts of sodium cholate solubilized rhodopsin were found to be dependent upon the concentration of sodium cholate present in the samples. This dependence is illustrated in Figure 14 and indicates these spectral changes occurred very sharply in the concentration range of 5-6 mg/ml

Figure 13. UV-Visible Absorption Spectrum of Sodium Cholate Solubilized Rhodopsin.

The rhodopsin solution was in 0.10 M potassium phosphate buffer, pH 7.0, containing 20 mg/ml sodium cholate and 20 mM hydroxylamine. The solid line (———) represents the spectrum recorded in the dark and the broken line (-----) represents the spectrum recorded after 10 minutes bleaching with white light. The spectra are presented on a scale normalized to 1 absorbance unit at 280 nm.

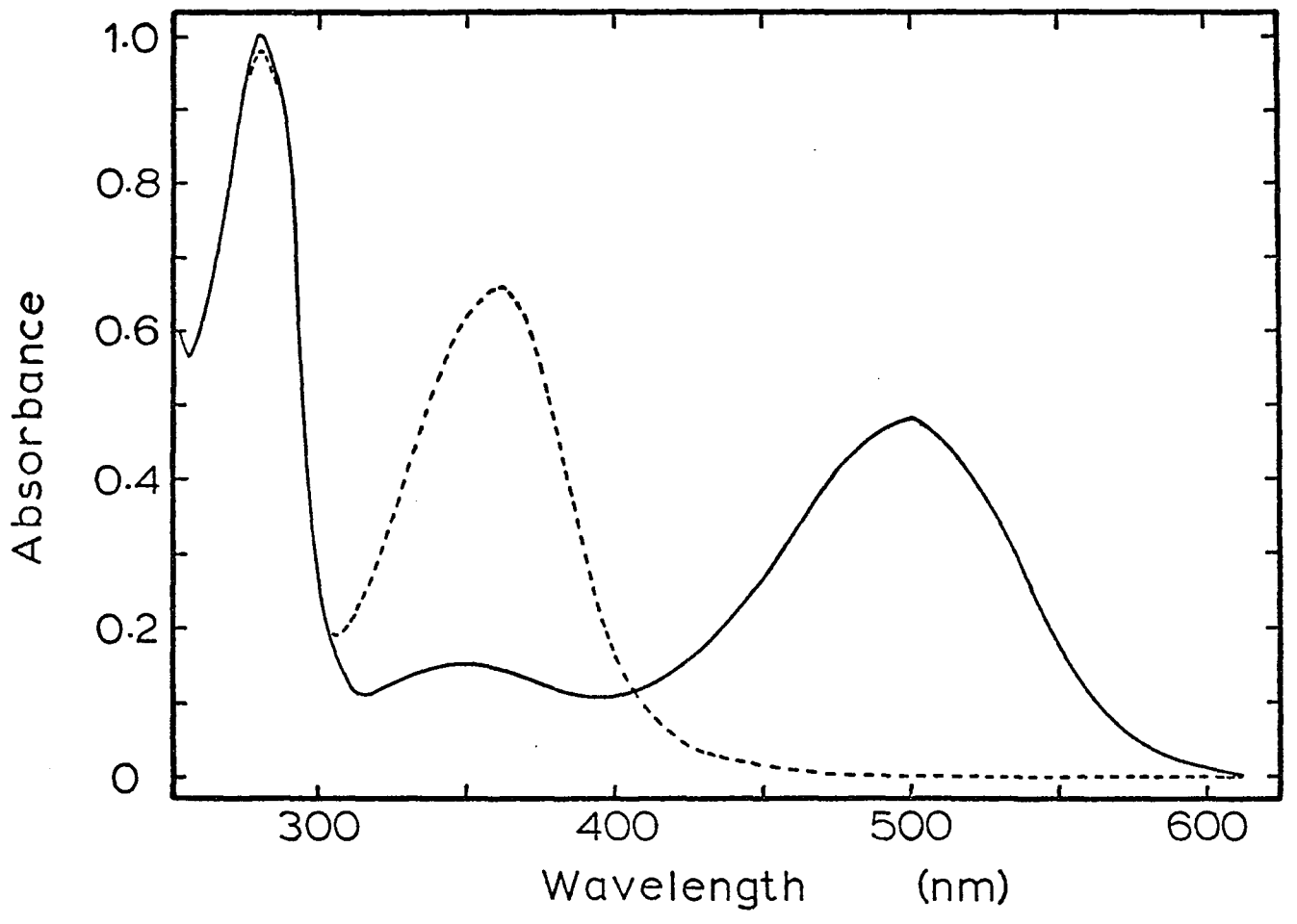


Figure 13. UV-Visible Absorption Spectrum of Sodium Cholate Solubilized Rhodopsin.

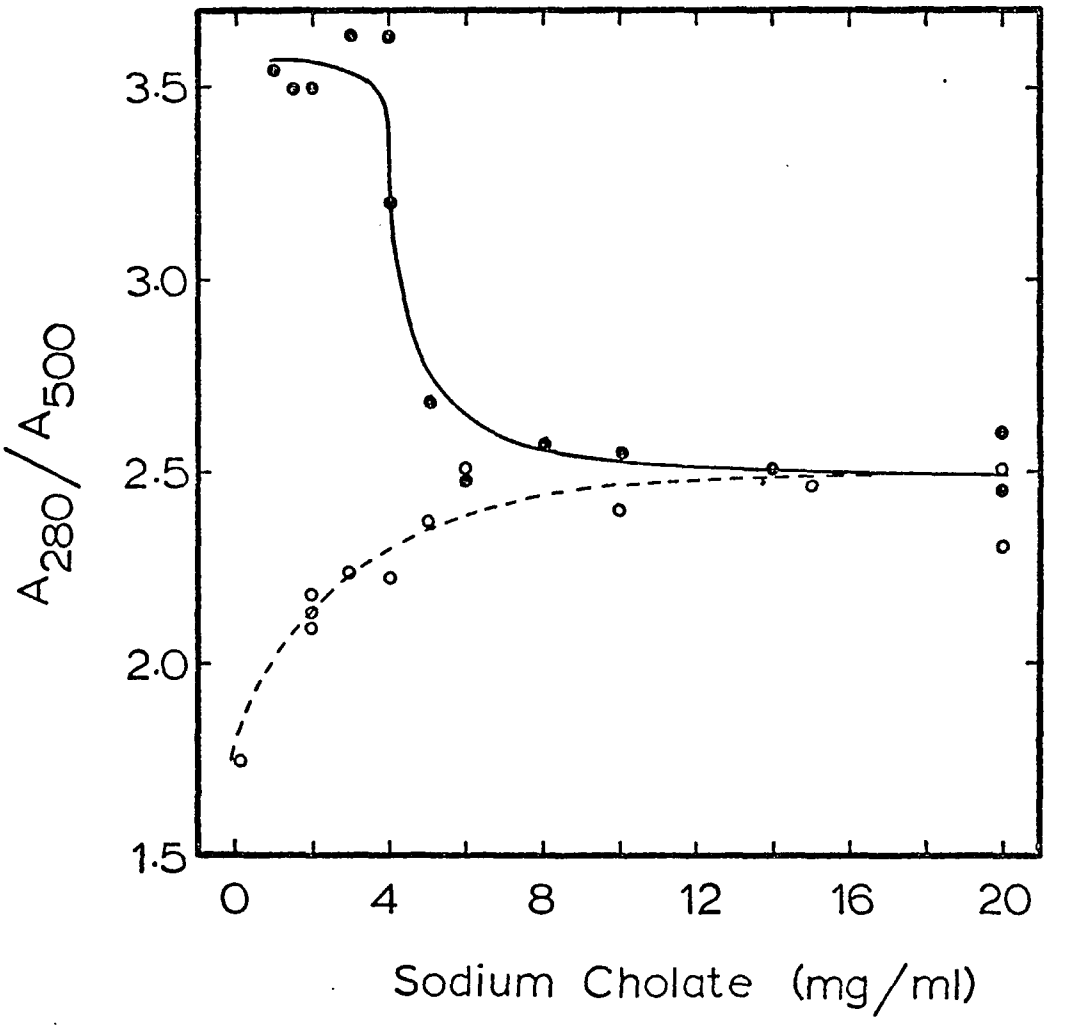


Figure 14. Dependence of the A_{280}/A_{500} Ratio of Rhodopsin Upon the Sodium Cholate Concentration.

The initial extracts of rhodopsin are represented by (—●—); and ammonium sulfate fractionated rhodopsin samples are represented by (---o---).

sodium cholate. The increased A_{280}/A_{500} ratio of rhodopsin at low (1-4 mg/ml) sodium cholate concentrations was probably a result of the aggregation of rhodopsin since these solutions appeared visibly turbid while solutions of rhodopsin at higher sodium cholate concentrations (8-20 mg/ml) were optically clear. This conclusion was further strengthened by the observation that rhodopsin in 0.10 M potassium phosphate buffer, pH 7.0, containing 20 mg/ml sodium cholate was not sedimented by a 1 hour centrifugation at 105,000 x g. Rhodopsin in the same buffer but containing only 2 mg/ml sodium cholate was totally sedimented by an identical centrifugation.

The circular dichroism spectrum (250-400 nm) of sodium cholate solubilized rhodopsin is presented in Figure 15. The rhodopsin sample (14.3 μM) used for this experiment was an unpurified initial sodium cholate (20 mg/ml) extract, had an A_{280}/A_{500} ratio of 2.48, and was in pH 7.0, 0.10 M potassium phosphate buffer. Qualitatively the circular dichroism spectra of both 2% digitonin solubilized rhodopsin (Figure 5) and sodium cholate (20 mg/ml) solubilized rhodopsin are very similar. Both bands, 338 and 280-285 nm, were also lost upon bleaching the rhodopsin sample, similar to that observed for the digitonin solubilized rhodopsin.

The initial extracts of sodium cholate solubilized rhodopsin contained 60.8 ± 7.5 moles of phosphate per mole of rhodopsin (average ± standard deviation of 8 determinations from 4 separate rod outer segment preparations). This

Figure 15. Circular Dichroism Spectrum of Sodium Cholate Solubilized Rhodopsin.

The rhodopsin sample (14.3 μM) was in 0.10 M potassium phosphate buffer, pH 7.0, containing 20 mg/ml sodium cholate and 1 mM dithioerythritol. The broken line (-----) represents the circular dichroism spectrum recorded in the dark and the solid line (———) represents the circular dichroism spectrum recorded after bleaching the sample with white light for 20 minutes. The rhodopsin sample used for this experiment was an unpurified extract from lyophilized rod outer segment membranes and had an A_{280}/A_{500} ratio of 2.48.

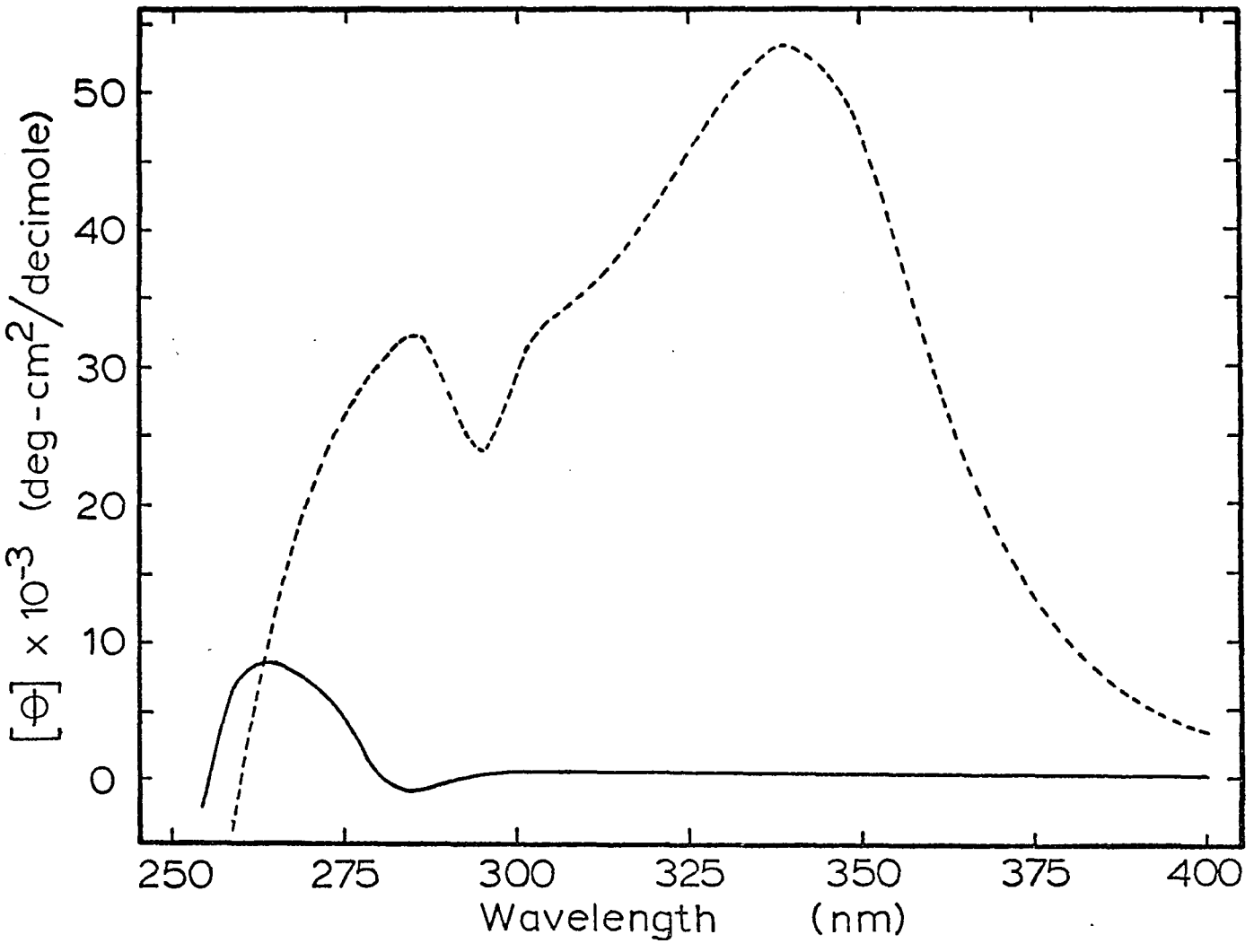


Figure 15. Circular Dichroism Spectrum of Sodium Cholate Solubilized Rhodopsin.

value represents an upper limit to the amount of associated phospholipids since the phosphate analysis performed as indicated in the Methods section analyzes for all nondialyzable organic phosphate which includes that from phospholipids and phosphorylated proteins.

The initial extracts of sodium cholate solubilized rhodopsin were further purified by the ammonium sulfate fractionation procedure outlined in the Methods section. This procedure resulted in a significant purification of the rhodopsin with respect to both proteins, as suggested by the lowering of the A_{280}/A_{500} ratio, and phospholipids. Phosphate analysis of rhodopsin after a single ammonium sulfate fractionation showed that the amount of nondialyzable organic phosphate was decreased to 16 ± 5 moles of phosphate per mole of rhodopsin (average ± standard deviation of 6 determinations from 2 separate rod outer segment preparations). Further purification was achieved by employing a second ammonium sulfate fractionation which reduced the organic phosphate to rhodopsin molar ratio to 6.0 ± 0.4 (average ± standard deviation of 3 determinations from 1 rod outer segment preparation). The yields of rhodopsin after a single ammonium sulfate fractionation ranged from 80-90% based upon the absorbance at 500 nm. It was extremely important to carefully follow the procedure outlined in the Methods section since the failure to include reducing agents

(mercaptoethanol or dithioerythritol) in the solutions resulted in the low recovery of rhodopsin. Failure to include the fractionation step at 25% ammonium sulfate saturation resulted in the partial precipitation of rhodopsin between 30 and 36% saturation in ammonium sulfate.

The spectral characteristics of the ammonium sulfate fractionated rhodopsin were in some respects similar to the unfractionated rhodopsin samples and in other respects were surprisingly different. The observed A_{280}/A_{500} ratios of ammonium sulfate fractionated rhodopsin samples (at sodium cholate concentrations of 20 mg/ml) were always lower than that observed in the same samples before fractionation. Hydroxylamine and sodium borohydride displayed similar reactivities towards both the ammonium sulfate fractionated rhodopsin and the initial sodium cholate extracts of rhodopsin. These observations suggested that the fractionation procedure did not seriously disrupt the integrity of the retinal binding site. The dependence of the A_{280}/A_{500} ratio of ammonium sulfate fractionated rhodopsin upon the sodium cholate concentration was substantially different from that observed for unfractionated rhodopsin. Figure 14 (broken line) illustrates the dependence of the A_{280}/A_{500} ratio upon sodium cholate concentration for ammonium sulfate fractionated rhodopsin and compares it with unfractionated rhodopsin (Figure 14, solid line). Why the A_{280}/A_{500} ratio of the ammonium

sulfate fractionated rhodopsin decreased with decreasing sodium cholate concentration while the opposite behavior was noted for the unpurified extracts is not entirely clear. The most reasonable explanation seems to be involved with the reduced organic phosphate content of the fractionated rhodopsin samples. It is possible that the interactions between sodium cholate and rhodopsin are in some manner dependent upon the organic phosphate content of the rhodopsin preparation.

Circular dichroism spectra were also recorded for the ammonium sulfate fractionated rhodopsin samples and they were generally similar to the spectrum shown in Figure 15. One difference was noted however in the band at 280-285 nm. This band tended to show an increase in the molecular ellipticity for the ammonium sulfate fractionated samples. The initial sodium cholate extracts of rhodopsin had a value for $[\theta]_{280}$ of approximately 32,000 deg-cm^2/decimole, and after 2 ammonium sulfate fractionations the value of $[\theta]_{280}$ increased to 45,000-50,000 deg-cm^2/decimole. The band at 338 nm did not display any significant change in molecular ellipticity after ammonium sulfate fractionation. Since two ammonium sulfate fractionations reduced the organic phosphate to rhodopsin ratio from approximately 60:1 to 6:1 the changes observed in the circular dichroism spectra were probably the result of the reduced organic phosphate content.

Sulfhydryl Group Reactivity of Sodium Cholate Solubilized Rhodopsin

The assay for reactive sulfhydryl groups in sodium cholate solubilized rhodopsin (non-ammonium sulfate fractionate) with 5,5'-dithiobis-(2-nitrobenzoic acid) carried out as described in the Methods section indicated the number of available sufhydryl groups was dependent upon both the sodium cholate concentration and whether or not the sulfhydryl reagent was added before or after bleaching the rhodopsin sample. Table 3 presents the sulfhydryl reactivity of dark and/or bleached rhodopsin and the dependence of this reactivity upon 5,5'-dithiobis-(2-nitrobenzoic acid) addition and sodium cholate concentration. The important points in Table 3 are that the number of reactive sulfhydryl groups was dependent upon both the surfactant concentration and whether the sulfhydryl reagent was added before or after bleaching. Figure 16 illustrates the sulfhydryl reactivity of bleached rhodopsin in the presence of varying concentrations of sodium cholate and demonstrates that the number of titratable sulfhydryl groups increased with increasing sodium cholate concentrations.

Equilibrium Properties of Rhodopsin Recombination in Sodium Cholate

The recombination of rhodopsin from opsin and exogenous 11-*cis* retinal in sodium cholate solutions was carried

Table 3. Sulfhydryl Group Reactivity of Rhodopsin.

Sodium Cholate (mg/ml)	DTNB Addition[a]	Moles reactive -SH/Mole Rhodopsin		
		Dark[b]	Revealed by[c] Bleaching	Total
20	Dark	1.9-2.2	1.9	3.8-4.1
20	Bleached	-	-	1.8-1.9
2	Dark	1.0-1.2	0	1.0-1.2
2	Bleached	-	-	0.35

Rhodopsin solutions, 1 ml, containing 0.10 M potassium phosphate buffer, pH 7.0, were treated with 5, 5'-dithiobis-(2-nitrobenzoic acid), (DTNB), under the conditions noted. In all experiments the rhodopsin concentration was approximately 5 μM and the final concentration of DTNB was 0.2 mM.

a. Indicates that DTNB was added to the solution either in the dark or after 10 minutes bleaching with white light.

b. The number of sulfhydryl groups assayed in the dark.

c. The number of sulfhydryl groups which were revealed upon bleaching when DTNB was present in the solution before bleaching.

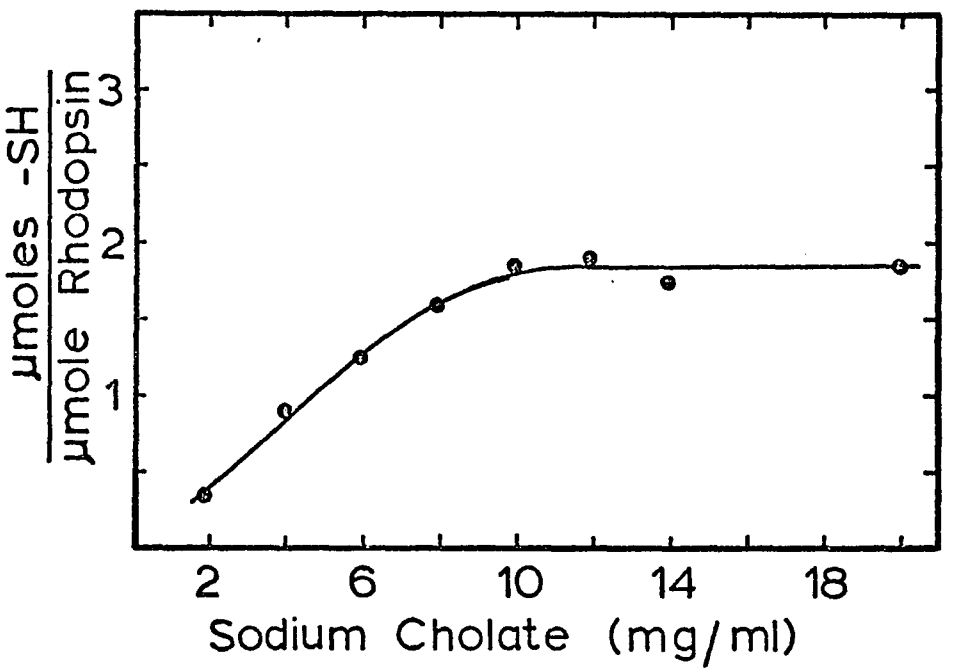


Figure 16. Dependence of the Availability of Opsin Sulfhydryl Groups Upon Sodium Cholate Concentration.

out as described in the Methods section and as indicated in each of the following experiments.

The concentration of sodium cholate present in the reaction mixtures was found to strongly influence the overall extent of rhodopsin recombination. Figure 17 demonstrates that at high sodium cholate concentrations (10-20 mg/ml) the recombination reaction between 11-*cis* retinal and opsin was significantly inhibited while at low sodium cholate concentrations (1-5 mg/ml) the reactions were not inhibited at all or only very slightly. In the above experiments the initial extracts of sodium cholate solubilized rhodopsin were used without further purification and these solutions contained approximately 60 moles of nondialyzable organic phosphate per mole of rhodopsin. Each reaction mixture consisted of 5-6 μM rhodopsin in 0.10 M potassium phosphate buffer, pH 7.0, containing 1 mM dithioerythritol. The bleaching and subsequent recombination of each sample was carried out as described in the Methods section.

The second equilibrium property of the recombination reaction of sodium cholate solubilized rhodopsin studied was the effect of pH upon the overall extent of recombination. Figure 18 illustrates the pH dependence of the recombination reaction in sodium cholate and indicates that the pH optimum for the maximum extent of recombination lies between pH 7.0 and 7.4. The data in Figure 18 were collected using rhodopsin

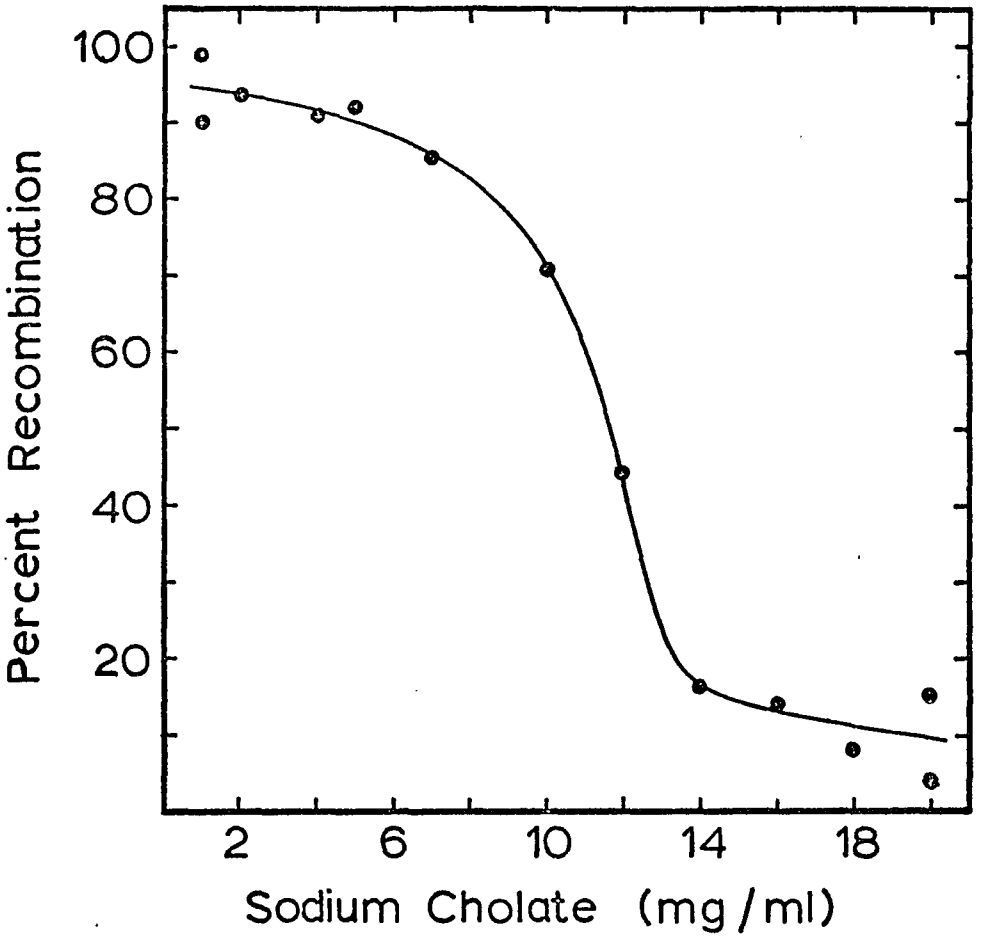


Figure 17. Rhodopsin Recombination as a Function of Sodium Cholate Concentration.

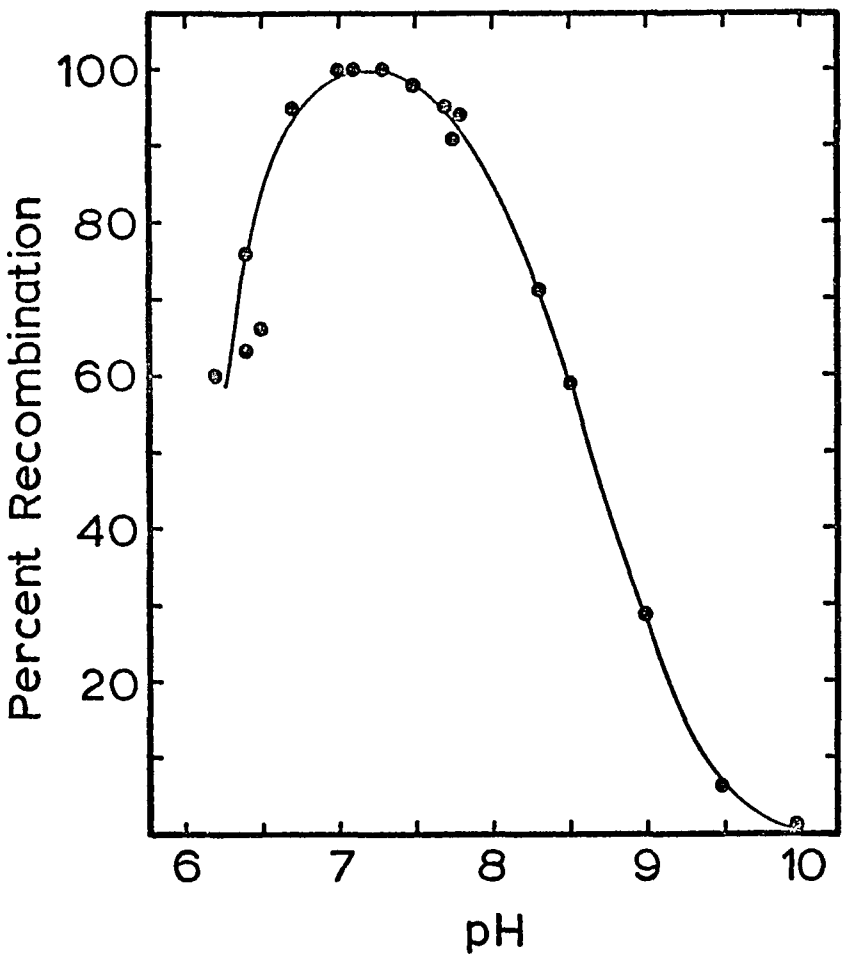


Figure 18. pH Dependence of the Extent of Rhodopsin Recombination in Sodium Cholate.

Recombination reactions were carried out as described in the text and the concentration of sodium cholate was 2-3 mg/ml. The data is presented on a normalized scale where the observed percent recombination at pH 7.0 was set equal to 100%. These data were collected using rhodopsin solutions from five separate rod outer segment membrane preparations.

solutions from 5 separate rod outer segment preparations and are presented on a normalized scale where the observed percent recombination at pH 7.0 was set equal to 100%. The observed extent of recombination in these experiments at pH 7.0 typically varied from 80-100% of that theoretically expected. The rhodopsin solutions used in these experiments were initial sodium cholate extracts and were not further purified. The recombination experiments were carried out as indicated in the Methods section and under the conditions indicated in the legend of Figure 18.

Kinetic Properties of Rhodopsin Recombination in Sodium Cholate

pH Dependence. The effect of pH on the rate of the recombination reaction of sodium cholate solubilized rhodopsin was assessed by measuring the pseudo first-order rate constant of the reaction at a variety of pH values. In these experiments initial sodium cholate extracts of rhodopsin were used without further purification. The reactions were carried out as described in the Methods section and each reaction mixture consisted of 0.10 M potassium phosphate buffer containing 1 mM dithioerythritol, 2 mg/ml sodium cholate, 6 μM opsin, and 53 μM 11-cis retinal. The kinetic plots (ln ΔA_{500} versus time) at various pH's for this reaction are shown in Figure 19. These plots are linear between pH 6.5 and 8.0 indicating that the reaction follows pseudo first-order

Figure 19. Effect of pH on the Pseudo First-Order Plots of Rhodopsin Recombination in Sodium Cholate.

Recombination reactions were carried out at the following pH values: pH 7.95 (—•—); pH 7.5 (—+—); pH 7.0 (—o—); pH 6.5 (---▪--). Solutions of rhodopsin (6 μM) in 0.10 M potassium phosphate buffer containing 1 mM dithioerythritol and 2 mg/ml sodium cholate were bleached with white light for at least 20 minutes. The recombination of each sample was then initiated by the addition of exogenous 11-cis retinal to a final concentration of 53 μM.

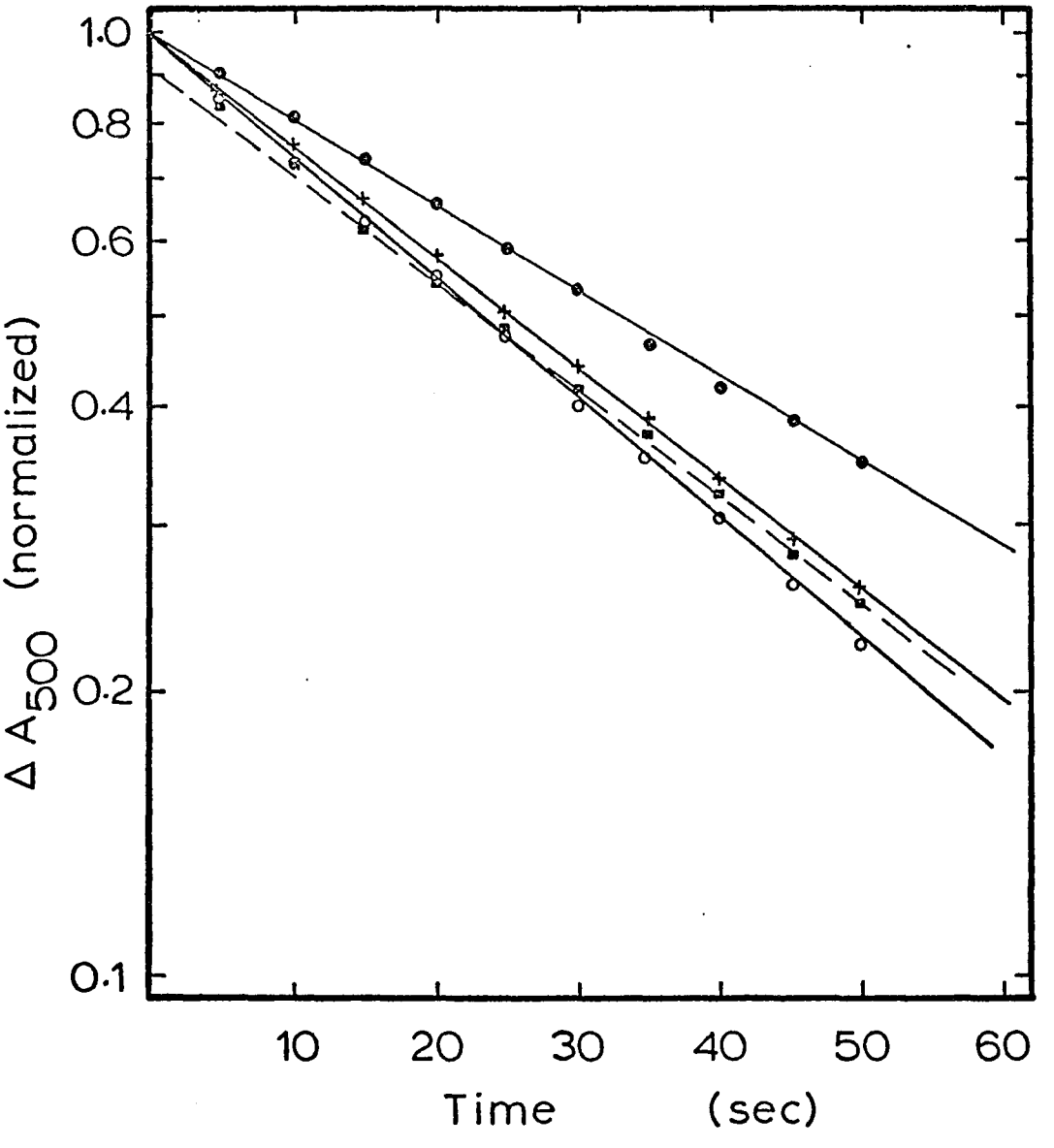


Figure 19. Effect of pH on the Pseudo First-Order Plots of Rhodopsin Recombination in Sodium Cholate.

kinetics. The observed rate constants are plotted against the reaction mixture pH in Figure 20 which shows that the recombination reaction in sodium cholate has an optimum rate between pH 6.8 and 7.2.

Second-Order Rate Determinations. The determination of the second-order rate constant for the recombination of sodium cholate solubilized rhodopsin was carried out by using the pseudo first-order analysis already described in the section on isorhodopsin recombination.

Each reaction mixture was incubated at 25 °C and consisted of 0.10 M potassium phosphate buffer, pH 7.0, containing 1 mM dithioerythritol, 2 mg/ml sodium cholate, 5-6 μM opsin (measured as rhodopsin before bleaching), and the indicated concentration of exogenous 11-cis retinal. Under these conditions the reactions gave yields near 100% of that theoretically expected and the pseudo first-order plots were typically linear through 2-4 half-times demonstrating the reactions were following pseudo first-order kinetics. Typical pseudo first-order plots for the recombination of sodium cholate solubilized rhodopsin at pH 7.0 and at different 11-cis retinal concentrations are shown in Figure 21. The second-order plot for this reaction is shown in Figure 22 and is linear up to an 11-cis retinal concentration of approximately 70-80 μM and above this concentration the value of k_{obs} appears to become independent of the

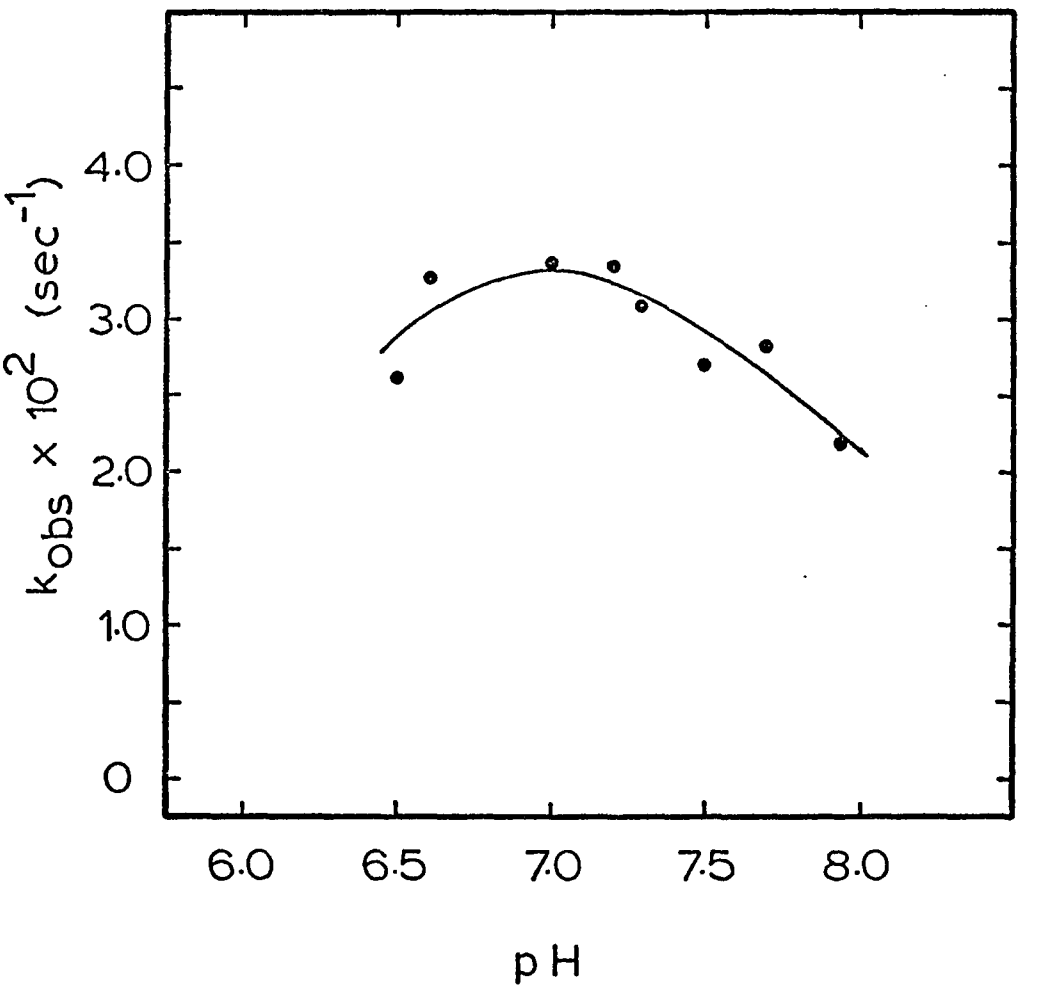


Figure 20. pH Dependence of the Rate of Rhodopsin Recombination in Sodium Cholate.

The experimental conditions were the same as in Figure 19.

Figure 21. Pseudo First-Order Plots for the Recombination of Rhodopsin in Sodium Cholate.

Solutions of rhodopsin (5-6 μM) in pH 7.0, 0.10 M potassium phosphate buffer containing 2 mg/ml sodium cholate and 1 mM dithioerythritol were bleached with white light for at least 20 minutes. The recombination of each sample was then initiated by the addition of exogenous 11-*cis* retinal to the indicated final μM concentration. Reaction mixtures were incubated at 25 °C.

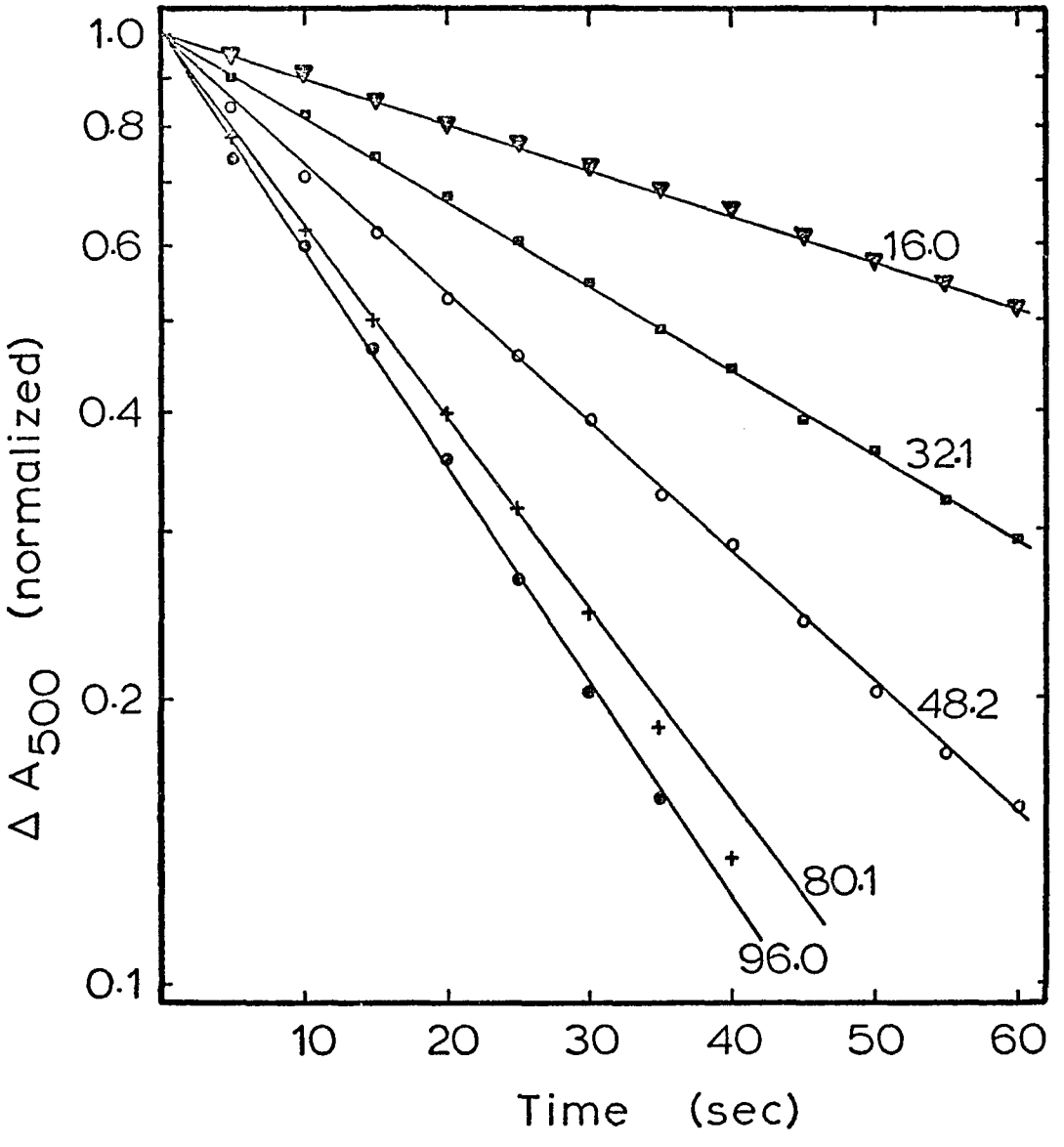


Figure 21. Pseudo First-Order Plots for the Recombination of Rhodopsin in Sodium Cholate.

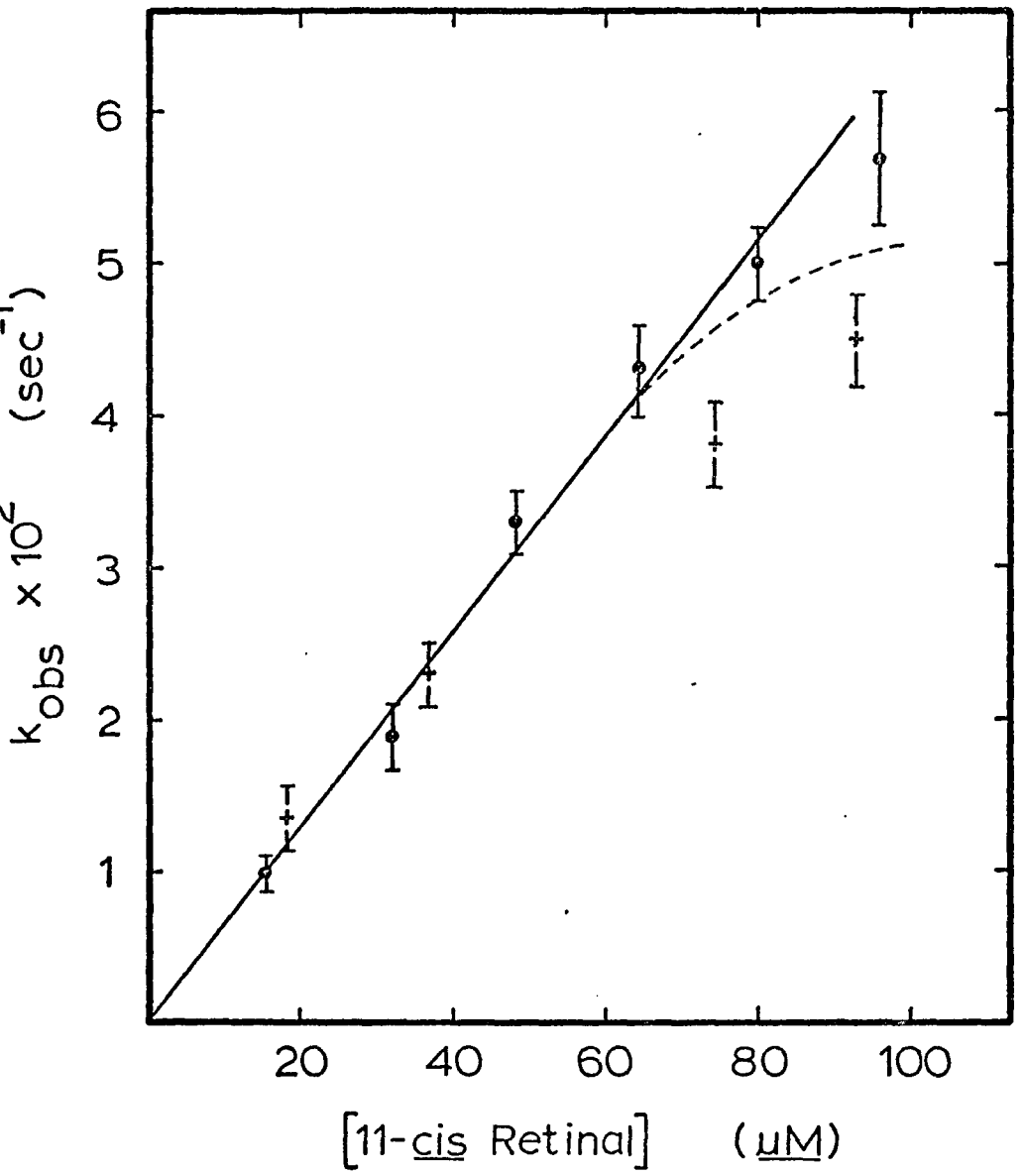


Figure 22. Second-Order Plot for the Recombination of Rhodopsin in Sodium Cholate.

The experimental conditions were the same as indicated in Figure 21. Each point and error bar represents the average and range of at least two kinetic runs. The different symbols represent rhodopsin samples from different preparations of rod outer segment membranes.

ligand concentration. The linear portion of the data in Figure 22 yields a value of 660 $\underline{M}^{-1}$ sec^{-1} for the second-order rate constant (k_1) for the rhodopsin recombination reaction in sodium cholate. These data indicate the recombination of sodium cholate solubilized rhodopsin at low concentrations of 11-cis retinal follows the kinetic mechanism given in equation 4 (with cis representing 11-cis retinal). At higher concentrations of 11-cis retinal (80-110 $\mu\underline{M}$) the data in Figure 22 suggests the reaction mechanism may have changed since the rate did not increase linearly with increasing ligand concentration.

Because the shape of the k_{obs} versus 11-cis retinal plot suggested a change in the reaction mechanism at high ligand concentration, the kinetic data was replotted in a reciprocal form according to equation 10 (with cis representing 11-cis retinal). Figure 23 illustrates the reciprocal plot for the recombination of sodium cholate solubilized rhodopsin. These kinetic data support the view that the recombination reaction (in sodium cholate) proceeds via a mechanism involving an intermediate as shown in equation 8. The reciprocal plot (Figure 23) yields values of 0.18 sec^{-1} for k_2 and 2.25 x 10^{-4} $\underline{M}$ for the quantity $(k_{-1} + k_2)/k_1$ at pH 7.0 and 25 °C. If the recombination of sodium cholate solubilized rhodopsin proceeds through an intermediate as suggested by the kinetic data, and if the rate of the initial

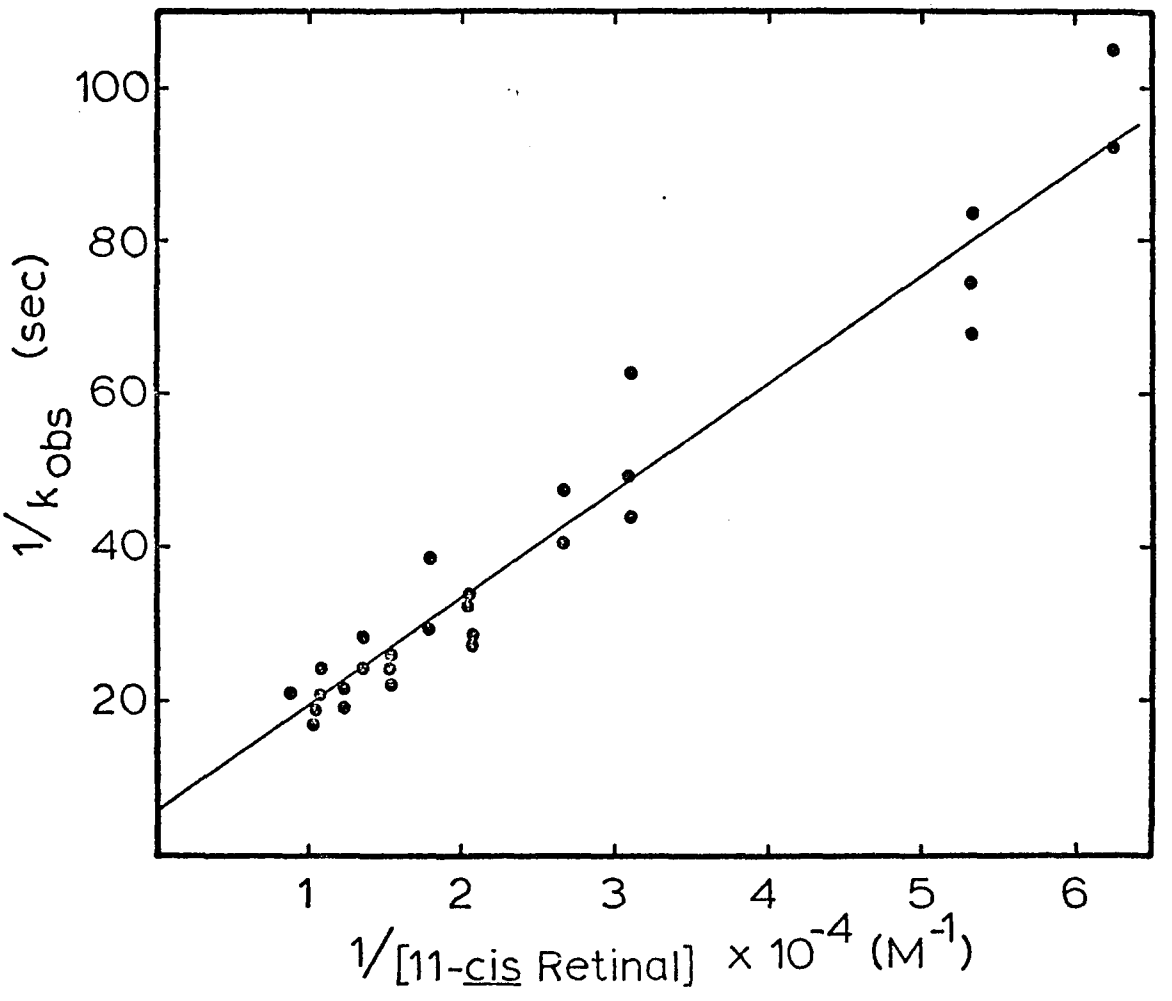


Figure 23. Reciprocal Plot for the Recombination of Rhodopsin in Sodium Cholate.

The experimental data and conditions were the same as presented in Figure 22.

part of the reaction at low ligand concentrations represents the rate of formation of the intermediate then it is possible to estimate the magnitude of the rate constant k_{-1}. If the value of k_{-1} is considered small when compared to k_2, then the quantity $(k_{-1} + k_2)/k_1$ simplifies to k_2/k_1. The value of k_2/k_1 is equal to 2.7×10^{-4} M (calculated from k_1 = 660 M^{-1} sec^{-1}, Figure 22; and k_2 = 0.18 sec^{-1}, Figure 23) which is very close to the experimental value of $(k_{-1} + k_2)/k_1$ equal to 2.25×10^{-4} M. This calculation indicates that k_{-1}, the rate constant for the breakdown of the intermediate to reactants, is small and negligible compared to k_2.

As stated earlier, the initial sodium cholate extracts of rhodopsin could be further purified by ammonium sulfate fractionation. It was of interest to determine if the removal of organic phosphorus from rhodopsin in any manner altered the kinetics of recombination. Figure 24 illustrates the results of a second-order rate determination which used ammonium sulfate fractionated rhodopsin. The rhodopsin samples used in these experiments contained 19-23 moles of organic phosphate per mole of rhodopsin as assayed by the procedure outlined in the Methods section. The reaction mixtures contained 5.5 μM rhodopsin, 0.10 M potassium phosphate buffer at pH 7.0, 1 mM dithioerythritol, 2 mg/ml sodium cholate, the indicated concentrations of 11-*cis* retinal, and were maintained at 25 °C. The pseudo first-order

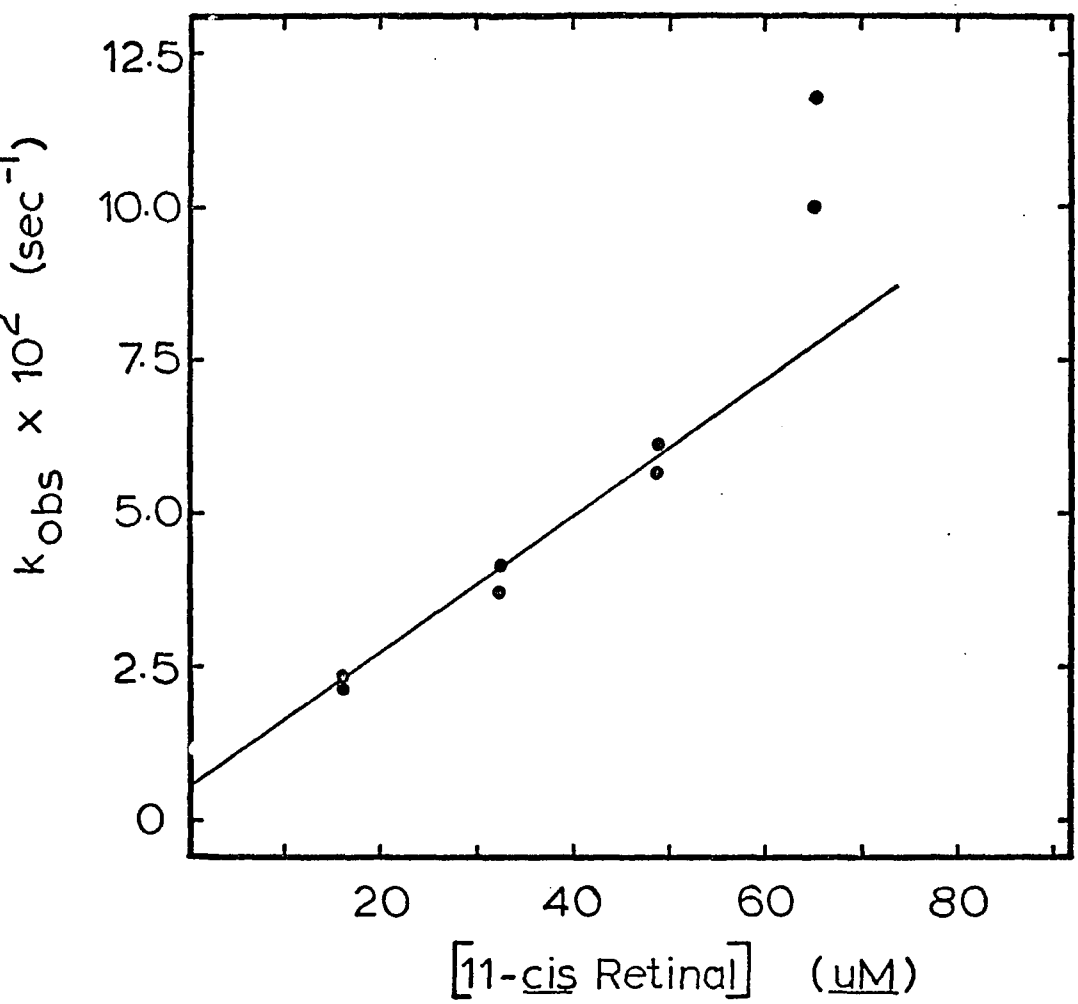


Figure 24. Second-Order Plot for the Recombination of Ammonium Sulfate Fractionated Rhodopsin.

The rhodopsin samples used in these experiments were fractionated one time with ammonium sulfate as indicated in the Methods section. The recombination experiments were carried out at pH 7.0 and 25 °C.

plots (not shown) were linear over 2-3 half-times of the reaction. The second-order rate constant evaluated from the linear portion of the curve in Figure 24 was equal to 1.11×10^3 $\underline{M}^{-1}$ sec^{-1}. The specific reason for the more than 2-fold increase in the rate constant for rhodopsin recombination after ammonium sulfate fractionation is not known at this time. The differences observed in the kinetics of recombination between the initial extracts of rhodopsin and ammonium sulfate fractionated rhodopsin samples were most likely the result of the removal of the organic phosphorus material. Since the dependence of the A_{280}/A_{500} ratios of these two types of rhodopsin preparations upon sodium cholate were quite different (Figure 14) it seems likely that the environment and probably the structure of rhodopsin is highly dependent upon the amount of associated organic phosphorus material. This point requires further work which must begin with the isolation and identification of the organic phosphorus material which was removed by the ammonium sulfate fractionation procedure.

Summary. The results of the study of the recombination reaction of sodium cholate solubilized rhodopsin are summarized in Table 4. The kinetic data obtained for the recombination of sodium cholate solubilized rhodopsin suggests the reaction may proceed through some type of intermediate. Purification of the rhodopsin preparation with ammonium

Table 4. Kinetic Properties of the Recombination of Sodium[a] Cholate Solubilized Rhodopsin.

	Rate	Extent
pH Optimum	7.0	7.0-7.4

	k_1	k_{-1}
Rate Constants for[b] Recombination (Eq. 4)	660 $\underline{M}^{-1}$ sec^{-1}	≈0
Fractionated with $(NH_4)_2SO_4$	1110 $\underline{M}^{-1}$ sec^{-1}	-

	k_2	$(k_{-1} + k_2) / k_1$
Rate Constants for[c] Recombination (Eq. 8)	0.18 sec^{-1}	2.25×10^{-4} $\underline{M}$

a. All kinetic experiments were at 25 °C. The initial extracts of rhodopsin were used without further purification unless otherwise stated. Rhodopsin samples contained 0.10 $\underline{M}$ potassium phosphate buffer, pH 7.0, 1 $\underline{mM}$ dithioerythritol, and 2 mg/ml sodium cholate.

b. Rate data calculated according to the reaction mechanism shown in equation 4.

c. Rate data calculated according to the reaction mechanism shown in equation 8.

sulfate fractionation resulted in a significant increase of the rate of recombination.

Isolated Rod Outer Segment Membranes

Lyophilized rod outer segment membranes were prepared according to the procedures described in Method B of the Methods section and were used as the basic starting material in all of the studies reported here.

Preparation and Properties

A typical difference spectrum of the isolated rod outer segment membranes used in this study is shown in Figure 25. The dashed line is the baseline absorbance of equal aliquots of the isolated membranes in 0.10 M potassium phosphate buffer, pH 7.0, containing 1 mM dithioerythritol and 20 mM hydroxylamine. The solid line (Figure 25) represents the difference spectrum after the reference sample was bleached with white light for 15 minutes. In the studies which follow the isolated rod outer segment membranes were suspended at a rhodopsin concentration of 5-6 μM as indicated in the Methods section. The lyophilized rod outer segment membranes used in these experiments, upon bleaching in the presence of hydroxylamine, typically yielded a ΔA_{500} of 0.20-0.22 when suspended at a concentration of 1 mg dry material per ml of solution.

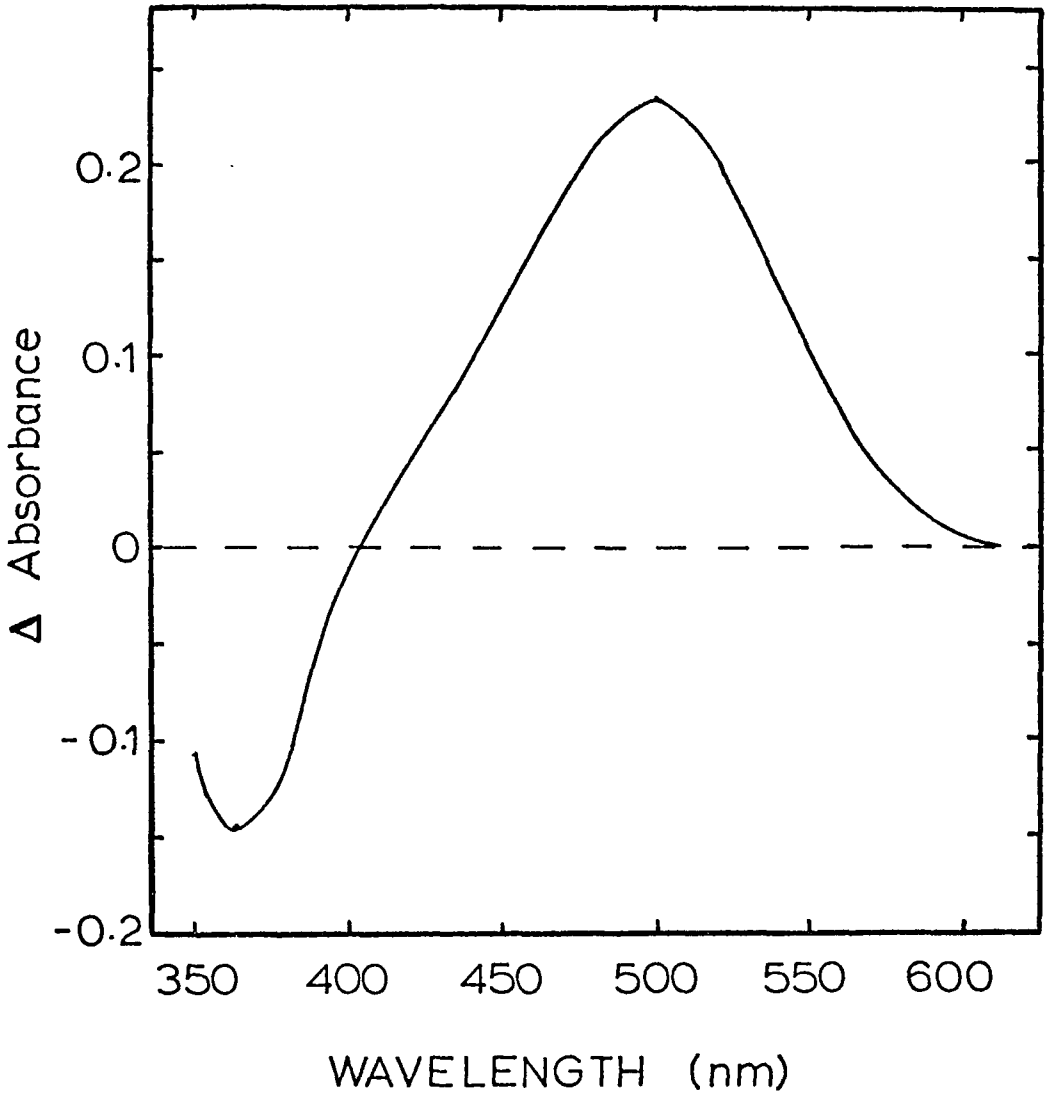


Figure 25. Difference Spectrum of Isolated Rod Outer Segment Membranes.

Equilibrium Properties of Rhodopsin Recombination

The recombination of rhodopsin as present in the isolated rod outer segment membranes was carried out as described in the Methods section and as indicated in the following experiments.

The observed percent recombination in isolated rod outer segment membranes at pH 7.0 was typically in the range of 90-95% of that theoretically expected. The dependence of the overall extent of rhodopsin recombination in the isolated membranes upon pH is shown in Figure 26 and indicates the pH optimum for the extent of reaction is very broad and lies between pH 5.5 and 7.2. The data illustrated in Figure 26 were determined using 3 separate rod outer segment preparations.

Kinetic Properties of Rhodopsin Recombination

The kinetic properties of the rhodopsin recombination reaction in isolated rod outer segment membranes was investigated by the same methodology employed for the solubilized protein. The results of these experiments are presented in the following sections.

pH Dependence. The effect of pH on the rate of the recombination reaction was assessed by measuring the pseudo first-order rate constant for the reaction as a function of pH. Representative kinetic plots (ln ΔA_{500} versus time) of these experiments are presented in Figure 27. These plots

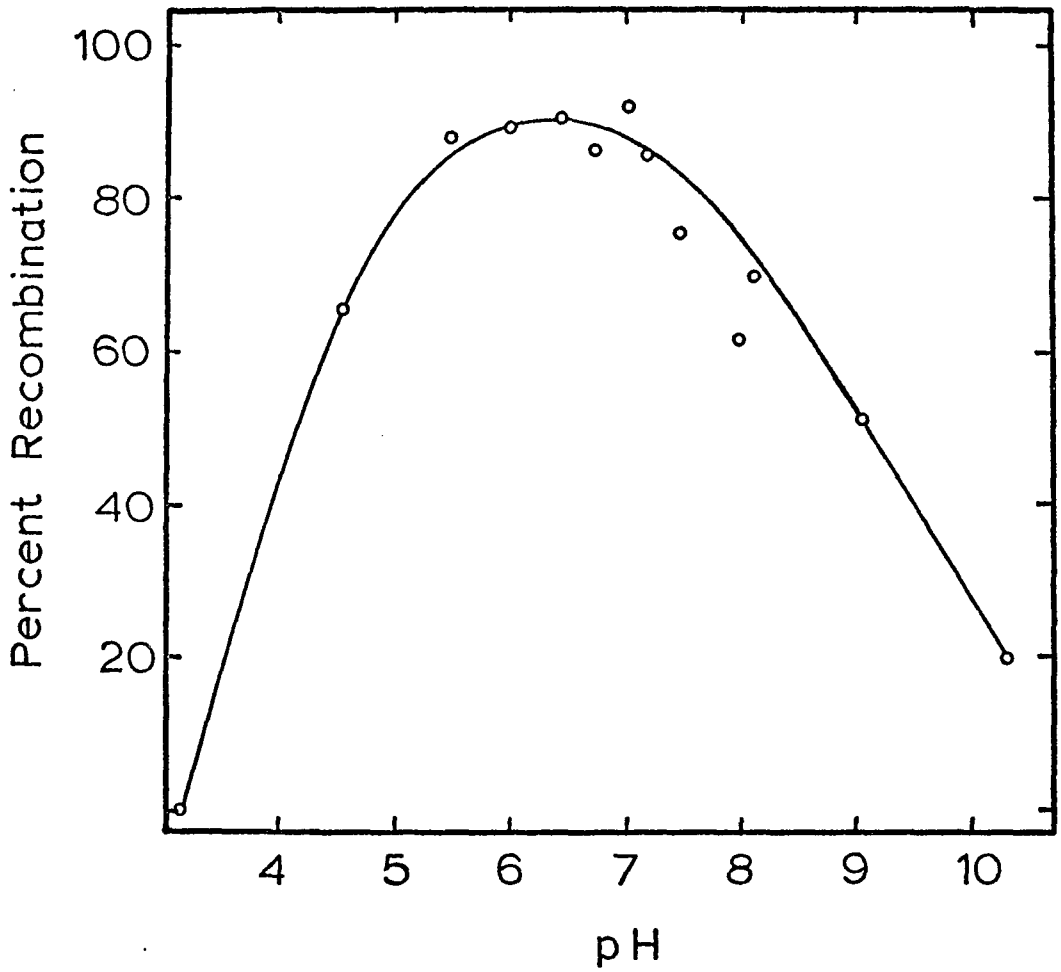


Figure 26. pH Dependence of the Extent of Rhodopsin Recombination in Isolated Rod Outer Segment Membranes.

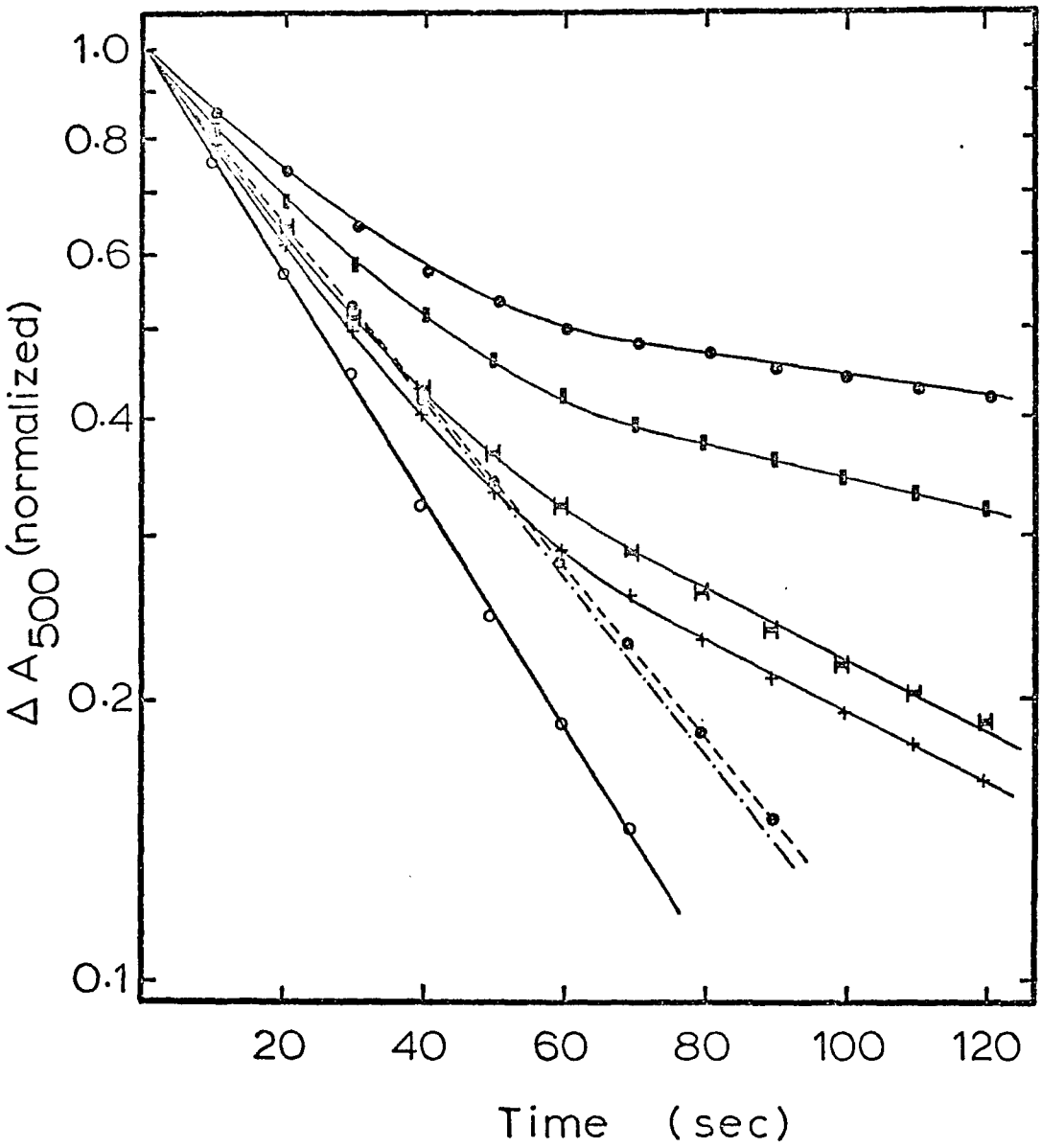


Figure 27. Effect of pH on the Pseudo First-Order Plots of Rhodopsin Recombination in Isolated Rod Outer Segment Membranes.

pH 4.6 (—●—); pH 6.0 (—+—); pH 6.45 (---●--);
pH 7.0 (—○—); pH 7.4 (-·-·-·); pH 8.0 (—▮—);
pH 8.5 (—■—).

were linear between pH 6.45 and 7.4 indicating that the reaction followed pseudo first-order kinetics while at higher or lower pH values the plots were biphasic. The biphasic curves in Figure 27 were resolved into a fast phase and a slow phase. The fast phase of the reaction below pH 6.45 was essentially independent of pH and had values of 0.045-0.05 sec^{-1}. The fast phase of the reaction at pH values greater than 7.4 also appeared to be independent of pH and had values of 0.04-0.06 sec^{-1}. At those pH values where the kinetic plots were biphasic, the fast phase represented approximately 40-60% of the total reaction. There was no obvious correlation between pH and the percentage of the fast phase. The dependence of the observed rate constant (k_{obs}) upon pH is illustrated in Figure 28 and indicates the reaction had a sharp pH optimum at pH 7.0. In Figure 28 the values of k_{obs} were obtained from the linear ($\ln \Delta A_{500}$ versus time) plots for the pH range 6.45-7.4 and from the slow phases of these plots at pH values lower than 6.45 or higher than 7.4.

Second-Order Rate Determination. The determination of the second-order rate constant for rhodopsin recombination in isolated rod outer segment membranes was carried out using a pseudo first-order analysis already described in earlier sections. In the experiments described below each reaction mixture was maintained at 25 °C and contained 0.10 M potassium phosphate buffer (pH 7.0), 1 mM dithioerythritol, rod outer

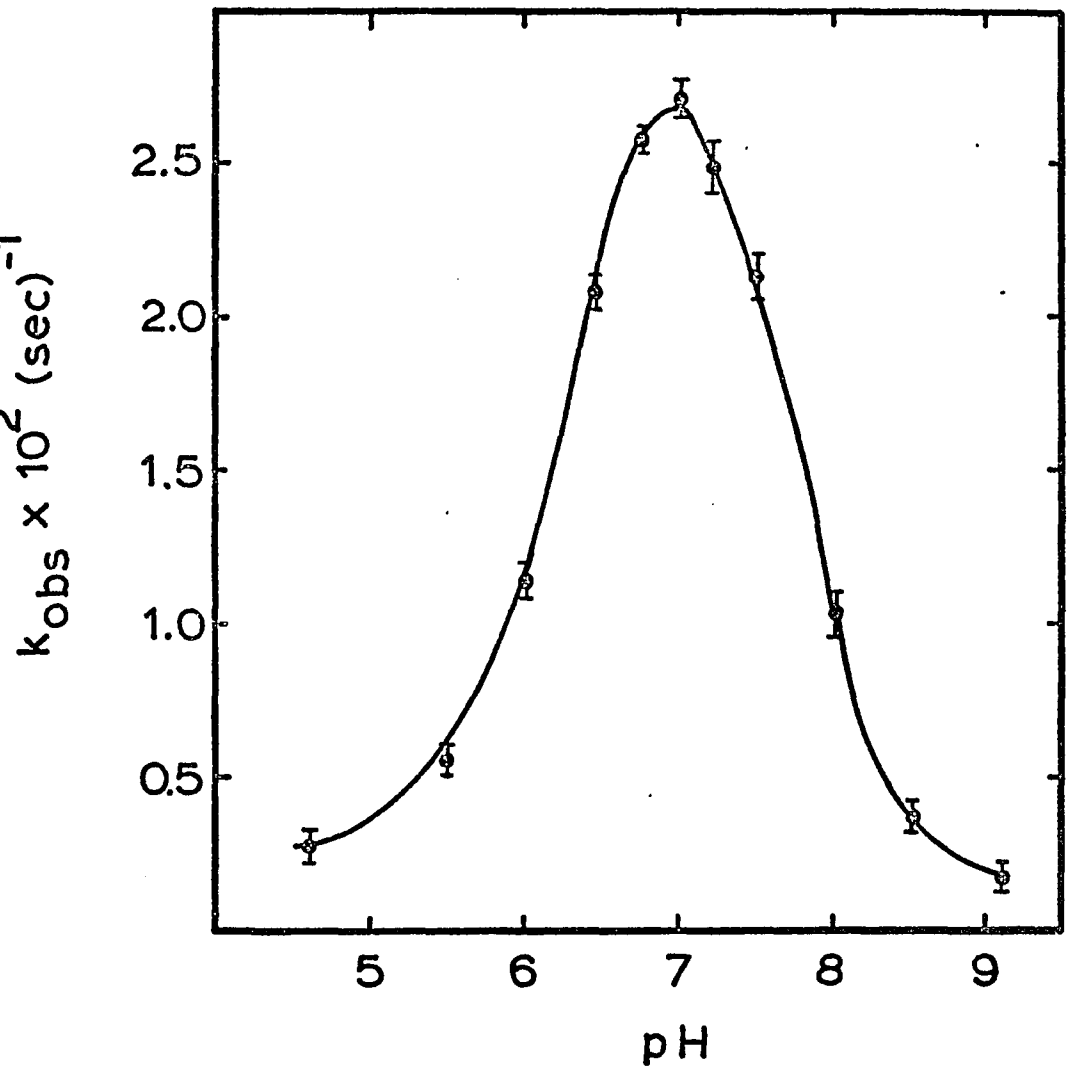


Figure 28. pH Dependence of the Rate of Rhodopsin Recombination in Isolated Rod Outer Segments.

The experimental data and conditions were the same as presented in Figure 27.

segment membranes equivalent to a rhodopsin concentration of 5-6 μM, and the indicated concentrations of 11-cis retinal. Typical pseudo first-order plots (pH 7.0) for the reaction are illustrated in Figure 29 and are linear over 2-3 half-times of the reaction.

The second-order plot for the recombination of rhodopsin (in isolated rod outer segment membranes) is presented in Figure 30 and is linear up to an 11-cis retinal concentration of approximately 50 μM. At concentrations of 11-cis retinal above 50 μM the value of k_{obs} appeared to become independent of ligand concentration. The data in Figure 30 suggested the reaction was possibly proceeding through an intermediate as shown in equation 8. In order to test this possibility the data in Figure 30 were replotted in a reciprocal form (Figure 31) according to the mechanism involving an intermediate. As can be seen in Figure 31, the experimental data forms a linear plot and supports the idea that the recombination reaction (in isolated rod outer segment membranes) proceeds via a mechanism involving an intermediate as shown in equation 8. The reciprocal plot (Figure 31) yields values of 5.5×10^{-2} sec^{-1} for k_2 and 5.9×10^{-5} M for the quantity $(k_{-1} + k_2)/k_1$ at pH 7.0 and 25 °C.

The observed layover at high 11-cis retinal concentrations of the kinetic data in Figure 30 does not appear to be due to a lack of solubility of the exogenous 11-cis retinal. This important point is demonstrated by the

Figure 29. Pseudo First-Order Plots for the Recombination of Rhodopsin in Isolated Rod Outer Segment Membranes.

Concentrations of 11-cis retinal were as follows: 17.5 μM (—●—); 34.9 μM (—○—); 52.3 μM (—■—); 87.1 μM (—□—). In these experiments isolated rod outer segment membranes equivalent to a rhodopsin concentration of 5-6 uM were suspended in 0.10 M potassium phosphate buffer, pH 7.0, containing 1 mM dithioerythritol. After bleaching with white light for at least 20 minutes the recombination of each sample was initiated by the addition of 11-cis retinal to the final concentration indicated above. Reaction mixtures were maintained at 25 °C.

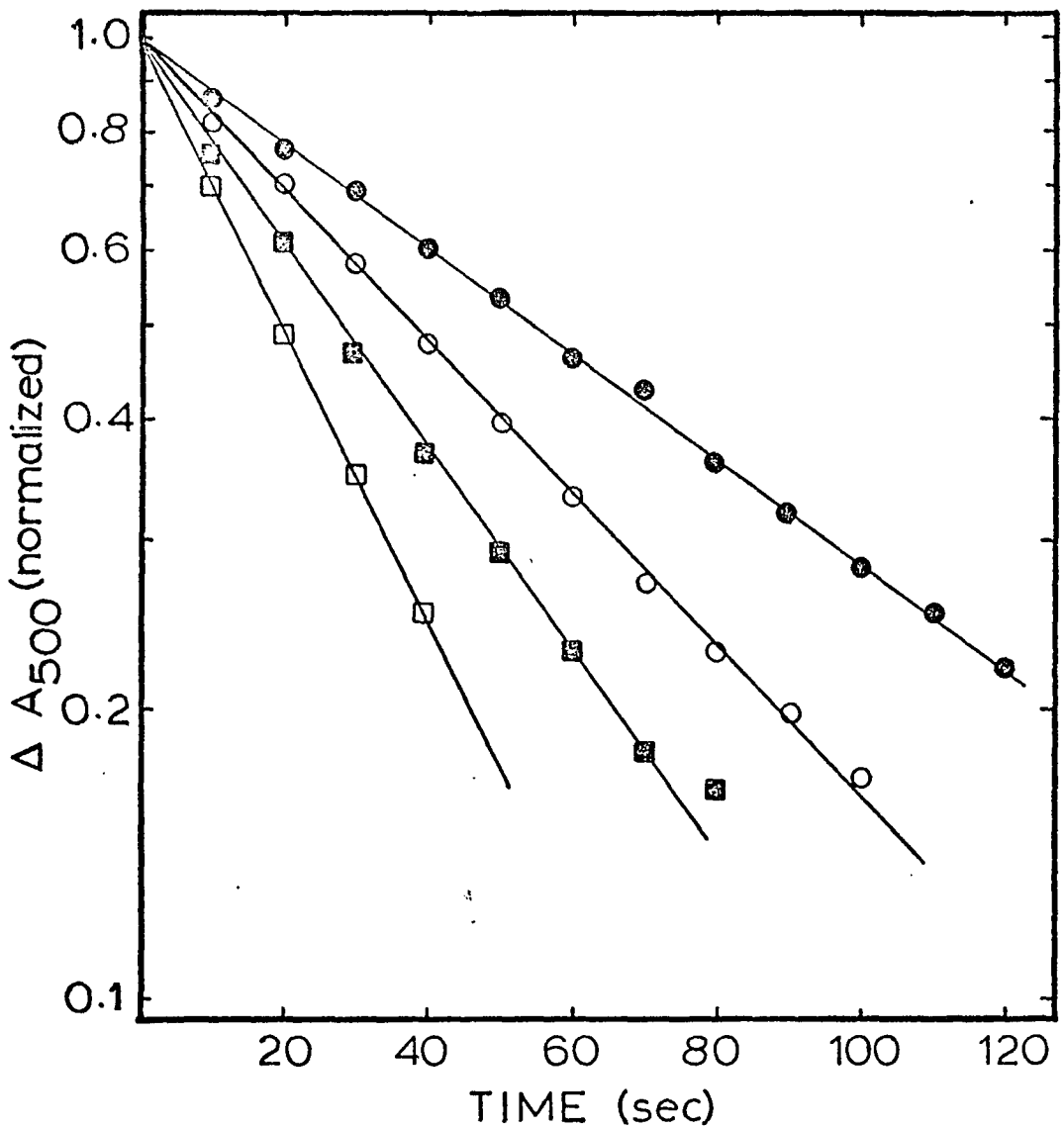


Figure 29. Pseudo First-Order Plots for the Recombination of Rhodopsin in Isolated Rod Outer Segment Membranes.

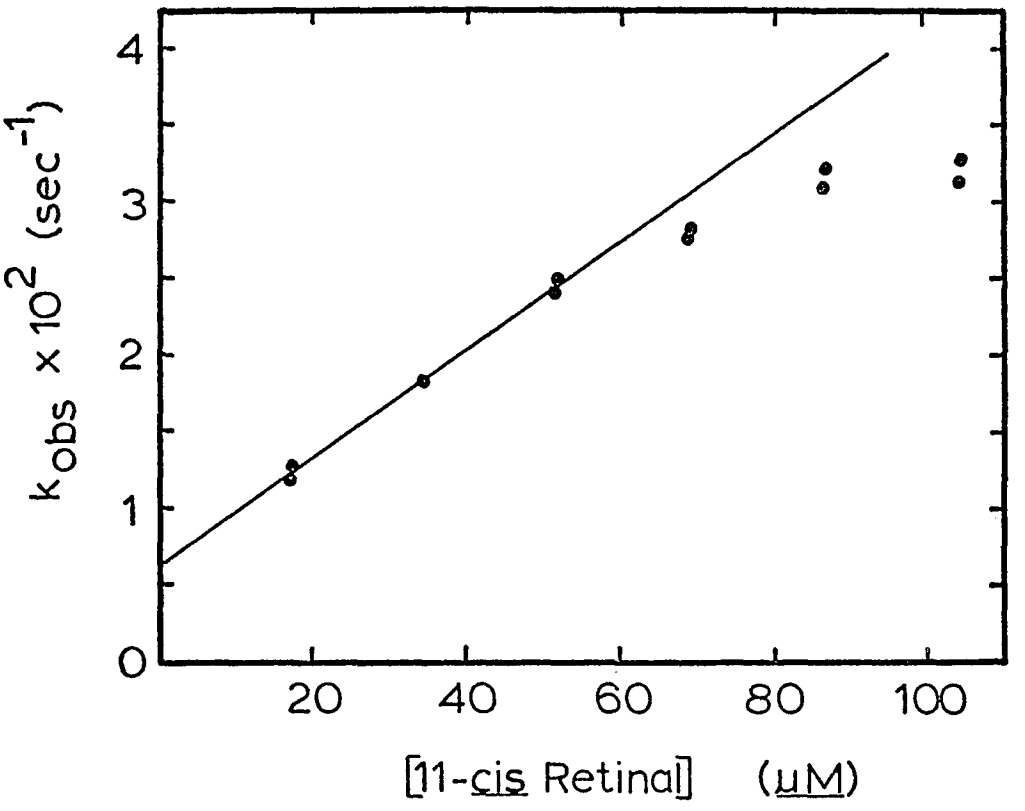


Figure 30. Second-Order Plot for the Recombination of Rhodopsin in Isolated Rod Outer Segment Membranes.

The experimental data and conditions were the same as indicated in Figure 29.

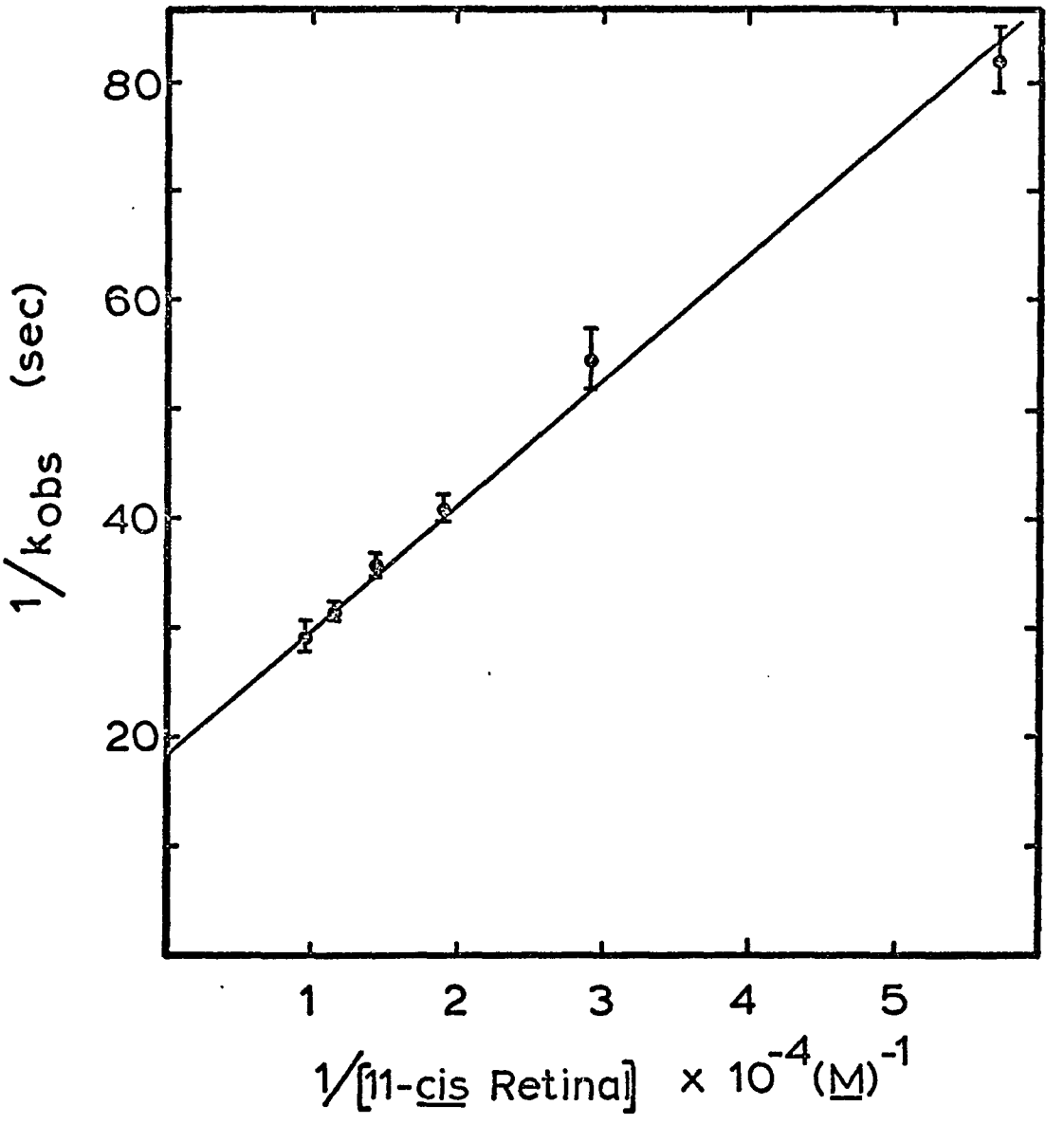


Figure 31. Reciprocal Plot for the Recombination of Rhodopsin in Isolated Rod Outer Segment Membranes.

The experimental data and conditions were the same as indicated in Figure 29.

experiment presented in Figure 32. Isolated rod outer segment membranes were homogenized and suspended in 0.10 M potassium phosphate buffer, pH 7.0, containing 1 mM dithioerythritol. The isolated rod outer segment membranes were suspended at a rhodopsin concentration equivalent to 6 μM and were not bleached or photolyzed during the course of the experiment. The baseline absorbance (difference spectrum) of the suspended membranes was determined from 625 to 350 nm and then increasing concentrations of exogenous 11-cis retinal were added and the difference spectrum recorded after each addition. Figure 32 demonstrates that the absorbance at 380 nm (the absorbance maximum of the added 11-cis retinal in the presence of the rod outer segment membranes) increased linearly with increasing concentrations of 11-cis retinal up to a concentration of 130 μM. These results along with the observed pH dependence of the rate of recombination (Figure 28) strongly suggest that neither the rate of transport of 11-cis retinal to the apoprotein opsin nor some limited solubility of 11-cis retinal in the presence of the membranes is a significant problem or a limiting factor in these studies. It is possible that some type of compartmentation or partioning of 11-cis retinal is taking place in the rod outer segment membranes but there is no positive evidence that this type of phenomena actually is occurring.

Figure 32. Solubility of Exogenous 11-cis Retinal in the Presence of Rod Outer Segment Membranes.

Rod outer segment membranes at a rhodopsin concentration equivalent to 6 uM were suspended in pH 7.0, 0.10 M potassium phosphate buffer containing 1 mM dithioerythritol. The increase in absorbance at 380 nm after the addition of increasing concentrations of 11-cis retinal was determined as described in the text. Experimental points represented by the closed circles (—•—) were determined with 1 cm pathlength cells and the experimental points represented by the crosses (—+—) were determined with 2 mm pathlength cells. For those experiments which utilized 2 mm pathlength cells the observed absorbance was multiplied by 5 to yield the absorbance which would have been observed with 1 cm pathlength cells.

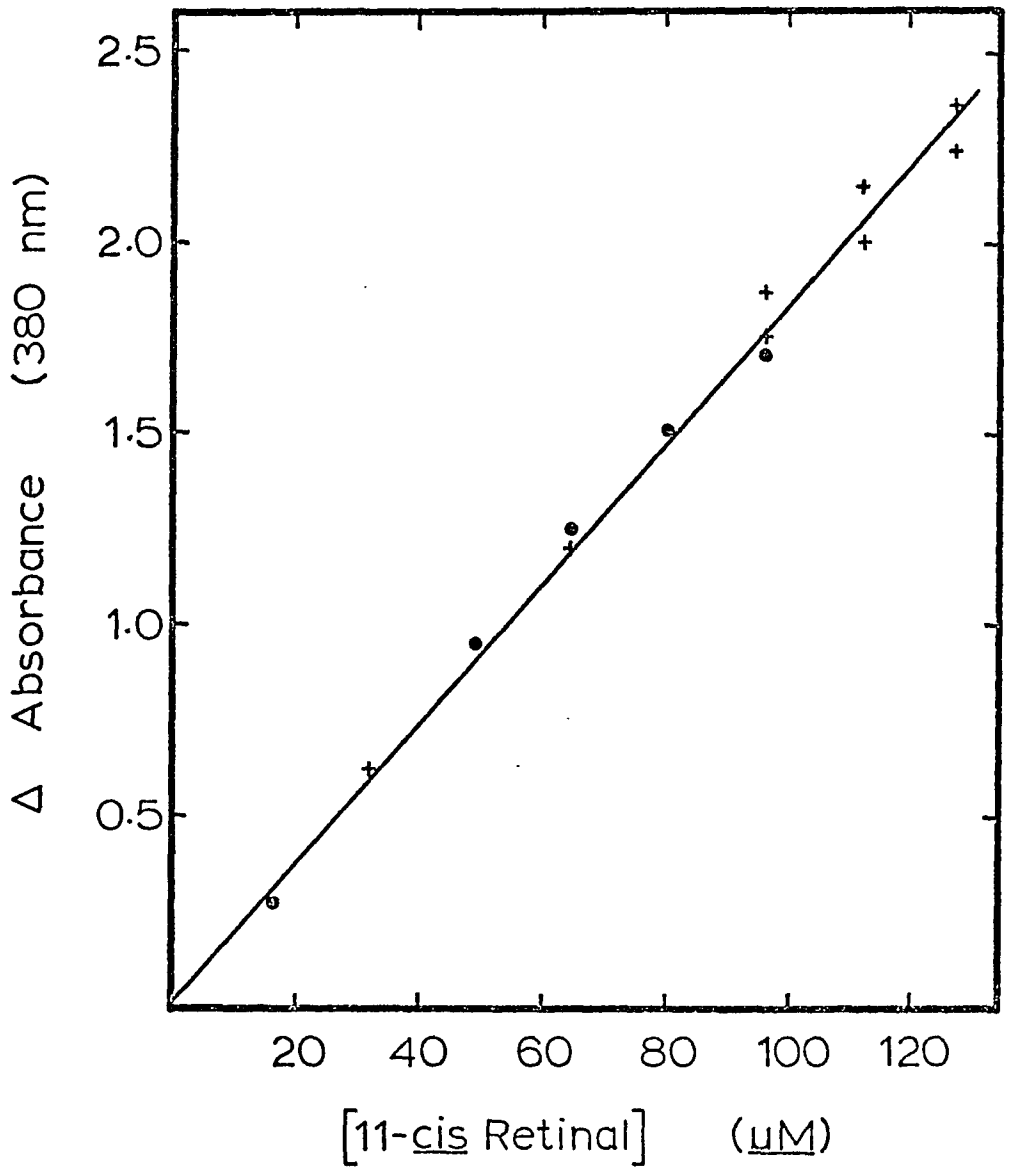


Figure 32. Solubility of Exogenous 11-cis Retinal in the Presence of Rod Outer Segment Membranes.

Summary. The results of the kinetic studies of the recombination reaction of membrane bound rhodopsin are summarized below and in Table 5. The kinetic data obtained with isolated rod outer segment membranes was consistent with the idea that the reaction proceeds through a mechanism which involves the formation of a complex or an intermediate. It was also found that at pH values below 6.45 or above 7.4 the kinetic plots (ln ΔA_{500} versus time, Figure 27) were biphasic and appeared to be composed of a fast pH independent phase and a slower pH dependent phase.

Table 5. Kinetic Properties of the Recombination of[a] Rhodopsin in Isolated Rod Outer Segment Membranes.

	Extent	Rate
pH Optimum	5.5-7.2	7.0
	k_2	$(k_{-1} + k_2) / k_1$
Rate Constants for[b] Recombination (Eq. 8)	0.055 sec^{-1}	5.9×10^{-5} M

a. Kinetic experiments for the determination of the pH optimum were carried out at 28 °C while all other kinetic experiments were at 25 °C.

b. Rate data calculated according to the reaction mechanism shown in equation 8.

CHAPTER 4

DISCUSSION AND CONCLUSIONS

The results of this work and the conclusions reached from these results will be discussed in the following sections. The properties of sodium cholate solubilized rhodopsin and the kinetic results for the recombination by rod outer segment membranes, sodium cholate solubilized rhodopsin, and digitonin solubilized rhodopsin will be discussed and a mechanism proposed to explain these results.

It is important to point out that all work on rhodopsin, reported here as well as elsewhere, is complicated by two factors. One of these is that rhodopsin is tightly bound to or in the rod outer segment membrane. The isolated protein rhodopsin is also completely insoluble in aqueous solution. These problems have to some extent been overcome by the use of various surfactants or detergents which render rhodopsin soluble. The specific perturbations (if any) of rhodopsin induced by these surfactants or by solubilization are not easily determined. The second and more technical limitation is the light sensitivity of rhodopsin. Rhodopsin can be handled under dim red light ($\lambda > 600$ nm), however, manipulations which require good visual accuracy are difficult under these conditions. The above limitations make quantitative

studies on rhodopsin difficult and the errors associated with these studies are greater than would be expected in studies on proteins which are soluble and not photosensitive.

The use of detergents or surfactants to solubilize rhodopsin is justifiable from the point of view that the solubilized protein is more easily studied by the various physical and chemical probes currently available. The effects these detergents may have on the structure and function of rhodopsin are not entirely known but have been commonly assessed by two properties of rhodopsin. These properties, the characteristic absorption spectrum and the recombination capacity of photolyzed rhodopsin (opsin), are to differing degrees sensitive to the detergent used for solubilizing rhodopsin. Since the only known functional aspect of rhodopsin involves the recombination of the photolyzed protein (opsin) with 11-cis retinal, this reaction must naturally serve as an indicator of the native structure of rhodopsin. Recent investigations on rhodopsin have utilized this reaction in determining which preparations of the visual pigment were active and hence "native" and what types of perturbations or modifications were allowable without destroying this activity. These investigations were largely qualitative in that only the relative retention or loss of the recombination activity was determined. One of the original intentions of this work was to put this functional aspect of rhodopsin on a more quantitative basis through

the use of a kinetic analysis of the recombination reaction. It was felt that this type of analysis should be sensitive to minor changes in the structure of rhodopsin and would also reveal those aspects of rhodopsin recombination which were common to each type of preparation. Through the analysis of these common features of the recombination reaction, it was the goal of this work to derive a plausible mechanism for this reaction.

Sodium Cholate Solubilized Rhodopsin

The characterization of sodium cholate solubilized rhodopsin is considered to be one of the more significant results obtained in this work since it has not been previously described in the literature. This form of rhodopsin is also fully capable of recombination with 11-cis retinal under the conditions defined in this work.

It has already been stated earlier that only two solubilized forms of rhodopsin were previously known to retain their recombination capacity. One of these forms, digitonin solubilized rhodopsin, has been known for a number of years (Tansley 1931; Hubbard and Wald 1952) and essentially all of the work involving rhodopsin recombination studies have utilized this preparation. It was through the use of this preparation that Hubbard and Wald (1952) demonstrated 11-cis retinal was the naturally occurring chromophore of rhodopsin.

Digitonin solubilized rhodopsin was also employed in studies on the pH dependence of recombination (Radding and Wald 1956) and in studies on the sulfhydryl group requirements for recombination (Wald and Brown 1952; DeGrip, Van De Laar et al. 1973; Zorn 1974). Tween-80 solubilized rhodopsin also retains its recombination capacity although at a lower level than digitonin solubilized rhodopsin (Zorn and Futterman 1973). Both digitonin and Tween-80 have difficulties associated with their use as solubilizing agents for rhodopsin. The main problems with digitonin have been its insolubility, the lack of good analytical methods to determine the surfactant concentration, and the difficulties in purifying rhodopsin in high yields once solubilized in this surfactant. The difficulties associated with Tween-80 include the following: The surfactant has a high absorbance in the ultra-violet, high concentrations (20%) were required to extract rhodopsin from the rod outer segment membranes and the resulting rhodopsin solutions had low recombination yields (Zorn and Futterman 1973).

The use of sodium cholate to solubilize rhodopsin from rod outer segment membranes, as described in this work, eliminates many of the above problems. Sodium cholate is inexpensive, easily purified, rapidly removed from rhodopsin solutions by dialysis, and accurately quantitated by the technique of Boyd et al. (1966); factors which greatly

increase its usefulness as a solubilizing agent for rhodopsin. A large amount of information is also available regarding the physical-chemical properties of sodium cholate including characterizations of its micelle size and behavior under a variety of conditions (Small 1968; Fontell 1971). Sodium cholate extracted rhodopsin from lyophilized rod outer segment membranes in high yields (0.5-0.7 μmoles of rhodopsin per 100 bovine retinas) and at a relatively low surfactant concentration (20 mg/ml or 2% wt/wt). The recombination yields of the resulting rhodopsin solutions were high (80-100%, Figures 17-18) under well defined conditions of pH and surfactant concentrations (pH 7.0-7.5, Figure 18; 1-5 mg/ml, Figure 17). Solutions of rhodopsin in sodium cholate were rapidly and easily fractionated with ammonium sulfate. This fractionation procedure resulted in considerable purification of rhodopsin with respect to organic phosphate compounds and reduced the phosphate to rhodopsin molar ratio from 60:1 to 6:1. The recombination of sodium cholate solubilized rhodopsin was found to be dependent upon the concentration of sodium cholate (Figure 17) and varied from 80-100% at 1-5 mg/ml sodium cholate to 5-20% at 14-20 mg/ml sodium cholate.

The sulfhydryl reactivity of sodium cholate solubilized rhodopsin was found to be quite different than that reported for other detergent solutions of rhodopsin. The

results obtained for sodium cholate solubilized rhodopsin (20 mg/ml; Table 3) are in good agreement with determinations made on digitonin solubilized rhodopsin where approximately 2 sulfhydryl groups are available in the dark and upon bleaching 2 additional sulfhydryl groups are exposed (DeGrip, Van De Laar et al. 1973; Kimble and Ostroy 1973). Data obtained using digitonin solubilized rhodopsin indicated the total number of reactive sulfhydryl groups (dark plus bleached) was the same whether the sulfhydryl reagent was added before or after bleaching (Kimble and Ostroy 1973). This was not the case with sodium cholate solubilized rhodopsin where the total number of titratable sulfhydryl groups were dependent upon whether the sulfhydryl reagent was added before or after bleaching. The data in Table 3 suggests that upon bleaching sodium cholate (20 mg/ml) solubilized rhodopsin goes through a transition where additional sulfhydryl groups are temporarily exposed. At low sodium cholate concentration (2 mg/ml) where the photolyzed rhodopsin solutions undergo their greatest recombination, no additional sulfhydryl groups are exposed upon bleaching (Table 3). DeGrip, Van De Laar et al. (1973) have reported similar results in their study on the sulfhydryl reactivity of isolated rod outer segment membranes where 2 sulfhydryl groups were available in the dark and no additional sulfhydryl groups became available after photolysis. The important point to be made from these

studies on the sulfhydryl groups of rhodopsin and opsin is that the availability of these groups is dependent upon the concentration of sodium cholate present in the reaction mixtures.

The high recombination capacity of sodium cholate solubilized rhodopsin suggests that solutions of rhodopsin containing 1-4 mg/ml sodium cholate are in a highly native form at least with respect to the recombination activity (Figure 17). The nearly complete lack of recombination capacity of rhodopsin solutions containing 20 mg/ml sodium cholate must be a reflection of an induced conformational state of rhodopsin which is non-regenerable. The number of available sulfhydryl groups in rhodopsin solutions, both dark and light exposed, was found to increase with increasing sodium cholate concentration (Figure 16, Table 3). Since the availability of specific amino acid residues to chemical modification is dependent upon their particular environment, the above results suggest that conformational differences do exist between those forms of rhodopsin which are capable of recombination and those which are not. It is important to note that these changes in the recombination capacity and sulfhydryl reactivity of sodium cholate solubilized rhodopsin were apparently the direct result of changing only the surfactant concentration. Sodium cholate was also most effective at extracting and solubilizing

rhodopsin when the surfactant concentration was equal to or above its reported critical micelle concentration range of 5-19 mg/ml (Fontell 1971). The data illustrated in Figure 17 demonstrated that bleached sodium cholate solubilized rhodopsin was most active in the recombination reaction with 11-cis retinal when the concentration of sodium cholate was below its reported critical micelle concentration range. These data indicate that the solution behavior of sodium cholate plays an extremely important role in determining the activity and hence conformation of rhodopsin and/or opsin.

Circular dichroism studies on sodium cholate solubilized rhodopsin were carried out with the expectation that the different conformations (active and inactive at low and high sodium cholate concentrations) of rhodopsin would be characterized by different spectra. The experimental results, however, did not demonstrate any significant differences in the 280-285 nm region for either bleached digitonin or sodium cholate solubilized rhodopsin. These bands were nearly totally lost upon bleaching rhodopsin solutions containing from 6 to 20 mg/ml sodium cholate or 2% digitonin. Bleached rhodopsin solutions which contain 2% digitonin or 6 mg/ml sodium cholate were at least 70% active in recombination while bleached rhodopsin solutions containing 20 mg/ml sodium cholate were essentially inactive.

The similarities observed in the circular dichroism spectra of opsin samples in both digitonin and sodium cholate indicate the conformational differences between active and inactive opsin were not detectable by this technique. These results are of further interest since the circular dichroism bands at 280-285 nm are due primarily to tyrosine and/or tryptophan residues in an asymmetric environment. The disappearance of these bands upon the bleaching of rhodopsin indicates that the groups which gave rise to the circular dichroism bands have moved from an asymmetric environment to a symmetric environment. Although it cannot be stated with absolute certainty, this change in the environment of these groups may represent their movement from an internal and solvent shielded environment to an external and more solvent accessible position.

Sodium cholate does have some limitations or disadvantages in its use as a solubilizing agent for rhodopsin. The most severe limitation is the fact that cholic acid has a pK of 6.4 and at pH values below about 6.5 the surfactant is essentially insoluble thus making experiments difficult, if not impossible, to do in the acid pH range. Rhodopsin which was not fractionated with ammonium sulfate to reduce the amount of associated organic phosphorus compounds was not truly soluble at low sodium cholate concentrations. A single ammonium sulfate fractionation, which reduced the

organic phosphous to rhodopsin molar ratio to about 16:1, rendered the sodium cholate-rhodopsin mixtures partially soluble as judged by high speed centrifugation. The reason for the above observations is not known at this time but most likely is related to the effect of the organic phosphate compounds upon the interactions between rhodopsin and sodium cholate. It is entirely possible that these observations could be used to an advantage in studies on the processes involved in the solubilization of rhodopsin by sodium cholate.

Proposed Mechanism and Kinetic Results

In this section a reaction mechanism is proposed to account for the kinetic results obtained for the recombination reaction of rhodopsin. The mechanism proposed is based on the known chemical and physical properties of rhodopsin, the aldimine linkage between the apoprotein opsin and 11-*cis* retinal (Bownds 1967; Fager et al. 1972), the known reaction mechanisms for the reactions between simple amines and aldehydes in model systems (Jencks 1959), and the kinetic results obtained with isolated rod outer segments in this work. The kinetic results obtained with sodium cholate solubilized rhodopsin are also considered, but done so with the realization that the surfactant could have a significant effect on the stability and nativeness of rhodopsin. The reaction mechanism to be described is consistent with the

known properties of rhodopsin and is supported by the kinetic studies reported in this work but like all reaction mechanisms it must be considered as a minimal description of the reaction.

The mechanism proposed to describe the recombination reaction between 11-cis retinal and opsin which yields rhodopsin is illustrated schematically in Figure 33. The proposed reaction mechanism involves the nucleophilic attack of an ϵ-NH_2 group (from an opsin lysine residue) on the carbonyl carbon atom of 11-cis retinal to form the intermediate addition compound (V). The dehydration of this intermediate compound, facilitated by the donation of a proton from the group $-BH^+$, results in the formation of the protonated aldimine product (VI) which is rhodopsin. The structure depicted by (IV) is meant to represent the apoprotein opsin in a conformation where the groups -B: and $-NH_3^+$ are in a hydrophobic environment which is the retinal binding site. This conformation of opsin (IV) is referred to as the closed conformation of opsin and is the form of opsin which is active in binding the ligand 11-cis retinal. This closed form of opsin (IV) is shown to be in a pH dependent equilibrium with a form of opsin which is not active in binding the ligand and is referred to as the more open form of opsin which is depicted by structure (III). It is important to keep in mind that open and closed are relative terms and do not indicate either totally unfolded or totally

Figure 33. Proposed Mechanism for the Recombination of Rhodopsin.

R-CHO represents 11-cis retinal and structure (VI) represents the product rhodopsin. The group -B: is postulated to be a histidine residue of opsin.

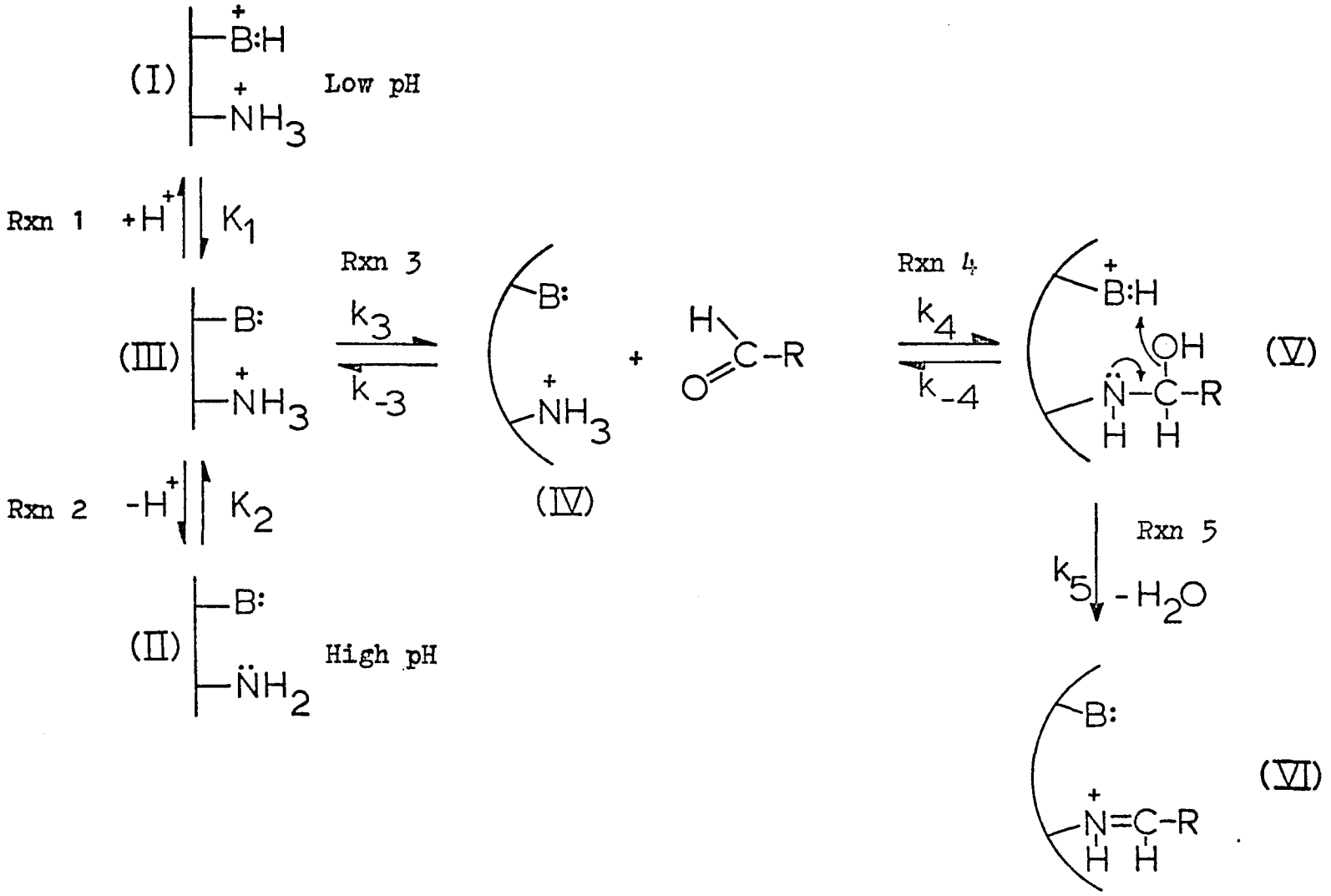


Figure 33. Proposed Mechanism for the Recombination of Rhodopsin.

compacted structures. At low pH the group -B: becomes protonated (pK_1) which results in another form of opsin which is not active in binding the ligand 11-cis retinal and this inactive form is represented by structure (I). At high pH the $-NH_3^+$ group becomes deprotonated (pK_2) to yield another inactive form of opsin depicted by structure (II). These inactive forms of opsin (I, II, and III) are presumed to be in equilibrium with each other through protonation/deprotonation reactions. The mechanism presented by Figure 33 describes the experimental data collected on the recombination of rhodopsin and, in part, (reactions 4 and 5) is also consistent with mechanisms which describe the reactions between carbonyl compounds and simple amines (Jencks 1969). In the following paragraphs this mechanism and the experimental results from the study of the recombination reaction are discussed in detail.

The kinetic study of the recombination reaction in isolated rod outer segment membranes yielded the following experimental results. At pH 7.0 the k_{obs} versus ligand plot (Figure 30) was linear at low ligand concentrations indicating the overall rate was directly proportional to the 11-cis retinal concentration. At higher ligand concentrations the observed rate constants were independent of the ligand concentration, indicating the reaction order had changed and the reaction was now proceeding through a rate limiting first-order step. The reciprocal plot utilizing

this data was linear (Figure 31) indicating the reaction pathway involves an intermediate (V) or the formation of a complex. The kinetic plots in the study of the pH dependence of the recombination of rhodopsin in isolated rod outer segment membranes, under pseudo first-order conditions, were found to be biphasic at acidic or basic pH (Figure 27). These biphasic kinetic plots were composed of a fast pH independent phase and a slower pH dependent phase. At pH values near neutrality (6.5-7.4) the kinetic plots (ln ΔA_{500} versus time, Figure 27) were linear. The values of the observed rate constants for the pH independent fast phase of the reaction were slightly higher (15-20%) than the value of k_{obs} at pH 7. This difference probably was not significant since the determination of the fast phase rate constants involved at least a 15% error. These results suggest that the rate limiting step of the reaction represented by the pH independent fast phase (at high or low pH) is the same as the rate limiting step being observed at pH 7.

The above experimental results are consistent with the reaction mechanism illustrated in Figure 33. At pH 7 the experimental results indicated the reaction order had changed from a second-order process, at low ligand concentrations, to a first-order process at higher ligand concentrations. These results suggest that at high concentrations of 11-cis retinal the rate limiting step of the

reaction is the step involving the dehydration of the intermediate compound (V). The observed kinetics at low 11-cis retinal concentrations were second-order suggesting that at low ligand concentrations the formation of the intermediate compound (V) was rate limiting. Another and kinetically indistinguishable possibility is the dehydration step, k_5, is rate limiting at pH 7 and the formation of the intermediate (V) occurs in a prior rapid equilibrium step. If this were the case, at low ligand concentrations the observed rate would be directly proportional to the ligand concentration since the concentration of (V) would be small and it would be converted to product as rapidly as it was formed. This situation would result in a k_{obs} versus ligand concentration plot which was linear at low ligand concentrations indicating the overall reaction was second-order and k_{obs} would be equal to k_5 times the equilibrium concentration of the intermediate (V). At higher ligand concentrations all of the opsin would be expected to be in the intermediate form (V) and the observed rate would simply be the rate of the breakdown of the intermediate into product (k_5) and would be independent of the ligand concentration. Although the two possibilities mentioned above are not kinetically distinguishable in these experiments, the following reasons strongly suggest the dehydration step is the overall rate limiting step of the reaction at pH 7. If k_4 were rate limiting then the rate of conversion of (IV) to

(V) would only increase with increasing ligand concentration and would not level off as was observed. The conversion of the open inactive form of opsin depicted by structure (III) to the closed active form (IV) also does not appear to be rate limiting at pH 7. If the conversion of (III) to (IV) were rate limiting, the kinetic plots ($\ln \Delta A_{500}$ versus time) would become biphasic once the rate of formation of (IV) became less than the rate of dehydration of (V). At pH 7 no biphasic kinetic plots were observed over the 11-cis retinal concentration range from 20 to 110 μM suggesting that k_3 was greater than k_5.

Further support for assigning k_5 as the rate limiting step of the reaction comes from an extensive study of the reactions between carbonyl compounds (acetone, furfural, benzaldehyde, pyruvate, or cyclohexanone) and simple amines (hydroxylamine, o-methylhydroxylamine, or semicarbazide) (Jencks 1959). The mechanism shown in equation 11 accounted for all of the above reactions.

$$R\text{-}CHO + NH_2R' \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} R\text{-}\overset{OH}{\overset{|}{C}}\overset{H}{\overset{|}{=}}\overset{H}{\overset{|}{N}}\text{-}R' \overset{k_2 \quad H^+}{\underset{k_{-2}}{\rightleftharpoons}} R\text{-}CH{=}N\text{-}R' + H_2O \qquad (11)$$

For the reaction between hydroxylamine and acetone the values of k_1 and k_{-1} were 1 x 10^3 M^{-1} sec^{-1} and 1 x 10^3 sec^{-1} respectively, indicating the intermediate was in rapid

equilibrium with the reactants. The dehydration step was the rate limiting step of the reaction at neutral pH and had a value of approximately 0.13 sec^{-1} (not including the contribution due to the H^+ concentration). At neutral pH the reactions between the above carbonyl compounds and amines were characterized by the same rate determining step, that being the dehydration of the intermediate compound. In the reaction between hydroxylamine and acetone at the pH optimum (pH 4.5) Jencks (1959) found that at low ligand concentrations the observed rate constants increased with increasing ligand concentration and then, at higher ligand concentrations, leveled off with no further increases in the observed rate constants. It is interesting to note that the maximal rate of reaction was at pH 4.5 which is 1.5 pH units below the pK of hydroxylamine. This is initially surprising since the amino group must be unprotonated before it can act as a nucleophile towards the carbonyl compound (Jencks 1959). This pH-rate behavior was the result of a change in the rate determining step of the reaction as the pH was increased. At low pH the rate limiting step of the reaction was the attack of the free (unprotonated) amine upon the carbonyl compound while at high pH the rate limiting step of the reaction was the acid catalyzed dehydration of the intermediate compound (Jencks 1959).

At pH values other than 7 the ln ΔA_{500} versus time plots (Figure 27) were biphasic for the recombination reaction in isolated rod outer segment membranes and indicated two first-order processes were occurring. Since the observed rate constants for the fast pH independent phase were nearly the same as the observed rate constant at pH 7, the results suggest the fast phase of the reaction at high or low pH represented the dehydration step. This interpretation suggests that at any given pH a certain fraction of the opsin exists in the closed active form (IV), this fraction being controlled by the interconversion between (III) and (IV), the pH, and the values of the equilibrium constants K_1 and K_2. The initial fast and pH independent phase of the reaction observed in the ln ΔA_{500} versus time plots (Figure 27) represents the reaction between the added 11-cis retinal and that fraction of the opsin present in the active form (IV). After the fraction of opsin present in the active form (IV) was consumed by this fast reaction, a slow and pH dependent phase of the reaction took place. This slow and pH dependent phase must represent the rate at which additional active opsin (IV) becomes available for reaction with 11-cis retinal. It is not possible from these kinetic studies to establish whether the slow phase of the reaction (represented by the wings of the k_{obs} versus pH plot, Figure 28) represents the rate of conversion of (III) to (IV) or the rate of conversion of (I) or (II) to (III).

The kinetic aspects of the recombination reaction using sodium cholate solubilized rhodopsin were in many respects similar to those observed for the reaction in the isolated membranes. At pH 7.0 the k_{obs} versus ligand plot (Figure 22) was linear at low 11-cis retinal concentrations indicating the overall rate was directly proportional to the ligand concentration, similar to that observed for the reaction in the membranes. The reciprocal plot for the reaction (Figure 23) was linear but had more experimental error than was observed for the same type of plot from the rod outer segment membrane data (Figure 31). The kinetic plots ($\ln \Delta A_{500}$ versus time, Figure 19) for the recombination of sodium cholate solubilized rhodopsin, under pseudo first-order conditions, were linear over the pH range studied, pH 6.5 to 7.5, and within an estimated error of ± 15%, the values of k_{obs} were independent of pH (Figure 20). These observations suggest that the rate limiting step being monitored for the recombination of rhodopsin (in sodium cholate under pseudo first-order reaction conditions) is, within experimental error, independent of pH between pH 6.5 and 7.5.

The above experimental results obtained with sodium cholate solubilized rhodopsin are, in the most part, also consistent with the reaction mechanism illustrated in Figure 33. The layover of the second-order plot (Figure 22) and the linear reciprocal plot (Figure 23) suggests the

dehydration step, k_5, of the reaction was rate limiting at pH 7 and the intermediate compound (V) was formed in a prior rapid equilibrium step. The reasons for assigning the rate limiting step of the reaction as the dehydration of the intermediate compound are the same as previously discussed for the rod outer segment experimental data. The values of k_{obs} were essentially independent of pH from pH 6.5 to 7.5 and suggest, in the context of the proposed mechanism, the solubilization of rhodopsin in sodium cholate may have altered the interconversion between (III) and (IV) and/or the equilibriums between forms (I) and (III) or (II) and (III).

In summary the experimental kinetic data for the recombination of rhodopsin in isolated rod outer segment membranes or in sodium cholate solutions are consistent with the mechanism proposed for the recombination reaction (Figure 33). Although it is not possible to unambiguously assign rate constants for all the steps involved, the following are suggested by the kinetic data. In isolated rod outer segment membranes (0.10 M potassium phosphate at pH 7.0, and 1 mM dithioerythritol at 25 °C) the dehydration step, k_5, has a rate constant of 0.055 sec^{-1} and is the rate limiting step under these conditions. The pseudo equilibrium expression $(k_{-4} + k_5)/k_4$ which is a measure of the affinity of the opsin for 11-cis retinal has a value of 5.9×10^{-5} M.

For sodium cholate solubilized rhodopsin (pH 7.0, 0.10 M potassium phosphate buffer containing 2 mg/ml sodium cholate and 1 mM dithioerythritol) the value of k_5 is 0.18 sec^{-1} and is the rate limiting step of the reaction under these conditions. The pseudo equilibrium expression $(k_{-4} + k_5)/k_4$ had a value of 2.25×10^{-4} M. From a comparison of the values of $(k_{-4} + k_5)/k_4$ for these two forms of opsin it appears that the membrane bound opsin has a higher affinity for 11-cis retinal than does sodium cholate solubilized opsin. This result indicates the solubilization process with sodium cholate caused a decrease in the affinity of opsin for 11-cis retinal.

Conclusions

The results of this work on the visual pigment rhodopsin have led to the following conclusions with respect to the physical-chemical properties of rhodopsin and the recombination of rhodopsin from opsin and 11-cis retinal. The reaction between membrane bound opsin and 11-cis retinal to form rhodopsin is described by a complex reaction mechanism and involves an amino acid residue on opsin which acts as a general acid and/or a general base. The solubilization of rhodopsin with sodium cholate yields a form of rhopsin which is capable of recombination with 11-cis retinal under defined conditions. The mechanism of recombination of sodium cholate solubilized rhodopsin (unpurified) closely

parallels that for membrane bound rhodopsin with only small alterations in the rate constants. The solubilization process with sodium cholate did reduce the apparent affinity of opsin for 11-cis retinal by nearly a factor of 4 and may indicate the partial disruption of the structure of opsin caused by sodium cholate.

Sodium cholate solubilized rhodopsin is a good model for studying the recombination reaction if the above restrictions are kept in mind. The structure of bleached sodium cholate solubilized rhodopsin (opsin) is different in the forms which are active and inactive in recombination. These structural differences are detectable by the availability of opsin sulfhydryl groups to chemical modification and are, in some manner which remains unknown, mediated by the concentration of sodium cholate in the solutions. The solubility characteristics and the recombination kinetic properties of sodium cholate solutions of rhodopsin are dependent upon the amount of associated phospholipids. In summary, sodium cholate solubilized rhodopsin represents a reasonable system in which to study the recombination reaction since to a large extent the mechanism of recombination is similar to the mechanism of recombination for rhodopsin in the isolated rod outer segment membrane.

Proposed Research

One of the results of this work has been the partial characterization of a newly solubilized form of rhodopsin. Several properties of this sodium cholate solubilized rhodopsin were described and some of these suggest that further work can be done on the physical-chemical properties of this preparation of rhodopsin. The solubility properties of rhodopsin were found to be dependent upon both the sodium cholate concentration and the amount of associated organic phosphorus compounds. An extremely important question which needs to be answered by further work deals with the isolation and identification of the organic phosphorus material which was removed from sodium cholate solutions of rhodopsin with the ammonium sulfate fractionation procedure. This material is most likely phospholipid but an absolute identification is needed. If this material is phospholipid then the results of this work suggest that rhodopsin may be present in mixed micelles composed of sodium cholate, rhodopsin, and phospholipids. Careful chemical and physical analysis of these mixtures under a variety of conditions and at known rhodopsin-sodium cholate-phospholipid ratios should yield a considerable amount of information about the interactions of these three components. Useful probes for this type of study could include analytical ultracentrifugation, gel filtration, absorption spectroscopy, and circular

dichroism spectroscopy. These types of investigations have the potential of establishing the composition and size of the micelles under different conditions, and the effects that altered micelle size and/or composition may have upon the absorption and circular dichroism spectrum of rhodopsin. Another interesting observation was that sodium cholate in potassium phosphate buffer extracts rhodopsin in high yields while sodium cholate in Hepes buffer was incapable of extracting rhodopsin. The reason for the above observation is not known since studies involving sodium cholate-Hepes solutions were not extensively pursued. In one experiment rod outer segment membranes were extracted 3 times with 50 mM Hepes, pH 7.0, containing 20 mg/ml sodium cholate and essentially no rhodopsin was extracted as indicated by the lack of absorbance at 500 nm. After the above procedure the membranes were washed with water and then extracted with 20 mg/ml sodium cholate in pH 7.0, 0.10 M potassium phosphate buffer. This second extraction solubilized rhodopsin which contained only 14 moles of organic phosphorus per mole of rhodopsin instead of the usual 60 moles of organic phosphorus. These preliminary results indicated that treatment with Hepes-sodium cholate followed by extraction with sodium cholate in phosphate buffer was an effective method of preparing rhodopsin which contains a reduced amount of associated organic phosphorus compounds. It is not known if

sodium cholate forms micelles in both Hepes and potassium phosphate buffers, but presumably there must be some differences between sodium cholate in the two buffers to account for the different solubilization properties which were observed. Experiments to determine the aggregation size of sodium cholate in these two buffer systems would reveal whether or not micelles were formed. With this information it may be possible to describe some of the features or processes required for the solubilization of rhodopsin from the rod outer segment membrane.

The mechanism proposed for the recombination reaction should be most useful in designing experiments to further elucidate the details of this reaction. One obvious set of experiments involves establishing the identity of the group -B: which was postulated to be a histidine residue. Unfortunately, this will not be an easy task since the number and types of chemical modifications which are relatively specific for histidine are limited. The establishment of the basic kinetic properties of recombination in sodium cholate solutions and in isolated rod outer segment membranes should allow the completion of a set of experiments designed to describe the specificity of the retinal binding site. It is well established that of all the retinal stereoisomers only the 9-*cis* or 11-*cis* forms will react with opsin to form rhodopsin or a rhodopsin analogue. It is not known if the outer stereoisomers of retinal or

if the various stereoisomers of retinol (the alcohol form) are capable of entering the retinal binding site. By carrying out experiments in the same manner as is done for inhibitor studies on enzymes it should be possible to establish what retinal and/or retinol isomers compete with 11-*cis* retinal for its binding site in opsin.

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