The Role of the Positively Charged Amino Acids in Bacteriorhodopsin

By

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ABSTRACT

This study has used chemical modification techniques to investigate the role of the only two types of positively charged amino acids, lysines and arginines, in the membrane-bound protein, bacteriorhodopsin (bR). The results of these chemical modifications have significance both for the structure of bR and for the molecular mechanism of light-activated photocycling and proton pumping.

The implications for the secondary structure of bR are:
LYS 40 is totally exposed to the aqueous phase, while the other five reactive lysines are partially buried in the hydrophobic domain; LYS 30, LYS 129 and LYS 159 are buried by only one or two residues within the hydrophobic domain; all but two arginines are totally exposed to the aqueous phase; and at least three ionic bridges form between arginines and carboxyl groups. These results were used to construct a new model of the secondary structure of bR. The implication for the tertiary structure of bR is that Model A (126) is the preferred model of fitting the bR sequence into the helices seen by electron diffraction. The implication for the quaternary structure is that one structural role of retinal is to increase the intermolecular distances between bR molecules in a trimer. This focuses attention on the bR trimer as the primary structural unit.
Regarding the molecular mechanism of photocycling and proton pumping, two specific arginines were responsible for the slowdown of the photocycle. This result was obtained by a quantitative analysis of the inhibition of $M_{412}$ decay as a function of fraction of arginines modified by 2,3-butanedione. Possible roles of the two important arginines, including controlling the conformational changes of the protein by ionic linkages to carboxyl groups and participating directly in proton pathways, are discussed. Bifunctional imidoester modification of lysines revealed that a conformational change of the bR protein is needed for photocycling, since cross-linking lysines slowed the photocycle. 2,3-Butanedione modification of arginines supported this result, since this modification caused a marked slowdown of the photocycle and also protein conformational changes as evidenced by tryptophan fluorescence and circular dichroism spectroscopy.
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CONCLUSIONS

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REFERENCES
This dissertation is dedicated to my parents.
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LIST OF ABBREVIATIONS

bR, Bacteriorhodopsin
bR_{568}, Initial Light-Adapted 568 nm Chromophore of bR
PM, Purple Membrane
K_{590} (also K), 590 nm Photocycle Intermediate
L_{550} (also L), 550 nm Photocycle Intermediate
M_{412} (also M), 412 nm Photocycle Intermediate
M', M' Photocycle Intermediate
O, O Photocycle Intermediate
EA, Ethyl Acetimidate
MA, Methyl Acetimidate
MB, Methyl Butyrimidate
MI, Monofunctional Imidate
DMS, Dimethyl Suberimidate
DMA, Dimethyl Adipimidate
TEA, Triethanolamine
BD, 2,3-Butanedione
PGO, Phenylglyoxal
EDC, 1-Ethyl-3-(3-dimethylaminopropyl) Carbodiimide
I\(^-\), Iodination Reagents
MES, 2-(N-Morpholino)ethane Sulfonic Acid
EPR, Electron Paramagnetic Resonance
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LIST OF REAGENTS (See end of list for locations of chemical companies)

Chemical Modification Reagents

Ethyl Acetimidate HCl, Pierce Chemical Co.
Methyl Acetimidate HCl, Aldrich Chemical Co.
Methyl Butyrimidate HCl, Aldrich Chemical Co.
Dimethyl Adipimidate HCl, Pierce Chemical Co.
Dimethyl Suberimidate HCl, Pierce Chemical Co.
Pyridoxal Phosphate, 98% crystalline, Sigma Chemical Co.
Fluorescamine, No. 23755 Pierce Chemical Co.
2,3-Butanedione, 99%, B8, 530-7 Aldrich Chemical Co.
Phenylglyoxal, Monohydrate, 14,243-3 Aldrich Chemical Co.
Ninhydrin, No. 6984 Mallinckrodt, Inc.
Lactoperoxidase, lyophilized from milk, 60-80 units/mg protein, L-2005 Sigma Chemical Co.
Glucose Oxidase, Type V from Aspergillus niger, 200 units/ml protein, G-6500 Sigma Chemical Co.
Sodium Iodide, S-8379 Sigma Chemical Co.
l-Ethyl-3-(3-dimethylaminopropyl) Carbodiimide HCl, Sigma Chemical Co.

Gel Electrophoresis Reagents

Acrylamide, Electrophoresis Purity Reagent, Bio-Rad Labs.
Bis-N,N'-Methylene-bis-acrylamide, Electrophoresis Purity Reagent, Bio-Rad Laboratories
Ammonium Persulfate, Electrophoresis Grade, Bio-Rad Labs.
N,N,N',N'-Tetramethylethylenediamine, Electrophoresis Grade, Bio-Rad Laboratories
Bromophenol Blue, No. 40095 Chroma-Gesellschaft, Schmid and Co.

Coomassie Brilliant Blue, Electrophoresis Grade, R-250 Bio-Rad Laboratories

Trypsin Inhibitor, Type 1-P, from beef pancreas, crystallized and lyophilized, Sigma Chemical Co.

Lysozyme, Grade 1, dialyzed and lyophilized, L-6876 Sigma Chemical Co.

α-Chymotrypsin, Type 1-S from bovine pancreas, 3X crystallized and lyophilized, C-7762 Sigma Chemical Co.

D-Amino Acid Oxidase, crystalline from porcine kidney, suspension, 3.2 M (NH₄)₂SO₄ solution, pH 6.5, A-8759 Sigma Chemical Co.

Bovine Serum Albumin, essentially fatty acid free, A-6003 Sigma Chemical Co.

β-Galactosidase, Grade IV, purified from E. coli, crystalline suspension in 2.2 M (NH₄)₂SO₄, pH 6.0, G-8504 Sigma Chemical Co.

Salts and Buffers

Sodium Chloride, No. 7581 Mallinckrodt, Inc.

Magnesium Sulfate, No. 6066 Mallinckrodt Chemical Works, Inc.

Potassium Chloride, No. 6858 Mallinckrodt, Inc.

Calcium Chloride, No. 4160 Mallinckrodt, Inc.

Sodium Citrate, No. 0754 Mallinckrodt Chemical Works, Inc.

Ferrous Chloride, I-90 Fisher Scientific Co.

Manganese Sulfate, No. 6192 Mallinckrodt Chemical Works, Inc.

Sodium Hydroxide, No. 7708 Mallinckrodt, Inc.
Ferrous Sulfate, No. 5056 Mallinckrodt Chemical Works, Inc.
Sodium Acetate, No. 3460 J.T. Baker Chemical Co.
Sodium Nitrite, S-346 Fisher Scientific Co.
Cupric Nitrate, J.T. Baker Chemical Co.
Sodium Phosphate, Monobasic, No. 7892 Mallinckrodt, Inc.
Sodium Phosphate, Dibasic, No. 7914 Mallinckrodt, Inc.
Sodium Borate, J.T. Baker Chemical Co.
Sodium Carbonate, No. 7521 Mallinckrodt Chemical Works, Inc.
Cupric Sulfate, No. 4840 Mallinckrodt Chemical Works, Inc.
Sodium Potassium Tartrate, S-387 Fisher Scientific Co.
Potassium Dichromate, No. 6758 Mallinckrodt Chemical Works, Inc.
Trizma Base, No. T-1503 Sigma Chemical Co.
Sodium Citrate Hydrolysis Buffer, 0.2 N, pH 7.0, No. 27216
   Pierce Chemical Co.
Sodium Citrate Elution Buffer, 0.2 N, pH 3.25, No. 27223
   Pierce Chemical Co.
Sodium Citrate Elution Buffer, 0.2 N, pH 4.25, No. 27224
   Pierce Chemical Co.
Sodium Citrate Elution Buffer, 0.2 N, pH 7.9, No. 27225
   Pierce Chemical Co.
N-(N-Morpholino)ethane Sulfonic Acid, No. M-8250
   Sigma Chemical Co.
Potassium Chloride, No. 8360 Mallinckrodt, Inc.

Other Reagents

Inolex Peptone, Inolex Corporation
AF-72 Antifoam Emulsion, General Electric Co.
L28 Purified Agar, Oxoid

Deoxyribonuclease I, from bovine pancreas, DN-25
    Sigma Chemical Co.

Sucrose, No. 8360 Mallinckrodt, Inc.

Sulfuric Acid (technical grade), 93%, No. 2900
    Mallinckrodt Chemical Works

1,10-Phenanthroline, No. P-9375 Sigma Chemical Co.

Flavin Mononucleotide, No. F-2255 Sigma Chemical Co.

HCl, 37%, No. 2616 Mallinckrodt, Inc.

Triethanolamine, T-345 Fisher Scientific Co.

Hydroxylamine, Grade 1, H-9876 Sigma Chemical Co.

Dodecyl Sodium Sulfate (Sodium Lauryl Sulfate), 95%,
    S-7003 Matheson, Coleman and Bell

Acetone, Reagent Grade, Lawrence Berkeley Laboratories

Ethanol, Reagent Grade, Lawrence Berkeley Laboratories

Folin and Ciocalteu's Phenol Reagent, 2N, F-9252 Sigma
    Chemical Co.


Glacial Acetic Acid, No. 2504 Mallinckrodt Chemical Works

Methyl Alcohol, No. 3016 Mallinckrodt, Inc.

Glycerol, No. 72841 Merck and Co.

Sephadex G-25 or G-10, Pharmacia Fine Chemicals

α-D(+)−Glucose, G-5000 Sigma Chemical Co.

Rhodamine Dye, Exciton Chemical Co., Inc.

Deuterium Oxide, D-4501 Sigma Chemical Co.

Asolectin, 95% Purified Soy Phosphatides, 1-09107
    Associated Concentrates
Locations of Chemical Companies

Pierce Chemical Co., Rockford, Illinois
Aldrich Chemical Co., Milwaukee, Wisconsin
Sigma Chemical Co., St. Louis, Missouri
Bio-Rad Laboratories, Richmond, California
Schmid and Co., Stuttgart, Germany
Mallinckrodt, Inc., Paris, Kentucky
Mallinckrodt Chemical Works, Inc., St. Louis, Missouri
Fisher Scientific Co., Fair Lawn, New Jersey
Inolex Corporation, Glenwood, Illinois
General Electric (GE), Cleveland, Ohio
Oxoid, England
Duke Scientific Co., Palo Alto, California
J.T. Baker Chemical Co., Phillipsburg, New Jersey
Matheson, Coleman and Bell, East Rutherford, New Jersey
Merck and Co., Rahway, New Jersey
Pharmacia Fine Chemicals, Piscataway, New Jersey
Exciton Chemical Co., Inc., Dayton, Ohio
Associated Concentrates, Woodside, New York
LIST OF INSTRUMENTS AND EQUIPMENT (See end of list for locations of instrument and equipment companies)

General

RC-5B Sorvall Centrifuge, DuPont Instruments
L2-75B Ultracentrifuge, Beckman
Model 2400 DU Spectrophotometer, Beckman
Model 2451-A Automatic Cuvette Positioner, Gilford Instrument Labs, Inc.
Model 6051 Instrument Recorder, Gilford Instrument Labs, Inc.
Model 2412 Gel Cuvette, Gilford Instrument Labs, Inc.
Aminco DW2 Recording Spectrophotometer, American Instrument Co.
Cary 14R Recording Spectrophotometer, Applied Physics Corp.
Type F Circulating Water Bath, Haake
FK2 Circulating Water Bath, Haake
Model 165 Argon Laser, Spectra-Physics
Model CR-12 Argon Laser, Coherent Radiation
Model TE-104RF Housing, Products for Research
Mod Comp II/222, Modular Computers, Inc.
Phase-R-DK-1100, Phase-R Lasers
Cary 60 Circular Dichroism Spectrophotometer, Applied Physics Corp.
No. 6263 12 volt DC Power Supply, Harrison Labs
Monochromators, Bausch and Lomb
Model CS-64H40 DC Power Supply, NJE Corp.
Model 1010 Waveform Recorder, Biomation
Type 564 Storage Oscilloscope, Tektronix, Inc.
PDP 11-34 Computer, Digital
X-Y Plotter, Varian

MGW Lauda RC3, Model T-2, Brinkman Instruments Inc.

Model 350 Sonifier Cell Disruptor, Branson Sonic Power Co.

Model 3500 Digital pH Meter, Beckman

Model 9176 Recorder, Varian

Model E-109A EPR Spectrophotometer, Varian

PDP 8-A Computer, Digital

Framework Molecular Models, Prentice Hall Inc.

Light Sources

"Cool-White" Fluorescent Lights, General Electric Co.

FCR Quartzline Lamp, 12 volts, 100 watts, General Electric Co.

CYC Projector Lamp, 115-125 V, 300 watts, General Electric Co.

EHT Quartz Bromine Lamp, Sylvania GTE

Model 707 Headliner Projector, Bell and Howell Co.

Projector Lamp, 300 watt, General Electric Co.

Filters

Filter No. 3389, Corning Glass Works

Filter No. 3-67, Corning Glass Works

412 nm Interference Filter, Baird Atomic Inc. (Baird Corp.)

Locations of Instrument and Equipment Companies

Chemapec, Mannedorf, Switzerland

General Electric (GE) Co., Cleveland, Ohio

DuPont Instruments, Newtown, Connecticut

Beckman, Spinco Division, Palo Alto, California

Yellow Springs Instruments, Yellow Springs, Ohio
Weston Instruments Inc., Newark, New Jersey
Corning Glass Works, Corning, New York
Bausch and Lomb, Rochester, New York
Sylvania GTE, Winchester, Kentucky
Fisher Scientific Co., Indiana, Pennsylvania
Perkin Elmer, Norwalk, Connecticut
Bio-Rad Labs, Richmond, California
Heath Company, Benton Harbor, Michigan
Hamilton Co., Reno, Nevada
Beckman, Fullerton, California
Gilford Instrument Lab, Inc., Oberlin, Ohio
American Instrument Co., Silver Springs, Maryland
Applied Physics Corp., Monrovia, California
Haake, Berlin, West Germany
Spectra-Physics, Mountain View, California
Coherent Radiation, Palo Alto, California
Products for Research, Danvers, Connecticut
Modular Computers, Inc., Fort Lauderdale, Florida
Phase-R Lasers, California
Harrison Labs, Berkeley Heights, New Jersey
NJE Corp., Kenilworth, New Jersey
Biomation, Cupertino, California
Tektronix, Inc., Portland, Oregon
Digital, Maynard, Massachusetts
Varian, Palo Alto, California
Brinkman Instruments, Inc., Westbury, New York
Branson Sonic Power Co., Danbury, Connecticut
Bell and Howell Co., Chicago, Illinois

Baird Atomic Inc., (Baird Corp.), Bedford, Massachusetts

Prentice-Hall, Inc., Englewood Cliffs, New Jersey
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INTRODUCTION

Bioenergetics studies how biological systems utilize and store energy. A major advance in this field was the formulation of chemiosmotic hypothesis by Peter Mitchell (205) for which he was awarded the Nobel prize in 1978 (206) and which has been verified by numerous investigators (207-211). The chemiosmotic theory states that primary energy conversion produces a proton motive force (pmf) across a coupling membrane of low permeability to protons, which has both an electrical and a protonic chemical component (205). The pmf then acts to form ATP in mitochondria, chloroplasts and bacteria. Thus, energy conversion and storage can be summarized in two steps:

Step 1: Energy Input → Transmembrane Electrochemical Potential
Step 2: Transmembrane Electrochemical → Chemical Storage of Potential Energy

The next step in bioenergetics is the elucidation of the detailed molecular mechanisms of the conversion processes in Steps 1 and 2 for specific systems. Bacteriorhodopsin (bR) is an ideal model system in which to study energy conversion in Step 1, since, unlike mitochondria and chloroplasts, only one protein is required to convert light energy into a proton gradient. Numerous studies have shown that when activated by light, bR pumps protons to form a pH gradient in bR lipid vesicles or in the cell of H. halobium (76,212-214). In addition, the easily isolated purple membrane (PM), contains only the bR protein in multiple copies (215). The question of interest now is how does bR convert light energy to pump protons vectorially across the membrane?
bR has been extensively studied in the last ten years and much has been learned (for recent reviews, see 216-218). Structurally, electron diffraction at 7 and 3.5 Å resolution has revealed that there are seven alpha helices in bR that span the membrane of PM (126,128). These helices, along with a small amount of unusual saturated lipids (219), form a two dimensional crystalline array (216). The primary sequence of the bR molecule has been determined (23,24). What remains to be known for certain is the secondary, tertiary, and quaternary structure of bR in the PM; i.e., how the primary sequence fits into the alpha helices, in what order, and how bR molecules form a trimer arrangement in the PM. Previous to this study, the secondary and tertiary structures of bR have been estimated by considering the scattering density of the helices seen by electron diffraction, possible interhelical charge neutralization, and the connectivity of non-helical link regions (112). Another model was based on the visibility of the interhelical linking segments in the electron diffraction pattern (126). The techniques of chemical modification used in this thesis provide additional information which helps to decide between different structural models. Information about the secondary structure is gained by modifying bR with water soluble group-specific reagents, and information about the tertiary and quaternary structure is gained by chemical cross-linking.

Although the function of bR has been well studied (216-218), many questions remain to be answered. For example, the primary photo-event is still controversial. Experiments by Mathies and Stryer (221) have revealed that when retinal is excited by light, there is a shift of negative charge towards the retinal Schiff base. However, it is
not known if this leads to an initial isomerization of the retinal (222), or if the induced dipole in retinal produces a conformational change in the protein (223). Another question regarding the primary photoevent is where a proton moves in the protein. Peters et al. (224) have demonstrated a deuterium isotope effect on a picosecond time scale, but there is disagreement as to whether the Schiff base proton or another proton on the protein is affected (117,225,226). Thus the initial mechanism of light energy storage by the chromophore and bR protein is not well understood.

A consensus of opinion has emerged about the overall photocycle of bR, although details are still in dispute (31,33,60,176,227-229). In the diagram below is a framework upon which investigators in this field have added back photoreactions, new intermediates and other pathways of thermal decay. The photointermediates are presented by the wavelength of their maximum absorption.

There is general agreement that retinal in the bR\textsubscript{568} form is joined to a lysine in the protein by a protonated Schiff base linkage and that the M\textsubscript{412} state is a deprotonated Schiff base linkage. It is also known that the retinal chromophore is not removed from the protein during
photocycling as in rhodopsin (139). In addition, conformational changes of the retinal in the form of an isomerization and reisomerization are thought to occur during a single photocycle. However, absorption studies (80,147,148), resonance Raman studies (30,63, 230,231) and extraction techniques (39,149,232) disagree as to which retinal isomer, all-trans or 13-cis, is present in the various intermediates. In addition, one recent study (69) suggests that retinal moves from LYS 41 to LYS 216 during photocycling. The use of chemical modification and chemical cross-linking to constrain movement of the protein and/or retinal is one method to obtain more information about the conformational changes involved in the photocycle.

Another unresolved question is whether the proton that leaves the Schiff base in the $M_{412}$ form is actually pumped through the protein and across the PM. A few years ago, it was thought that a maximum of one proton was pumped in each photocycle; this agreed with the idea that the Schiff base proton is pumped, and a number of models based upon this idea have been proposed (184,233). However, recent experiments have shown that the number of protons pumped appears to be closer to two, which is difficult to reconcile with simpler models (136,171). One structure that most models assume and thus could explain more than one proton pumped per photocycle is that there is a channel or pathway for protons within the membrane (233). Without such a channel, a very large conformational change of the protein would have to occur to carry protons a distance of 45 Å, which seems unlikely in a supposedly rigid, crystalline membrane. A molecular model of this channel containing hydrogen bonded chains between amino acid side chains of the bR protein has been proposed by Nagle and
Morowitz (175), who call this structure a proton wire. Such proton wires could be coupled in a variety of ways to the active site to pump protons vectorially. Chemical modification of amino acid side chains to alter their proton transport ability is an appropriate tool to study which, if any, side chains are involved in a proton wire.

After a chemical modification is carried out, the number of amino acids modified is quantitated. Then the photoactivity is tested. If a change in activity is caused by the chemical modification, it is essential to know if the change in activity is due to a different chemical reactivity of a specific amino acid or to a change in the structure of the protein and/or chromophore. Several spectroscopic techniques were used in this study to analyze structural changes after a chemical modification: absorption, tryptophan fluorescence, resonance Raman, retinal fluorescence and circular dichroism. The photoactivity of bR was most frequently measured by flash photolysis, which is an indirect indicator of protons pumped.
MATERIALS AND METHODS

I. Preparatory Procedures

A. Purple Membrane (PM) Growth and Purification

Initially, PM was kindly provided by Dr. Janos Lanyi of Ames Research Center, NASA, Moffett Field, California. Most of the PM used in these experiments, however, was prepared at Berkeley as described below. *Halobacterium halobium* strain S9 was grown according to (1) in 10-liter batches in a sterilized LF-14 Chemapac Fermenter. The growth media contained the following per liter: 250 gms NaCl, 20 gms MgSO$_4$·7H$_2$O, 2 gms KCl, 200 mgs CaCl$_2$·2H$_2$O, 3 gms Na$_2$Citrate·2H$_2$O, 1 ml of 3.58 gms/liter FeCl$_2$·4H$_2$O, 1 ml of 190 mg/liter MnSO$_4$·H$_2$O, 10 gms Inolex peptone, and 10 mgs NaOH for a final pH of 7.0. A small amount of AF-72 Antifoam Emulsion was added to prevent foaming during mixing. The solution was mixed with a magnetic stirrer until it was clear. The media was then autoclaved 20 minutes at 121° C.

The cooled media was inoculated either with a small amount of media containing colonies from 1 or 2 agar slants, or with a test tube or 250 ml starter culture (see below). Agar slants were prepared by adding 1.5 % agar to the culture media in sealed test tubes, autoclaving 20 minutes at 121° C and positioning the test tubes at a slant to gel at room temperature. Petri plates were filled with 1.5 % agar in media, autoclaved and left to gel covered at room temperature. 1 ml of late log phase liquid cell culture was spread on agar plates or slants. Alternatively, selected colonies from established plates (one month of growth at room temperature) were streaked on agar plates or slants. Agar slants were maintained in tightly sealed test tubes and agar plates were sealed in plastic bags. Slants and plates were grown at
room temperature in a tightly sealed box illuminated by GE "Cool-White" fluorescent lights. Established slants could be stored at 4° C for one year.

Starter test tube cultures were prepared by picking desired cultures from plates or slants and placing 5-7 ml of liquid culture medium in a loosely capped test tube. The test tube culture could be shaken on a shaker or grown in a test tube rack under illumination. 250 ml starter cultures were prepared from a test tube starter culture or from colonies picked from plates or slants in culture media. This starter culture was grown on a shaker platform with shaking at 180 rpm at 25-35° C. Illumination was by a band of 6 GE "Cool-White" fluorescent lights. This 250 ml culture was transferred to 5-10 liter batches of media when the cell growth had produced a light scattering of Abs.₆₆₀ = 0.4-0.5.

It was also possible to serial culture the S9 colonies, provided that not more than five serial transfers were made without starting with fresh colonies. To serial transfer a culture, 250 ml of a culture that had reached the stationary growth phase was diluted into 10 liters of new media.

Aeration in the Chemapec Fermenter was achieved by flowing compressed air into the fermenter at a flow rate of 2 liters/minute and monitoring oxygen concentration in the media polarographically with the Il 530 Industrial Dissolved Oxygen Monitoring System. The oxygen electrode was initially calibrated in distilled water that had been bubbled with compressed air for 10 minutes and set at 160 mm H₂O, or 21.1% atmospheric pressure. The oxygen was gradually depleted by the growing cells. Just before S9 cultures reached the stationary growth phase (Abs.₆₆₀ = 0.8-1.0 and about 4 days of growth), the air was turned off
and the anaerobic culture stirred about 2 more days. The fermenter was maintained at 37°C, stirred at 460 rpm, and surrounded by seven 18" GE "Cool-White" fluorescent lights mounted on a circular wooden stand 2 cm from the fermenter. A typical growth curve of *H. halobium* S9 appears in Figure 1.

After the cells had remained at the stationary phase of growth for two days, they were harvested by centrifugation at 2,000 x g for 60 minutes. The clear yellow supernatant was discarded. The cell pellet was frozen at -40°C for one day or 3-4 months. The thawed cell pellet was resuspended in 400 ml of cold distilled water and 5 mg Deoxyribonuclease I was added. This suspension was blended in a Waring Blender 5 x 5 seconds, and 600 ml of cold distilled water was added. This cell lysate was stirred for not more than one night in an 8°C room.

A preliminary low speed centrifugation at 7,000 x g for 10 minutes pelleted cell debris. The supernatant was then centrifuged at 100,000 x g for 30 minutes. After this spin the pellet was resuspended with a Pasteur pipette with a minimal volume of distilled water and the above centrifugations of the cell lysate supernatant were repeated until the entire cell lysate was pelleted together. After each spin the reddish supernatant was discarded. The combined pellet was resuspended in 30-40 ml of distilled water and centrifuged at 100,000 x g for 30 minutes. This pellet was resuspended to a concentration of 5 mg/ml bacteriorhodopsin (bR) protein, based on the molar extinction coefficient of 63,000 M⁻¹ cm⁻¹ at 568 nm and approximate molecular weight of bR of 26,000 (2).

The following sucrose step gradient was carried out to remove any remaining red membrane (3) at 175,000 x g for 16-20 hours:
Figure 1
Growth Curve of *Halobacterium halobium*, Strain S9

Oxygen as Percent Total Atmospheric Pressure

Absorbance

- 568 nm, ▲
- 660 nm, ○

Days of Growth

21.1
15.8
10.5
5.3

Air Turned Off

Cell Growth

Purple Membrane

Oxygen
0.5 ml 60% sucrose cushion (w/v)
2.0 ml 52% sucrose (w/v)
2.0 ml 45% sucrose (w/v)
2.0 ml 40% sucrose (w/v)
2.0 ml 38% sucrose (w/v)
2.0 ml 36% sucrose (w/v)

1.5 ml of the cell lysate combined pellet was loaded onto this gradient in six separate centrifuge tubes. The PM fraction sedimented primarily at 45% sucrose. The sucrose was removed from the collected PM fractions by repeatedly centrifuging 10 ml aliquots diluted with 15 ml distilled water at 100,000 x g for 20 minutes. The combined pellet was washed by centrifuging at 180,000 x g for 20 minutes and resuspending in 25 ml distilled water, three times. The final pellet was resuspended in distilled water to a bR concentration of 10 mg/ml.

The resulting PM was examined for microheterogeneity by disk gel electrophoresis in slabs using a 5% polyacrylamide stacking gel and 12.5% polyacrylamide separation gel according to the method of Laemmli (4) and found to be free of contaminating proteins. The overall yield was determined by comparing the final bR weight to the initial weight of the total protein in the cell lysate analyzed by a modified Lowry method (see Materials and Methods II.C.2. and (5)). The usual overall yield of PM per 10 liter culture batch was 250 mgs of bR protein, representing a 60% yield. The purification fold was determined by comparing the final weight ratio of bR/total protein to the initial weight ratio of bR/total protein. The resulting homogeneous bR protein was 10.2 times more pure than in the cell lysate step. This preparation was stable for more than 4 months if stored at 5 mg/ml in 2 M NaCl at 5°C.
I. B. Light Intensity Measurements

The method used to measure the light-intensity varied depending upon the geometry of the sample in the light source and also upon the measuring method available at the time of the experiment. The light intensity of the light source used to bleach bR was measured with a Yellow Springs Instrument Kettering Model 65 Radiometer, using Kodak neutral density filters. The sample was located at a distance of 8 inches from the light source in a water bath maintained at 25 to 30°C. The light source was covered by a 400 nm cut-off filter, Corning filter No. 3389, to eliminate UV light during illumination. The light intensity was measured in mW/cm².

The light source used to light-adapt bR before reading absorption spectra was a GE FCR quartzline lamp. The Variac was set at 120 for this purpose. The light intensity at the lamp was measured by placing the light sensor of a LI-COR LI-185 Quantum/Radiometer/Photometer next to the lamp but protected by Kodak neutral density filter 1.3, cutting off 95% of the light. The LI-COR Photometer was kindly loaned by the Chemical Biodynamics Laboratory of Lawrence Berkeley Laboratory. The accuracy of the LI-COR Photometer was tested against a recently calibrated Weston 75 Radiometer at Lawrence Berkeley Laboratory and found to read slightly higher, by a factor of 1.45, between 20 and 250 footcandles from a light source. The measurements were necessarily recorded in μEinstein/m²sec and converted to mW/cm² by converting at 592 nm. 592 nm was determined to be the converting wavelength by a calculation described in Appendix 1.

This GE FCR quartzline lamp was also used as the actinic light source for producing the M₄ photostationary steady state absorbance.
However, in order to measure the light intensity in this case, the LI-COR sensor was placed directly in front of the cuvette in the Aminco DW2 Recording Spectrophotometer, and the actinic light was moved back to compensate this 1 cm distance. Corning filter No. 3-67 separated the actinic light source from the LI-COR Photometer as will be described in Materials and Methods V.B.2. The converting wavelength in this case was determined in the manner described in Appendix 1, taking into account that Corning filter No. 3-67 is a low wavelength cut-off filter which has 50% transmission at 550 nm. The Variac setting was 120 for this measurement.

As a further check of the accuracy of the LI-COR Photometer to measure the light intensity inside the cuvette area during illumination of the 412 nm photostationary steady state absorbance, actinometry was performed according to the method of Jagger (6) in collaboration with Dr. O'Hara Augusto. This method measures the conversion of Fe$^{5+}$ to Fe$^{2+}$ produced by illuminating a potassium ferrioxalate solution in the presence of oxygen and then measuring the color produced at 510 nm. The number of moles of Fe$^{2+}$ caused by the illumination is determined by comparing to a standard curve made by reacting known quantities of ferrous sulfate with the actinometry solution. A typical standard curve is shown in Figure 2. The procedure for the reaction was to combine either 0.1 M ferrous sulfate or illuminated 6 mM potassium ferrioxalate solution (0.3-5.0 ml) with 0.1 N H$_2$SO$_4$ to a final volume of 10 ml. Then 2 ml of 0.1% phenanthroline and 8 ml of 1 N acetate buffer (pH 5.0) were added. The color was allowed to develop for 30 minutes and was then read in a Bausch and Lomb Spectronic 100 at 510 nm. Illuminated samples were run in duplicate at two time points. Illumination of the actinometry solutions was through two solution
Figure 2

Absorbance at 510 nm

Actinometry Standard Curve

FeSO$_4 \times 10^6$ Moles
filters transmitting only light of 436 nm, since the quantum yield of Fe$^{2+}$ produced by potassium ferrioxalate actinometry is well known at 436 nm (7). The solution filters were saturated sodium nitrite and saturated copper nitrate prepared according to Davies and Manning (8). A comparison of light intensities measured with the LI-COR probe and Jagger actinometry is given in Table 1. As shown, the Jagger actinometry indicated that the LI-COR probe might have been measuring too high by a factor of 3.7 instead of 1.45 as was originally thought from comparing to the Weston light meter.

The light source used to illuminate the suspension of bR lipid vesicles was a GE CYC Projector Lamp with Variac setting of 100. The light intensity at the cuvette was measured with the LI-COR Photometer in μEinsteins/M$^2$ sec. These units were converted to mW/cm$^2$ at 592 nm as described in Appendix 1.

The lamp used to illuminate the EPR cavity to study surface charge changes during photocycling was a Sylvania EHT Quartz Bromine Lamp without a Variac. The light intensity was first measured next to the lamp using the LI-COR Photometer protected by Kodak neutral density filter 1.3, cutting off 95% of the light intensity. The wavelength used for converting μEinsteins/M$^2$ sec to mW/cm$^2$ was 592 nm as determined previously for a quartzline lamp. In order to compare light intensity inside the EPR cavity with that next to the lamp, another type of actinometry was performed in collaboration with Dr. Rolf Mehlhorn and Anne Lewis, that measures the light-induced oxidation of a reduced spin probe. The procedure was to combine 10 μl of 10 mM flavin mononucleotide with 5 μl of 1 M TEMPOL, a spin probe that was prepared in the reduced form by Dr. Rolf Mehlhorn, (to be submitted to Photochemistry and Photobiology), in 1 ml of 30 mM potassium phosphate.
Table 1
Comparison of LI-COR Photometer with Potassium Ferrioxalate Actinometry

<table>
<thead>
<tr>
<th>Light Source</th>
<th>LI-COR Photometer</th>
<th>Actinometry</th>
<th>LI-COR Actinometry</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intensity Measured in $\mu$Einsteins/$M^2$ sec</td>
<td>Intensity Measured in $\mu$Einsteins/$M^2$ sec</td>
<td></td>
</tr>
<tr>
<td>For 412 nm Photostationary Steady State Absorbance (In Cuvette)</td>
<td>52</td>
<td>$14 \pm 1.3$ (4)</td>
<td>3.67</td>
</tr>
<tr>
<td>For EPR (Next to Lamp)</td>
<td>370</td>
<td>101.1 (1)</td>
<td>3.66</td>
</tr>
</tbody>
</table>
buffer, pH 7.0, that had been bubbled with nitrogen for 10 minutes. Individual samples were prepared by removing 40 μl of this solution and placing in sealed glass capillary tubes. During illumination the peak height of the three lines of the EPR spectrum increased. The peak height of the unilluminated capillary was determined first and subtracted from the peak height of the illuminated sample. Average values for duplicate samples illuminated 5 seconds and 2 seconds next to the lamp were obtained and compared to values of duplicate samples illuminated for the same times within the EPR cavity. The results of the TEMPOL actinometry shown in Table 2 indicate that the light intensity was only slightly higher outside the cavity than inside the cavity. The ratio and standard deviation from Table 2 were used to convert the light intensity measured outside the EPR cavity with the LI-COR Photometer into the light intensity inside the EPR cavity. The standard deviation of the Outside/Inside Ratio shown in Table 2 was calculated using the average percent deviation of the four EPR Peak Height/Gain Ratios listed in Table 2. A summary of all the measured and converted light intensities used for the experiments in this thesis appears in Table 3.

I. C. Centrifugation Procedures

Centrifugation speeds in Materials and Methods appear as "x g". For g forces smaller than 20,000 x g, the Sorvall RC-5B Centrifuge was used with Sorvall rotors GSA and GS3. For higher g forces, the Beckman L2-75B Ultracentrifuge was used with Beckman rotors SW41, 42.1 or 50 Ti. All centrifugation procedures were carried out with refrigeration at 5°C.
Table 2
TEMPOL Actinometry Measurements

<table>
<thead>
<tr>
<th>Position of Actinometry Capillary</th>
<th>Time of Illumination</th>
<th>EPR Peak Height Gain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Outside the Cavity</td>
<td>2 seconds</td>
<td>.0011 ± .00014</td>
</tr>
<tr>
<td></td>
<td>5 seconds</td>
<td>.00231 ± .0018</td>
</tr>
<tr>
<td>Inside the Cavity</td>
<td>2 seconds</td>
<td>.00084 ± .00016</td>
</tr>
<tr>
<td></td>
<td>5 seconds</td>
<td>.00154 ± .000375</td>
</tr>
</tbody>
</table>

Outside Inside Ratio
2 seconds = 1.31
5 seconds = 1.50
Average = 1.41 ± 0.47
### Table 3

**Summary of Light Intensities**

<table>
<thead>
<tr>
<th>Light Source</th>
<th>Converting Wavelength</th>
<th>LI-COR Photometer Intensity Measured in µEinstein/s/M² sec</th>
<th>Converted Intensity in mW/cm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>For Bleaching bR</td>
<td>592 nm</td>
<td>20,000</td>
<td>300 (YSI Measurement)</td>
</tr>
<tr>
<td>For Light Adaptation</td>
<td>592 nm</td>
<td>20,000</td>
<td>404</td>
</tr>
<tr>
<td>For 412 nm Photostationary Steady State Absorbance</td>
<td>632 nm</td>
<td>410</td>
<td>7.8</td>
</tr>
<tr>
<td>For pH Electrode</td>
<td>592 nm</td>
<td>2,850</td>
<td>58.6</td>
</tr>
<tr>
<td>For EPR</td>
<td>592 nm</td>
<td>8,000 (Next to Lamp)</td>
<td>162 (Next to Lamp)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>115 ± 38 (Inside Cavity)</td>
</tr>
</tbody>
</table>
I. D. Stability of bR's 568 nm Chromophore

The stability of the bR 568 nm chromophore structure to variations in pH was determined by combining 100 µl of light-adapted bR (0.3 mg/ml) with 1 ml of distilled water of varying pH's between 3.0 and 11.0. The pH of these solutions had been preadjusted with HCl or NaOH. The absorption at 568 nm of these bR solutions was read using the high sensitivity slide wire on the Cary 14R. Following this, the pH was redetermined using a Fisher Accumet Model 23-A pH meter at room temperature. The pH dependence in distilled water was determined on two separate sets of samples.

In order to determine the effect of buffer salt on the pH dependence of bR's chromophore at high pH, a similar assay to that described above was carried out in 0.2 M phosphate buffer.

II. Chemical Modification of Lysine Residues in bR

A. Imidoester Reaction

Monofunctional imidoesters react with protein amino groups to maintain lysine's positive charge and bifunctional imidoesters covalently cross-link lysines (9). The reaction is depicted in Figures 3 and 4 and is described in detail in Appendix 2.A.

1. Imidoesters Employed

The monofunctional imidoesters used to modify bR were ethyl acetimidate (EA, 4.3 Å functional chain length), methyl acetimidate (MA, 2.8 Å) and methyl butyrimidate (MB, 5.8 Å). The bifunctional imidoesters used to modify bR were dimethyl suberimidate (DMS, 11.3 Å) and dimethyl adipimidate (DMA, 8.3 Å). (See Appendix 2.A. for chemical formulae).
Figure 3. Imidoester reaction (taken from Kent, S., (9)). This figure depicts the imidoester reaction between the unprotonated form of a protein amino group and a monofunctional imidoester. The tetrahedral intermediate is fairly stable at pH's near 8.0, but not 10.0, as will be explained in Appendix 2. The desired product is the N-alkyl amidine.
Figure 4

Possible Mechanism for Cross-Linking by a Monofunctional Imidoester

![Chemical Diagram]

Protein—NH<sub>3</sub> + \( \text{CH}_3-\text{C}-\text{OR} \) → Protein—NH<sub>2</sub>

Protein Amino Group 1  Monofunctional Imidoester

Protein—NH<sub>2</sub> + NH<sub>3</sub> → \( \text{CH}_3-\text{C}-\text{NH-Protein 1} + \text{ROH} \)

Protein Amino Group 2  N-Alkyl Acetimidate Intermediate  Cross-Linked Protein(s)

Figure 4. Possible mechanism for cross-linking by a monofunctional imidoester (taken from Kent, S. (9)). At pH's near 8.0, where the N-alkyl intermediate is fairly stable, a second protein amino group may react with this intermediate as if it were an imidoester. This would cross-link amino groups within one bR protein or between bR proteins in the purple membrane.
II. A. 2. Reaction Conditions

a. Variation of Imidoester Concentration and pH

For most of the experiments, a series of stock solutions of imidoester was prepared by mixing a weighed amount of imidoester into an aqueous solution of 400 mM borate or carbonate buffer, adjusted to pH 10.3. Immediately after the addition of imidoester, the pH dropped to pH 10 or slightly below. Adjustment of the pH to 10 was then achieved by adding a few drops of dilute NaOH within a few seconds. Decreasing concentrations of imidoester were prepared by serially diluting the most concentrated stock into 400 mM buffer. The stock imidoester solutions were added to equal volumes of purified PM in distilled water to a final imidoester concentration between 0.1 and 200 mM. Twice as much monofunctional imidoester as bifunctional was used for an expected equivalent amidination. The molar ratio of monofunctional imidoester:bR varied from 9:1 to 17,000:1. Generally the reaction was allowed to proceed for 30 minutes at 22°C and then the reaction mixture was diluted with 200 ml cold distilled water and centrifuged at 180,000 x g for 20 minutes. The pellet was resuspended in 25 ml cold distilled water and centrifuged as above. This washing procedure was repeated once more and the final pellet was taken up in 1-2 ml distilled water.

In early experiments, the imidoester reaction was carried out in 20 mM triethanolamine (TEA) buffer, pH 8.0. Before chemical modification, bR samples in distilled water were light-adapted for 30 minutes by exposing to visible light of 25 mW/cm² (estimated intensity at the samples) at 20-25°C. The light source was a 150 watt tungsten GE light bulb, 6-10 inches above the samples in a shaking water bath. In order to ascertain if loss of the 568 nm chromophore occurred under these
conditions of light-adaptation, the 568 nm chromophore absorption was monitored at 15 and 30 minute intervals up to 2 hours. The chemical modification reaction procedure was to prepare a stock imidoester solution in 0.2 M TEA buffer, pH 8.0, with an imidoester concentration 10 times more concentrated than the final imidoester concentration, by weighing imidoester salt into the TEA buffer. An equivalent amount of NaOH had been previously added to the TEA buffer to raise the pH slightly above 8.0. Immediately after addition of imidoester to the TEA buffer, the pH was checked and readjusted if necessary to pH 8.0, and 0.5 ml of imidoester stock was added to 4.5 ml of bR (0.3 mg/ml) in distilled water. New stock was prepared after one hour and added similarly. After another hour, the reaction mixture was diluted with 20 ml cold distilled water and centrifuged at 180,000 x g for 20 minutes. The pellet was resuspended in 25 ml cold distilled water and centrifuged as above. This washing procedure was repeated once more and the final pellet was taken up in 1-2 ml distilled water. Alternatively, after the first centrifugation, excess reagent was dialyzed out overnight against 2 liters of distilled water.

II. A. 2. b. Successive Imidoester Treatments

The effect of four successive amidinations using 3.5, 5, 7 and 10 mM DMS was examined by treating bR samples four successive times. The effect of three successive amidinations using 5 and 10 mM DMA, and 10 and 20 mM MA was also examined.
II. A. 2. c. Variation of Temperature and Protein Concentration

The effect of varying the temperature between 5° and 60° C was also investigated. Water baths adjusted to various temperatures in this range were used to maintain the temperature constant during the 30 minute amidination reaction at pH 10. The effect of varying protein concentration between 0.005 and 0.4 mg/ml during amidination with 10 mM DMS was also investigated. The treated pellets were collected as above.

3. Imidoester Modification of bR in Bleached PM

The effect of amidinating bleached bR, i.e., with the retinal removed, was also investigated. bR was bleached essentially according to the method of Becher and Cassim (10). PM was added to a 1.0 M hydroxylamine solution (pH 7.0), so that the final concentration was 0.5-1.0 mg/ml. This solution was exposed to light of 300 mW/cm² at 25-30° C for 5 hours. The bleached PM was centrifuged at 100,000 x g for 30 minutes. The bleached PM pellet was washed three times by resuspending in 25 ml distilled water and centrifuging at 100,000 x g for 30 minutes. The final pellet was taken up in 5 ml distilled water and treated with imidoester at pH 10 as described above.

In order to regenerate bR's 568 nm chromophore after imidoester modification, one mole of all-trans retinal from a 100 mM stock solution in ethanol was added per mole of bR protein. Regeneration was carried out in dim light for 1/2 hour. The percent regeneration was determined by comparing the 568 nm peak height of a known amount of
regenerated bR protein determined by a modified Lowry procedure (see Materials and Methods II.C.2. and (5)) to the 568 nm peak height of the same amount of control bR, one hour and one week after regeneration.

II. B. Pyridoxal Phosphate Reaction

Pyridoxal phosphate reacts with protein amino groups as shown in Figures 5 and 6, changing lysine's charge from positive to net negative (11).

1. Reaction Conditions

Pyridoxal phosphate modification of bR was carried out with 0.81 mg/ml bR, 25 mM pyridoxal phosphate and 37.5 mM phosphate buffer at pH 7.2. Pyridoxal phosphate was first dissolved in 75 mM phosphate buffer to a final concentration of 50 mM and the pH adjusted to 7.2. This stock solution was next added to PM in distilled water and the reagent was left in place to prevent reversal of the modification.

2. Reagent Removal

The modified bR sample was analyzed as will be described for imidoester-treated bR samples, except that the reagent had to be removed for flash photolysis, 412 nm photostationary steady state absorption, and fluorescence measurements, since pyridoxal phosphate absorbs at 388 nm (ε=3.7)(11). Reagent removal had to be carried out quickly since the modification reverses in the absence of reagent (11). This was attempted by centrifuging at 100,000 x g for 15 minutes, removing the supernatant and resuspending the pellet
Figure 5

Pyridoxal Phosphate Reaction

Protein $\rightarrow \text{NH}_2$

Protein Amino Group

Protein $\rightarrow \text{NH}_2$

Pyridoxal Phosphate

$\lambda_{\text{MAX}} = 420 \text{ nm}$

Reduced Schiff Base Pyridoxamine

$\lambda_{\text{MAX}} = 340 \text{ nm}$
Figure Legend 5. Pyridoxal phosphate reaction (taken from Means and Feeney, (11)). One way that pyridoxal phosphate interacts with protein amino groups is by forming a Schiff base intermediate that can be reduced with NaBH₄ at neutral pH. The Schiff base intermediate can be identified spectroscopically since it absorbs at 420 nm.
Figure 6

Alternative Pyridoxal Phosphate Reaction

Protein—NH₃⁺

Protein—NH₂

Protein Amino Group

Protein—NH—C

Protein—NH—C

Protein—NH—C

Protein—NH—C

Pyridoxal Phosphate

Pyridoxal Phosphate

Aldamine

λ_MAX = 330 nm
Figure Legend 6. Alternative pyridoxal phosphate reaction (taken from Means and Feeney, [11]). Pyridoxal phosphate can also combine with protein amino groups to form an unreactive aldamine that absorbs at 330 nm. The aldamine can be transformed into the Schiff base form in Figure 5 by dehydration.
in 9 ml of 10 mM phosphate buffer at pH 7.0. The measurements were carried out within 15 minutes of resuspension. In order to determine the number of lysines modified by pyridoxal phosphate by a procedure which will be described, the pyridoxal phosphate adduct had to be first reduced before the reagent was removed. Reduction of the adduct was in 100 mM sodium borohydride, by adding 1.9 mgs of dry reagent to 0.5 ml samples and letting them react for 5-10 minutes before centrifugation.

II. C. Chemical Analysis of Lysine Modified bR
1. Fluorescamine Assay

After modification with a lysine-specific reagent, the extent of modification was examined by a procedure developed by Udenfriend et al. (12,13) that detects free amino groups in proteins (see Figure 7). bR protein was first solubilized as completely as possible using the anionic detergent dodecyl sodium sulfate.

The procedure was to make an aliquot of a bR solution in a test tube in duplicate, from 1 to 50 nanomoles lysine, usually in a volume of 0.1 ml or less. To this solution was added 1.5 to 2.0 ml of 0.1 M sodium borate buffer in 0.1 N NaOH (pH 9.5-10) and 50 μl 20% SDS detergent. The solutions were vortexed and allowed to stand at room temperature for 1/2 hour. A solution of fluorescamine powder dissolved in acetone (15-30 mg/100 ml) was rapidly added dropwise while holding the test tube on a vortex mixer. The fluorescence was read at 480 nm with excitation at 390 nm in a Perkin Elmer MPF-44A Spectrofluorimeter. Relative fluorescence of the modified sample was determined by dividing
Figure 7. Fluorescamine reaction (taken from Udenfriend, S. (12)). The reaction of fluorescamine with amino groups occurs with a $t_{1/2}$ of 100-500 milliseconds producing the fluorophor shown. Unreacted fluorescamine hydrolyzes with a $t_{1/2}$ of 5-10 seconds to yield non-fluorescent products.
the fluorescence of the modified sample by the fluorescence of the control bR at the same Lowry protein concentration on the standard curve. A typical fluorescamine standard curve appears in Figure 8. Calculation of the standard error associated with the relative fluorescence is described in Appendix 3.

In order to determine the number of lysines modified, the average relative fluorescence for a bR modified sample was first multiplied by the number of free amino groups for the control as determined by amino acid analysis (see Results Section II.A.1.). Since one lysine is attached to retinal by a Schiff base linkage, it will not react with fluorescamine (personal communication, Dr. Stanley Stein). Retinal is thought to remain attached to the protein even in the presence of SDS since Schiff bases are stable in basic solution. This number was subtracted from the number of lysines in the control to determine the number of modified lysines.

In order to confirm that the Schiff base did not react with fluorescamine, this test was performed on two sets of bR controls, one of which had been previously reduced with 100 mM NaBH₄ to covalently attach the Schiff base retinal to lysine. A difference in fluorescence between these two bR sets of controls would indicate a different number of lysines reacting with fluorescamine in reduced bR. As shown in Figure 9, there was no difference in these standard curves, suggesting that the Schiff base lysine does not react with fluorescamine.

II. C. 2. Lowry Protein Determination

Protein was determined by a modification of the Lowry test (5). To carry out the modified Lowry test, a standard curve was first constructed of 5 concentrations in duplicate of bovine serum
Figure 8

Typical Fluorescamine Assay

Fluorescence Units

Amount of bR Protein (µg)
Figure 9

Effect of NaBH₄ on the Fluorescamine Assay

Fluorescence Units

Without NaBH₄, ○
With NaBH₄, △

Microliters bR Solution (1.13 mg/ml in Water)
albumin from 10 to 50 µg in 0.1 ml in test tubes. Next, 1.0 ml of a mixture of freshly prepared Reagent A and Reagent B was added and allowed to remain at room temperature for 10 minutes. The mixture was prepared as follows:

49 ml of Reagent B

20 gms Na₂CO₃

4 gms NaOH, diluted with distilled water to one liter

1 ml of Reagent A

0.5% CuSO₄

1 % NaK Tartrate

Then 0.15 ml of a 1:1 dilution of Folin and Ciocalteu's Phenol Reagent was added and immediately vortexed. The reaction was allowed to remain at room temperature for 30 minutes. The blue color that developed was read at 750 nm. Absorbance of the bR samples was compared to the standard curve to determine the protein concentration. A typical Lowry protein standard curve is shown in Figure 10.

II. C. 3. Gel Electrophoresis

The extent of oligomerization of bR caused by bifunctional and possibly monofunctional imidoesters was determined by 10% polyacrylamide gel electrophoresis essentially according to the method of Weber and Osborne (14).

Usually 2 liters of gel buffer were prepared, containing 7.8 gms NaH₂PO₄·H₂O, 3.16 gms Na₂HPO₄·7H₂O and 2 mgs of SDS. For the 10% acrylamide solution, 22.2 gms of acrylamide and 0.6 mgs of Bis-N,N'-methylene-bis-acrylamide were dissolved in water to give 100 ml of solution. Glass gel tubes 10-12 cm long with an inner diameter of 6 mm were cleaned by soaking overnight in chromic acid solution and thoroughly rinsing.
(To prepare 1 liter chromic acid solution, 400 gms of potassium dichromate was covered with 300 ml of water and mixed. To this saturated solution was then added 700 ml of concentrated sulfuric acid with stirring.) For a run of 12 gels, 15 ml of gel buffer was deaerated about 30 minutes and mixed with 13.5 ml acrylamide solution. After further deaeration, 1.5 ml of freshly made ammonium persulfate solution (15 mg/ml) and 5-10 ul of N,N,N',N'-tetramethylethlene-diamine were added. After mixing, each tube which was positioned exactly vertically in a stand especially designed for this purpose, was filled with about 2 ml of the polyacrylamide solution. Before the gel hardened a few drops of water were layered on top of the gel solution. Gels were left to polymerize for at least one hour.

In order to solubilize the bR samples and standard proteins, a solubilization cocktail was prepared as follows:

2.0 ml Glycerol
1.0 ml SDS (20%)
1.0 ml Tris (1.0 M)
0.1 ml Bromophenol Blue (20%)
4.0 ml Distilled Water
8.1 ml Total Volume

To 10 μl of protein (20-30 μg) in distilled water, 10 μl of solubilization cocktail was added. For the DMS protein concentration curve and the DMS/DMA temperature experiment equal amounts of control and modified bR were placed on each gel and 10 μl of cytochrome C (1 mg/ml) was added as an internal standard to quantitate the amount of protein that entered the gel. The mixture of protein and solubilization cocktail was allowed to stand at room temperature for at least one-half hour. With every gel run, at least four of the following molecular weight standards
were included: trypsin inhibitor (MW: 6160), cytochrome C (MW: 11,700),
lysozyme (MW: 14,300), α-chymotrypsin (MW: 25,700), D-amino acid oxidase
(MW: 37,000), bovine serum albumin (MW: 68,000) and β-galactosidase
(MW: 130,000).

The gels were placed into a Bio-Rad Model 150A Electrophoresis
Apparatus, powered by a Heathkit Regulated H.V. Power Supply. The
two compartments of the electrophoresis apparatus were filled with
gel buffer which covered the gel tubes. 20 μl solubilized protein
sample was carefully layered on top of each gel with a Hamilton syringe.
Electrophoresis was carried out at 5-6 mamps per gel. For twelve
gels the current was set at 60-72 mamps and the samples were allowed
to enter the gels. After this occurred (in 5-10 minutes) the current
was switched off and any excess sample was removed with a syringe. Then
electrophoresis was restarted at the same current, 60-72 mamps. Elec-
trophoresis was allowed to continue until the ion front reached within
1/2 cm of the end of the gel, about 5-6 hours. After the gels had
been removed from the gel tubes by injecting a stream of water
through a fine needle between the gel and the gel tube, and then
ejecting the gel into a beaker of water by applying air pressure,
the ion front was marked with a needle containing indelible ink.

The protein bands on the gels were identified using a Coomassie
Blue (see below) staining procedure with 5 hours of staining in
Stain I followed by 5 hours in Stain II. After staining, the gels
were destained for two days in a Bio-Rad Gel Electrophoresis Diffusion
Destainer, that contained a destaining solution that was changed
2-3 times.
Stain I:

0.25 gm Coomassie Brilliant Blue
250 ml Isopropanol
100 ml Glacial Acetic Acid
Diluted to 1 liter with distilled water

Stain II:

0.025 gm Coomassie Brilliant Blue
100 ml Isopropanol
100 ml Glacial Acetic Acid
Diluted to 1 liter with distilled water

Destaining Solution:

250 ml Methanol
75 ml Glacial Acetic Acid
675 ml Distilled Water

After staining and destaining, the gels were scanned using a Beckman DU Spectrophotometer fitted with a Gilford Automatic Cuvette Positioner and a Gilford Instrument Recorder. A Gilford 2414 Gel Cuvette was used.

The molecular weights of the bR bands were determined by comparing to a standard curve of the molecular weight standards. The log of the molecular weights of the standards vs. the relative mobilities (distance traveled of the standard/total distance from the start of the gel to the ion front) were plotted to make the standard curve. A typical gel calibration curve is shown in Figure 11. The areas of the peaks on the gel scans were calculated by the method of triangulation. For the bR samples, the percentage of each peak's area
Figure 11

Typical Calibration Curve with Weber and Osborn Gel Electrophoresis

Molecular Weight

\[ \times 10^4 \]

\begin{align*}
\Delta & \quad \beta\text{-Galactosidase} \\
\Delta & \quad \text{Bovine Serum Albumin} \\
\Delta & \quad \text{D-Amino Acid Oxidase} \\
\Delta & \quad \text{Chymotrypsin} \\
\Delta & \quad 17,800 \text{ Molecular Weight Standard} \\
\Delta & \quad \text{Cytochrome C}
\end{align*}

Relative Mobility
of the total area of all the bands was determined. In order to quantitate the amount of total protein that should have entered the gel in the imidoester protein concentration curve experiment and the imidoester temperature experiment, the fraction of the 10 \( \mu g \) cytochrome C standard that had entered the gel was calculated by comparing to a gel of control bR with 10 \( \mu g \) cytochrome C. The ratio of bR protein to cytochrome C should have been the same in all the samples unless some of the bR protein did not enter the gel due to oligomerization. Equal amounts of control and oligomerized bR necessarily had to be placed on each gel in order to quantitate the percent entering in this way.

III. Chemical Modification of Arginine Residues in bR

A. 2,3-Butanedione (BD) Modification of bR

In the presence of 50 mM borate buffer, BD adds a negative charge to the positive charge of arginine (see Figure 12 and Appendix 2.B.).

1. Reaction Conditions of BD Modification

The BD modification of bR was usually carried out for three hours at room temperature, pH 8.2, 50 mM borate buffer, 0.4 mg/ml bR protein. BD was initially added to a 100 mM solution of borate buffer and the pH was readjusted to pH 8.2. Then this stock BD solution was added to purified PM in distilled water. After three hours the samples were diluted with 20 ml of cold 50 mM borate buffer, pH 8.2, and centrifuged at 160,000 \( \times g \) for 20 minutes. The supernatant was discarded and the pellet was resuspended in 25 ml 50 mM borate buffer
Figure 12

2,3-Butanedione Reaction

Protein Guanidinium Group 2,3-Butanedione 4,5-Dimethyl-4,5-dihydroxy-2-imidazoline

In the presence of borate buffer, Borate Complex
Figure 12. 2,3-Butanedione reaction (taken from Riordan, (15)).
The reaction of 2,3-butanedione with guanidinium groups occurs in less than three hours and the imidazoline adduct is maximally stabilized in the presence of 50 mM borate buffer, pH 8.2.
and recentrifuged as above. Washing of the pellet and recentrifugation were repeated once more. The final pellet was resuspended in a small volume of 50 mM borate buffer so that the final bR concentration was about 0.5 mg/ml.

II. A. 1. a. Variation of BD Concentration

The effect of varying the final concentration of BD in the reaction mixture over the range of 10-1000 mM was studied (molar ratio of BD:bR varied from 135:1 to 66,700:1). A series of stock solutions was first prepared, twice as concentrated as the final BD concentration in the reaction mixture, by adding varying amounts of stock BD (11.4 M) to several vials containing 5 ml 100 mM borate buffer, pH 8.2. Immediately after the addition of BD, and especially at the high concentrations, the pH became acidic and had to be readjusted to pH 8.2 with NaOH. At the high concentrations, a few drops of 4 M NaOH was sufficient.

b. Reagent Removal

One method of reagent removal was similar to that employed for the imidoester reaction. After three hours of reaction with BD, the modified sample was diluted to 25 ml with 50 mM borate buffer, pH 8.2, and centrifuged at 180,000 x g for 20 minutes. The pellet was resuspended in 50 mM borate buffer. The effect of additional centrifugations and washings was studied.

Alternatively, BD modified bR that had reacted for 3 hours was first centrifuged at 180,000 x g for 20 minutes to concentrate the sample. The pellet was resuspended in 50 mM borate buffer, pH 8.2, and then loaded onto a Sephadex G-25 column, according to (16), that was preequilibrated with borate buffer, pH 8.2, at 4°C.
To ascertain the presence of any remaining BD, an absorption spectrum using the Aminco DW2 Recording Spectrophotometer of the UV and visible regions was taken. Since BD's largest extinction is at 284 nm (ε=9.75) (see Appendix 2.B.), the ratio of 568/284 nm peaks was calculated and compared to this ratio in the control bR.

III. A. 1. c. Reaction Reversal

In order to determine if the BD modification had reversed after one of the following attempts to reverse it, the 412 nm decay kinetics were monitored. This indicator of bR's activity will be described fully in a later section of Materials and Methods (V.B.1.).

Duplicate samples from a concentration curve of BD modification were investigated for reversibility by a modification of (16). These samples had reacted with BD for three hours in 50 mM borate buffer pH 8.2, and were centrifuged at 180,000 x g for 25 minutes once. They were resuspended in 50 mM borate buffer pH 8.2 and flash photolysis activity was determined. They were then recentrifuged at 180,000 x g for 25 minutes. One-half of the duplicate samples was resuspended in 0.5 ml of 100 mM bicarbonate buffer, pH 8.2, and the other half was resuspended in 0.5 ml of 50 mM borate buffer, pH 8.2. These bR samples were combined with glycerol and loaded onto two Sephadex G-25 columns pre-equilibrated with these two buffers. After eluting the bR samples in the void volume, the bR eluates were examined by flash photolysis. The next step was to resuspend the bR samples from the bicarbonate column in 25 ml bicarbonate buffer and centrifuge at 180,000 x g for 25 minutes. This washing procedure was repeated.
two more times. The final pellets were resuspended in bicarbonate buffer and then flash photolysis activity was observed.

In addition, the effect of high salt and buffer on reaction reversal was studied. The simplest means to convey the procedure is with a diagram:

```
200 mM BD Reaction with bR in 50 mM borate buffer and 4 M NaCl, pH 8.2

Observed 412 nm decay (Sample #1)

Centrifuged at 100,000 x g for 30 minutes

Pellet

Resuspended in 500 ml 4 M NaCl; left to stand one day at 4°C. Observed 412 nm decay (Sample #3). Centrifuged as above.

1/2 Pellet

Left to stand two days at 4°C; centrifuged as above and resuspended in 500 ml 4 M NaCl. Observed 412 nm decay (Sample #2).

1/2 Pellet

Resuspended in 500 ml distilled water. Left to stand one day at room temperature. Observed 412 nm decay (Sample #4).

Resuspended pellet in 500 ml 4 M NaCl; left to stand one day at room temperature. Centrifuged as above.

Resuspended in 500 ml 4 M NaCl; left to stand one day at room temperature.

III. A. 2. BD Modification of bR in Cell Envelope Vesicles

Cell envelope vesicles of H. halobium were prepared according to (1) with a final protein concentration of 1.3 mg/ml.

The BD reaction was carried out in 4M NaCl in 50 mM borate buffer, pH 8.2,
for 3 hours at room temperature, using a final concentration of 500 mM BD. After three hours the reaction was stopped by diluting with 25 ml of 4 M NaCl with 50 mM borate buffer, pH 8.2, and centrifuged as described for the BD reaction, but always washing and resuspending in 4 M NaCl containing 50 mM borate buffer, pH 8.2, since the cell envelope vesicles need high salt to maintain their structure (1).

After treatment and washing, the cells were burst with distilled water and the purple membrane was concentrated by centrifugation at 180,000 x g for 20 minutes. The purple membrane was then purified free of red membrane by loading onto a sucrose gradient as described above in Materials and Methods I.A. This preparation was washed free of sucrose as described using distilled water and 10 mM borate buffer to stabilize the BD modification. The final pellet was stored in 10 mM borate buffer, pH 8.2.

III. B. Phenylglyoxal Modification of bR

In contrast to BD, the reaction product of phenylglyoxal (PGO) with arginine does not add a negative charge to the positively charged guanidinium group. The reaction is thought to proceed as in Figure 13 (17,18), and is described in Appendix 2.C.

1. Reaction Conditions

For PGO modification of bR the normal procedure was to react 0.9 mg/ml bR protein with 12.5 mM PGO in 50 mM bicarbonate buffer, pH 8.0, for at least 30 minutes.

2. Reagent Removal

Following each of the procedures listed below,
Figure 13

Phenylglyoxal Reaction

Protein $\text{Guani}^{-}\text{dinium Group}$  +  2 Phenylglyoxal $\rightarrow$ pH 8.0

Figure 13. Phenylglyoxal reaction (taken from Means and Feeney (19)). Two moles of phenylglyoxal are thought to react with one mole of protein guanidinium group in 30 minutes producing the adduct shown.
the UV and visible absorption spectra were taken as will be described in Materials and Methods V.A.1.

Experiment #1:

1) After the PGO reaction, bR samples were centrifuged 1-1/2 hours at 150,000 x g and resuspended in 25 ml 50 mM bicarbonate buffer, pH 8.2.

2) The samples were recentrifuged at 150,000 x g for 20 minutes and resuspended in 25 ml of 50 mM bicarbonate buffer, pH 8.2.

3) These bR samples were then dialyzed for two days at 4°C against 10 mM phosphate buffer, pH 8.0.

Experiment #2:

1) After the PGO reaction, bR samples were centrifuged at 150,000 x g for 40 minutes and resuspended in 50 mM bicarbonate buffer, pH 8.2.

2) These samples were then loaded onto a Sephadex G-25 column preequilibrated either with 10 mM bicarbonate buffer, pH 9.0, or 10 mM borate buffer, pH 9.0.

III. C. Chemical Analysis of Arginine Modified bR

1. Ninhydrin Fluorescence Test

One method that was used to detect arginine was a fluorescence test. This test involves the reaction of ninhydrin with guanidinium groups. In the presence of base, ninhydrin is converted to a molecule similar to PGO as follows:

\[
\text{Ninhydrin} + \text{KOH} \rightarrow \text{Ortho-carboxyphenylglyoxal}
\]
PGO is known to react with guanidinium groups as explained above (see Figure 13). The product obtained is fluorescent. It is necessary to wait at least 15 minutes before reading the fluorescence since 0-carboxyphenylglyoxal has a yellow color that interferes, but which hydrolyzes in 15 minutes (21).

Normally a standard curve was first prepared by placing 2 to 50 μg of bR in duplicate into test tubes and adjusting the volume to 1 ml with distilled water. Then 1 ml of 0.5% ninhydrin was added. After vortexing, 1 ml of 1.0 N KOH was added and the solution was again vortexed and left to stand at room temperature for 15-20 minutes. The fluorescence was read in a Perkin Elmer MPF-44A Spectrofluorimeter with an excitation wavelength of 390 nm and emission wavelength of 495 nm.

The best results were produced by including 150 μl ethanol in the 1 ml volume used for the bR standard curve and modified bR samples. These samples were then heated to 99° C for 1-2 minutes. The fluorescence produced by the chemically modified samples was compared to the fluorescence of the bR standard curve. The percent decrease in fluorescence was multiplied by the total arginines found by amino acid analysis of the control (see Results II.A.1.). This number was then subtracted from the total number of arginines in the control to yield the number of modified arginines.

III. C. 2. Amino Acid Analysis

The bR samples were prepared for amino acid analysis by removing most of the 200 mM borate buffer and 2 M NaCl used for storing these samples by diluting the samples with 10 ml distilled water and centrifuging twice at 140,000 x g for 40 minutes. The pellets
were resuspended in distilled water and 100 μg of each bR sample was placed into a pyrex vacuum seal vial.

The sample analysis was carried out by Dr. William Brown with the assistance of Mrs. Wei-Duan Lin Liao at Mellon Institute of Carnegie-Mellon University, Pittsburgh, Pennsylvania. The method of ion-exchange chromatography was essentially that of Moore, Spackman and Stein (22). Acid hydrolysis of the sample was carried out in 1 ml of Constant Boiling HCl for 24 hours at 110° C. The hydrolyzed residue was dissolved in 0.2 N NaCitrate buffer, pH 7.0. The instrument used for ion-exchange chromatography was a Durrum (Dionex) Model D-500 Amino Acid Analyzer. The D-500 uses a single ion-exchange column with three changes of buffer. The first buffer is 0.2 N NaCitrate, pH 3.25, the second buffer is 0.2 N NaCitrate, pH 4.25, and the third buffer is 1.1 N NaCitrate, pH 7.0. The peaks were computer analyzed by a PDP 8-A Computer and the results were presented in nanomoles. Nanomoles were converted to numbers of amino acids by two methods. The first method used a converting factor that was multiplied by the number of nanomoles for each amino acid.

Converting factor:

\[
\frac{\text{Number of amino acids (Ovchinnikov (23) and Khorana (24))}}{\text{Total number of nanomoles in this study}}
\]

A second method used the minimum standard deviation from whole numbers obtained with different converting factors, by setting lysine equal to between 6.5 and 7.0. Details of these procedures will be presented with Results II.A.1.

The number of modified arginines was determined by subtracting the number of arginines in the chemically modified samples from the number of arginines in the control.
III. C. 3. Possible Modification of Lysine Residues

The fluorescamine test (see Materials and Methods II.C.1.) was carried out on BD modified bR samples that had been washed three times as described above, in order to detect the possible modification of lysine residues by BD. This test was also carried out on PGO modified bR samples that had been diluted with distilled water after the PGO reaction.

IV. Double Chemical Modifications

A. EA or BD Followed by Iodination

EA modification of lysine residues was carried out as described above with 20 mM final EA concentration in 0.2 M carbonate buffer, pH 10.0, 0.2 mg/ml bR. BD modification was also carried out as described above with 200 mM final BD concentration in 50 mM borate buffer, pH 8.2, 0.4 mg/ml bR. The resulting chemically modified pellets were resuspended in distilled water and the protein determined by the Lowry method (see Materials and Methods II.C.2. and (5)).

Iodination was carried out in collaboration with Dr. Peter Scherrer with an iodinating mixture specific for tyrosine (25) since there are no histidyl or cysteinyl residues in bR. Iodination occurs as in Figure 14. The following sequential additions were made:

<table>
<thead>
<tr>
<th>Reactant</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>bR</td>
<td>1 mg/ml</td>
</tr>
<tr>
<td>NaPO₄, pH 7.2</td>
<td>50 mM</td>
</tr>
<tr>
<td>α-D-Glucose</td>
<td>20 mM</td>
</tr>
<tr>
<td>Water</td>
<td>To adjust final volume</td>
</tr>
<tr>
<td>Lactoperoxidase</td>
<td>0.13 units/ml</td>
</tr>
<tr>
<td>Glucose Oxidase</td>
<td>0.13 units/ml</td>
</tr>
<tr>
<td>NaI</td>
<td>0.78 μM</td>
</tr>
</tbody>
</table>
Figure 14

Iodination of Tyrosine

\[
\text{Protein} + I_3^- \rightarrow \text{Protein} + H^+ + 2I^-
\]

Figure 14. Iodination of tyrosine (taken from Means and Feeney, (25)). Iodination by lactoperoxidase and glucose oxidase in the presence of \(\alpha\)-D-glucose and NaI, pH 7.0, iodinates tyrosine in the positions ortho to the phenolate oxygen.
The time of iodination was varied from 0 minutes to 24 hours. To quench the reaction, 0.5 ml of the reaction mixture was mixed into 3 ml of 50 mM phosphate buffer, pH 7.2, containing 15 μl of 0.1 N sodium thiosulfate. The number of tyrosines modified was determined on a bR sample that had been iodinated only, by spectrophotometric NaOH titration according to Edelhoch (26) in collaboration with Dr. Peter Scherrer, and also by X-ray fluorescence by Dr. Stanley Seltzer. The reagent was removed in order to quantitate the extent of iodination, by centrifugation at 100,000 x g for 30 minutes, and then washing twice by centrifugation with distilled water. Before X-ray fluorescence the protein was separated from the lipid phase using a methylene chloride extraction procedure (27), to eliminate nonspecific binding of iodine to the lipid (27). These two methods were compared and the number of tyrosines in the doubly modified samples was assumed to be the same as a singly iodinated sample at the same 568 nm chromophore absorbance, since neither 20 mM EA nor 200 mM BD decreased the chromophore absorbance appreciably. For activity measurements, the reagent was left in place and the samples were stored at 4°C.

IV. B. EA Followed by 1-Ethyl-3-(3-dimethylaminopropyl) Carbodiimide (EDC), and EDC Followed by EA

bR was first treated with EA as described previously with 200 mM final concentration of EA in 0.2 M carbonate buffer, pH 10.0, for 30 minutes. The washed pellet was then treated with 50 mM EDC, a water soluble carbodiimide, in collaboration with Jeffrey Herz. The reaction is thought to occur as in Figure 15, with possible involvement of protein amino groups (28). The reaction was carried out in 100 mM 2-(N-morpholino)ethane sulfonic acid (MES) buffer, pH 5.6, for
Figure 15
Carbodiimide Reaction Involving Amino Groups

Protein $\begin{array}{c} \equiv \\ \equiv \end{array}$ + $\begin{array}{c} \equiv \\ \equiv \end{array}$ + $\begin{array}{c} \equiv \\ \equiv \end{array}$ $\rightarrow$

Protein Carboxyl Group  Carbodiimide

Protein $\begin{array}{c} \equiv \\ \equiv \\ ^{\text{NH}}R \end{array}$ + Protein $\begin{array}{c} \equiv \\ \equiv \end{array}$ $\rightarrow$

Unstable O-Acylisourea  Protein Amino Group or Any Nucleophile

Protein $\begin{array}{c} \equiv \\ \equiv \\ \equiv \end{array}$ + R'$\begin{array}{c} \equiv \\ \equiv \end{array}$ $\rightarrow$

Substituted Amide  Substituted Urea

Alternatively, rearrangement of the O-Acylisourea produces

Protein $\begin{array}{c} \equiv \\ \equiv \\ \equiv \end{array}$ $\rightarrow$

N-Acylurea

$\left[ \begin{array}{c} \text{CH}_3\text{CH}_2\text{N} \equiv \text{C} \equiv \text{N} \equiv \text{CH}_2\text{CH}_2\text{CH}_2\text{N} \equiv \text{CH}_3 \end{array} \right]$

1-ethyl-3-(3-dimethylaminopropyl) carbodiimide

EDC
24 hours at 25°C, with a bR concentration of 1 mg/ml. The carbodiimide solution was prepared in 100 mM MES buffer at pH 5.6 immediately prior to use. After the reaction, bR samples were dilute 10-fold with cold, distilled water and centrifuged at 100,000 x g for 30 minutes. The supernatant was discarded and the washing procedure was repeated twice. The final pellet was resuspended in distilled water. The number of carboxyl groups modified was quantitated by addition of a nucleophile, glycine methyl ester, in an EDC reaction without prior EA modification. The number of additional glycines/bR molecule as determined by amino acid analysis represents the number of EDC-modified carboxyl groups (28).

One sample that had been treated with 50 mM EDC was then divided and treated with increasing concentrations of EA as described in Materials and Methods II.A.

IV. C. BD Followed by EDC

BD modification was carried out as described in Materials and Methods III.A. with 500 mM final BD concentration. The three times washed, treated pellet was then treated with 50 mM EDC as described above.

V. Structural and Functional Analysis of Chemically Modified bR

A. Structural Techniques

1. Absorption Spectroscopy

Absorption spectra of washed, chemically modified and control bR samples were obtained using an Aminco DW2 Recording Spectrophotometer in the split beam mode by scanning a dilute solution (0.15 mg/ml) of bR in 10 mM phosphate buffer, pH 7 or 8,
from 250 to 700 nm at absorbance 1.0 with a chart speed of 2 nm/sec. All samples were preilluminated at least for 30 seconds with light of 404 mW/cm² to insure that the samples were completely light-adapted. The temperature of the samples was maintained at 20-22° C during spectral measurements with a HAAKE Circulating Water Bath. Changes in the 568 nm chromophore peak height were related to the amount of protein present as determined by the Lowry protein assay. Spectral shifts of the 568 nm wavelength maximum were noted.

Alternatively, the absorption at 568 nm was read using a Cary 14R Recording Spectrophotometer in a similar manner to the Aminco measurement. In the case of one chemically modified sample, with one lysine modified by DMS, the pH dependence of the light-adapted 568 nm chromophore was compared to the pH dependence of the 568 nm chromophore of the control sample between pH 1.85 and 10.5. 10 µl of control or DMS modified sample was diluted into 1 ml 10 mM phosphate buffer of varying pre-adjusted pH's. After the spectra were read, the pH's of these solutions were measured again with a Fisher Accumet Model 23-A pH meter at room temperature.

Absorption spectroscopy was also the first indication of any remaining reagent, or adducts formed. Occasionally, the absorption spectra of bR with the reagent in situ were taken.

V. A. 2. Tryptophan Fluorescence Spectroscopy

The tryptophan fluorescence spectra of bR control and chemically modified samples was studied in dilute protein solution (0.15 mg/ml) in 10 mM phosphate buffer, pH 7 or 8. The samples were at 22° C before and during the measurement in a Perkin-Elmer MPF-44A Spectrofluorimeter in the direct energy mode.
Temperature was controlled by a HAAKE FK2 Circulating Water Bath.

Excitation spectra were taken between 270 and 330 nm with emission at 315 nm, and emission spectra were taken between 300 and 340 nm with excitation at 287 nm.

In addition, the relative quantum yield of tryptophan fluorescence of chemically modified samples was also studied by comparing this fluorescence to the fluorescence of control bR at the same Lowry protein concentration. To do this, a standard curve was prepared of bR controls, and the tryptophan fluorescence (287 nm ex./315 nm em.) was read. Fluorescence quenching and spectral shifts were noted.

V. A. 3. Resonance Raman Spectroscopy

Chemically modified and control bR samples were examined by resonance Raman spectroscopy in collaboration with Mark Braiman and Greg Perreault. The samples examined by Mark Braiman were observed by one of the following methods:

1) with the sample in a 1 mm capillary, in which scattering from the initial 568 nm chromophore and subsequent photoproducts originated from a 48 mW 514.5 nm Argon laser or

2) by a flow technique (29) with the sample flowing in a tube passed the 12 mW 514.5 nm probe laser, so that scattering from the initial 568 nm ground state but not its photoproducts was seen. All experiments by Mark Braiman were carried out at room temperature, 20-22°C, and the flow rate in the flow experiments was about 300 cm/sec. The detection system was essentially that described in (30).

Samples examined by Greg Perreault were examined with 140 µW/µ² beam intensity at the sample in the backscattering (180 degree)
geometry for excitation and detection using a 457.9 nm laser beam from a model CR-12 Argon laser. This permitted use of optical glass cuvettes containing small magnetic stirrers to prevent settling and aggregation during long runs. The samples were sonicated briefly prior to observation. The cuvettes were sealed with parafilm to minimize loss of water by evaporation. The laser light was vertically polarized at the sample. No polarization scrambler was used in the collection system, since the range of the spectra was small. Scattered light from the sample was collimated and focused onto the entrance slit of a Spex 1401 double monochromator. Photons emerging from the exit slit were detected using an RCA C31032 GaAS photomultiplier tube thermoelectrically cooled to -40° C in a Model TE-104RF housing. Current pulses from the anode were amplified and selected using an amplifier/discriminator circuit set for gain = 100, threshold = 700 mV, pulse width = 30 nsec. Pulses from the discriminator were routed to a single channel printing scaler, a multichannel display scaler and a computer interface for counting and recording. The computer used was a Mod Comp 11/221 with a MAX III.F operating system. Multiple scans for each sample were added; noise spikes were removed before addition of the spectra either manually or using a program which searches for points outside 3 or 5 standard deviations. Spectra with many spikes were rejected.

V. A. 4. Circular Dichroism

Circular dichroism spectra of control and BD modified bR samples were obtained in collaboration with Dr. Bonnie Wallace. The samples were about 0.5 mg/ml in distilled water and were sonicated briefly prior to reading the spectra. A Cary 60 CD Spectrometer with model 6001 CD attachment was used. The spectra
were taken at 23° C, with a scan speed of 10 nm/minutes, 0.04 millidegrees full scale and time constant = 1. The spectra presented are bR spectra minus the spectrum of water.

V. B. Activity Measurements

1. Flash Photolysis Rise and Decay Kinetics of the M₄₁₂ Photocycle Intermediate

a. Conditions of Flash Photolysis and Apparatus

Flash photolysis was carried out at low concentrations of bR (0.15 mg/ml) and rise and decay kinetics of M₄₁₂ were studied as a function of pH, temperature and salt concentration. The buffer was usually 10 mM phosphate buffer, between pH 6.5 and 9.5. The temperature was usually maintained at 20, 22 or 24° C with a MGW Lauda RC3 Brinkman Circulating Water Bath. The pH dependence of M₄₁₂ flash photolysis kinetics was investigated in two separate experiments. In Experiment #1, a wide range of pH's was evaluated using the technique of successive approximations to analyze the 412 nm decay kinetics as the sum of two exponentials (see Materials and Methods V.B.1.b.). In Experiment #2, a narrow pH range was evaluated using a computer program to analyze the 412 nm kinetics of 20 averaged flashes at each pH (see Materials and Methods V.B.1.b.). Experiment #1 was carried out at 20° C and Experiment #2 was carried out at 20° C and 5° C. In both experiments the pH of a light-adapted bR solution in 10 mM phosphate buffer (0.3 mg/ml) was gradually increased by adding NaOH. The pH was read after flash photolysis spectroscopy with a Fisher Accumet Model 23-A pH meter. When the M₄₁₂ decay kinetics were analyzed as the sum of two exponentials using the successive approximations technique to be described in Materials and Methods V.B.1.b., the analysis was carried out on between
1 and 4 flashes for each sample. Since this analysis needed to be carried out manually, it was a time consuming procedure. In order to assess more quickly the reproducibility of the flash photolysis data, another technique was used. This was to cover the oscilloscope screen with a transparent plastic screen and record the first flash of the M decay of a bR sample on the transparency. Subsequent flashes, 10 or more, were compared to the trace. Under ideal conditions, this visual method revealed nearly identical traces on subsequent flashes. It also indicated experimental defects, such as a laser dye that needed to be replaced or "drift" in the electrical equipment.

The flash photolysis apparatus (see Figure 16) consisted of a Phase-R-DK-1100 dye laser with Exciton, Rhodamine dye, $10^{-4}$ M in ethanol, which emits a monochromatic flash of 575 nm of 0.25 joules with a rise time of 150 nanoseconds. The measuring light consisted of a General Electric quartzline lamp of 200 mW/cm$^2$ at the light, cooled by a small fan and powered by a 12 volt DC power supply. This light was passed through a Bausch and Lomb monochromator with a slit width of 19.8 nm set at 412 nm. After traversing the sample the measuring light was then passed through an identical Bausch and Lomb monochromator with a slit width of 19.8 nm set at 412 nm. This light then reached the photomultiplier which was powered by 500 volts from a Model CS-64H40 DC Power Supply from NJE Corporation. The signal was amplified by a Photomultiplier Tube Current Amplifier and rapidly stored in a Biomation Model 1010 Waveform recorder. Simultaneously, the signal was viewed on a Tektronix Type 564 Storage Oscilloscope. When the computer was used, each kinetic trace was transferred electrically to storage buffers in a PDP 11-34 Computer. A computer program allowed data averaging and curve peeling with the PDP 11-34 computer.
Figure 16

Flash Photolysis Apparatus

Biomation Waveform Recorder

Tektronix Storage Oscilloscope

PDP-11/34 Computer

Dye Pump for Laser

Circulating Water Bath for Laser

Phase-R-DK Laser

DC Power Supply for Measuring Light

DC Power Supply for Photomultiplier

Bausch & Lomb Monochromator Set at 412 nm

Bausch & Lomb Monochromator Set at 412 nm

Sample Cuvette

Light

GE Measuring Light

Fan

Bausch & Lomb Monochromator Set at 412 nm

Circulating Water Bath for Cuvette

Varian X-Y Plotter

412 nm

412 nm

412 nm

575 nm
Figure Legend 16. Flash photolysis apparatus (not drawn to scale).
The arrows in this scheme of the flash photolysis apparatus represent the light path. Company names of the instruments may be found in the List of Instruments and Equipment.
as will be described below in this section. 10, 20 or 50 flash spectra were usually averaged for data manipulation and occasionally kinetic traces were printed out using a Varian X-Y Plotter.

Before flash photolysis was set up in our laboratory, several trips were made to Ames Research Center, NASA, at Moffett Field, California, to perform flash photolysis. In collaboration with Dr. Roberto Bogomolni and Dr. Janos Lanyi, transmission changes of bR were measured with a single beam flash kinetic spectrophotometer constructed at AMES and described in (31).

V. B. 1. b. Analysis of Kinetic Traces

Originally the rise or decay of the 412 nm absorbance was copied onto a transparency or manually onto graph paper (see Figure 17). The changes at 412 nm have been referred to as changes in "absorbance" even though changes in "transmitted" light were actually measured. The following calculation shows that changes in absorbance were not significantly different from changes in transmitted light during flash photolysis of a dilute solution of bR. These two parameters are related by the equation

\[ \Delta A = \ln \left( \frac{\Delta I}{I_0} + 1 \right) \]

where \( \Delta A \) = change in absorbance,

\( \Delta I \) = change in transmitted light,

\( I_0 \) = light intensity without sample.

However, at very small \( \frac{\Delta I}{I_0}, \frac{\Delta I}{I_0} \approx \Delta A \).

Height of peak in flash decay = 3.125 cm

Height of oscilloscope screen = 20 cm

Amplifier sensitivity while measuring decay = x 10
Figure 17

Original Data of \( M_{412} \) Decay

DMA Repeated Treatment

Control 1, pH 7.5

Absorbance,
Relative Units

Time in Milliseconds

Figure 17. Original data of \( M_{412} \) decay, DMA repeated treatment, control 1, pH 7.5. This kinetic trace was transferred from the oscilloscope screen to graph paper point by point manually.
\[
\frac{3.125 \times 0.1}{20} = 0.0156 = \frac{\Delta I}{I_0}
\]

\[
\Delta A = \ln (0.0156 + 1) = 0.0155
\]

\[
0.0155 = 0.0156
\]

Changes in absorbance vs. time for the rise and decay of the 412 nm photointermediate were analyzed in relative units. The absolute height of the M_{412} intermediate produced by flash photolysis was not used to measure changes caused by chemical modification due to fluctuations in laser output during one experiment.

When the data was replotted onto semilog paper it became clear that the decay was not a single exponential decay whereas the rise was a single exponential. However, with the standard assumption that these decays are first order or pseudo first order, the decay may be expressed as a sum of exponentials

\[
A_\lambda (t) = \sum b_i e^{-k_i t}
\]

Equation 1

In this thesis most of the decays appeared to be the sum of two exponentials and were analyzed accordingly.

In order to rule out the possibility that the decay represented a second order reaction of the form

\[
M_{412} + M_{412} \rightarrow \text{New Intermediate}
\]

where the M state in one bR molecule might interact with the M state in a neighboring bR molecule, the data was graphed as \( \frac{1}{[M_{412}]} \) vs. time (32). Since this graph curved upwards rather than showing linearity, a second order decay of this type was ruled out.

In order to test for a second order decay of the following type:

\[
M_{412} + X \rightarrow \text{New Intermediate}
\]

it is necessary to graph \( \frac{1}{[M_0 + X_0]} \ln \left( \frac{[X_0][M]}{[M_0][X]} \right) \) vs. time (32). It is thought by some investigators in this field that X represents protons
which are in large excess, making the decay represent two pseudo first order decays. There is the possibility, however, that X is another intermediate in a neighboring bR molecule, but since the wavelength maximum and nature of the X that interacts with M are unknown at this time, and since the concentration of X was not monitored over time, this possibility cannot be ruled out or accepted on the basis of the data in this thesis.

Returning to the treatment of the kinetics as first order decays, the rate constants $k_i$ in Equation 1 relate to the rate of decay of various intermediates in the photocycle, and the amplitudes $b_i$ are related to the spectra of these intermediates. Therefore it is of interest to obtain the $k_i$'s and $b_i$'s from the data. Although the most sophisticated procedure developed to do this involves non-linear least squares fitting methods (33), for the sum of two exponential decays a curve peeling technique is adequate. To illustrate this curve peeling technique, let us first consider a known example. In Figure 18 are plotted three solid lines, calculated from the following equations:

$$A = b_1 e^{-k_1 t} + b_2 e^{-k_2 t}$$  \hspace{1cm} \text{Equation 2}

and $A_0 = b_1 e^{-k_1 t}$ (Equation 2-1) and $A_2 = b_2 e^{-k_2 t}$ (Equation 2-2),

where the $k_i$'s and $b_i$'s are chosen to be in the range of the experimental data. As can be seen from Figure 18, one should not obtain $k_i$'s and $b_i$'s from the line (shown dashed in Figure 18) which is drawn through the data points at long times and then subtracted from the data to draw the initial decay line (also shown dashed). In particular, in Figure 18, this incorrect procedure would yield errors of 10% in $k_1$, 26% in $k_2$, 14% in $b_1$ and 43% in $b_2$. Most of the data in this thesis has been analyzed manually by a successive approximations procedure to find lines $A_1$ and $A_2$ that will be described in the next paragraph. However, a few experiments in which the original decay data was not saved were
Figure 18

Testing of the Method

Absorbance, Relative Units

Data, Reconstructed from

\[ A = b_1 e^{-k_1 t} + b_2 e^{-k_2 t} \]

- \( A_1 = b_1 e^{-k_1 t} \), \( t_{1/2} = 15 \text{ msecs} \)
- \( A_2 = b_2 e^{-k_2 t} \), \( t_{1/2} = 85 \text{ msecs} \)
- \( t_{1/2} = 63 \text{ msecs} \)

Time in Milliseconds
analyzed by a computer program that used this incorrect procedure. The results of these experiments have been included in this thesis because qualitative changes in the decays are still seen with the computer curve peeling procedure.

The solid lines in Figure 18 served as a model for fitting two exponentials to the data by a successive approximations technique. This technique was first tried on the reconstructed data curve, $A = b_1 e^{-k_1 t} + b_2 e^{-k_2 t}$ in Figure 18 where the lines $A_1$ and $A_2$ are drawn from calculation. As can be seen in Figure 19 a line was first drawn asymptotic and just below the decay data at long times and extrapolated to the $y$-axis. The slope of this line is a first approximation of $k_2$ and the $y$-intercept is a first approximation of $b_2$. The values on this line were then subtracted from the data between 0 and 10 milliseconds and another straight line was drawn through these points and extrapolated out to long times. The slope of this line is a first approximation of $k_1$ and the $y$-intercept is a first approximation of $b_1$. This line was then subtracted from the data at times longer than the midpoint of the decay and a new $A_2$ line was drawn through these points and extrapolated back to the $y$-axis. This $A_2$ line gave a second approximation of $b_2$ and $k_2$. For the example shown, it was necessary to draw $A_1$ and $A_2$ three times each before the lines yielded kinetic constants close to the model. The improvement in the kinetic constants can be seen in the following table:

<table>
<thead>
<tr>
<th></th>
<th>$k_1$</th>
<th>$b_1$</th>
<th>$k_2$</th>
<th>$b_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>First Approximation</td>
<td>.0592</td>
<td>3.50</td>
<td>.0129</td>
<td>1.61</td>
</tr>
<tr>
<td>Second Approximation</td>
<td>.0471</td>
<td>3.74</td>
<td>.00906</td>
<td>1.35</td>
</tr>
<tr>
<td>Third Approximation</td>
<td>.0467</td>
<td>3.90</td>
<td>.00803</td>
<td>1.20</td>
</tr>
<tr>
<td>True Values</td>
<td>.0462</td>
<td>3.90</td>
<td>.0082</td>
<td>1.20</td>
</tr>
</tbody>
</table>
Figure 19

Successive Approximations Technique

Absorbance, Relative Units

Data, Reconstructed from $A = b_1 e^{-k_1 t} + b_2 e^{-k_2 t}$

First Approximation of $A_1$
Second Approximation of $A_1$
Third Approximation of $A_1$

First Approximation of $A_2$
Second Approximation of $A_2$
Third Approximation of $A_2$

Time in Milliseconds
A more clear graphical representation that the reconstructed data line, \( A = b_1 e^{-k_1 t} + b_2 e^{-k_2 t} \), in Figure 18 is actually the sum of only two exponentials is seen in Figure 20. In this figure, line A has been calculated out to times longer than what was usually measured where the contribution from the first decay is very small. At these and longer times, the second decay is seen to coincide with the reconstructed data. The reason that this data was not usually measured is because the signal to noise ratio at the longer times was small.

With the real decay data, the way to finally check the kinetic constants of lines \( A_1 \) and \( A_2 \) was to fit them to Equation 2 at several times (at least four). If a good fit had not been achieved, the approximations technique had to be continued. The use of this technique was made easier by first extrapolating the 412 nm decay data plotted on semi-log paper out to longer times. This provided an additional constraint for drawing the first approximation to the \( A_2 \) line. Normally, the \( A_2 \) line had to be redrawn three times, and the \( A_1 \) line twice, before a good fit of the data to Equation 2 was achieved.

V. B. 1. c. Effect of Deuterium Oxide

Chemically modified bR samples were concentrated by centrifugation at 200,000 x g for 15 minutes. The pellets were resuspended in 3 ml of \( \text{H}_2\text{O} \) or \( \text{D}_2\text{O} \) buffer, pH 7.0, containing 100 mM NaCl and 5 mM NaH\(_2\)PO\(_4\) and recentrifuged as above. This washing procedure was repeated once and the final pellet was resuspended in 3 ml of either the \( \text{D}_2\text{O} \) or \( \text{H}_2\text{O} \) buffer.
Figure 20

Testing of the Method

Absorbance,
Relative Units

Data, Reconstructed from \[ A = b_1 e^{-k_1 t} + b_2 e^{-k_2 t} \]

\[ A_1 = b_1 e^{-k_1 t}, \quad t_{1/2} = 15 \text{ msecs} \]

\[ A_2 = b_2 e^{-k_2 t}, \quad t_{1/2} = 85 \text{ msecs} \]

Time in Milliseconds
Figure Legend 20. Testing of the method. This figure shows the same data curve as Figure 18, calculated out to longer times to demonstrate that the second decay line $A_2$ coincides with the data curve when the contribution from the first decay line $A_1$ is small.
In the case of the pyridoxal phosphate modification, 25 mM pyridoxal phosphate in 37.5 mM phosphate buffer was included with each D$_2$O or H$_2$O wash, except the final pellet was resuspended in D$_2$O or H$_2$O buffer only. Flash photolysis was carried out on this pyridoxal phosphate sample immediately and reversibility of the reaction was monitored as a function of time. Flash photolysis kinetics at 412 nm of all of these samples were obtained in collaboration with Dr. Paul Sullivan.

V. B. 2. 412 nm Photostationary Steady State Spectroscopy

This measurement was carried out with dilute solutions of bR (10-125 µg/ml) in 10 mM phosphate buffer, pH 7 or 8, maintained at 20° or 24° C with a MGW Model T-2 Brinkman Circulating Water Bath in an Aminco DW2 Recording Spectrophotometer. Actinic illumination was provided by side illumination through a Corning No. 3-67 low wavelength cut-off filter having 50% transmission at 560 nm, 7.8 mW/cm$^2$ at the cuvette, with the photomultiplier protected by a Baird Atomic 412 nm transmission interference filter. The effect of changing the wavelength of the measuring light when reading the 412 nm photostationary steady state absorbance was examined by changing the measuring wavelength between 396 and 420 nm to determine the maximum absorbance. The sample in this experiment was bR (50 µg/ml) in 10 mM phosphate buffer, pH 7.0.

The molar ratio of the 412 nm intermediate per 568 nm chromophore was determined by calculating the molarities as follows: the molarity of the 412 nm intermediate was determined by dividing the 412 nm photostationary steady state absorbance by its molar extinction coefficient (34) minus the contribution from the 568 nm chromophore as
measured by Iwasa et al., (32,800 M$^{-1}$cm$^{-1}$)\(^{(34)}\), and the molarity of
bR\(_{568}\) was determined by dividing the 568 nm absorbance by the molar
extinction coefficient determined by Oesterhelt and Hess (63,000 M$^{-1}$cm$^{-1}$)
\(^{(2)}\). Usually this molar ratio was between 0.5 and 4% in control bR,
since the actinic light at the sample was weak. The molar ratio of
modified bR samples was presented as percent control, measured under
the same conditions as the bR samples.

In addition, the effect of D$_2$O upon the 412 nm photostationary
steady state absorbance was examined by using the samples prepared in
D$_2$O described in Materials and Methods V.B.1.c.

V. B. 3. Light-Induced pH Changes of bR Lipid Vesicles

Monitored by pH Electrode

One method that was used to prepare bR lipid
vesicles was to solubilize crude Asolectin to a final concentration
of 100 mg/ml lipid in 0.25 M sucrose or KCl and 7.5 mM 3-mercapto-
1,2-propanediol (1-thioglycerol). Solubilization was achieved by
suspending dried asolectin granules in the buffer and sonicating with
the Tapered Microtip attached to the Model 350 Sonifier Cell Disruptor
at 30% pulsed sonication output, power setting 3 for 1 hour under
Argon at 4° C. The titanium from the microtip was centrifuged out
after this step by spinning at 10,000 x g for 10 minutes. This
lipid solution was dialyzed overnight against distilled water to
remove 1-thioglycerol. The resulting clarified solution of lipid
vesicles could be stored for several months at 4° C in 1M KPi, pH 7.0,
with no loss of activity in the following pH assays.
To incorporate bR, one method was to combine about 0.4 mg bR with 8 mg asolectin stock, always maintaining a lipid:protein ratio of 20:1. Then, 0.3 ml of 4 M NaCl and 0.3 ml of 5 mM phosphate buffer, pH 7.0, were added. The volume was adjusted to 3.0 ml with distilled water, so the final buffer concentration was 0.5 mM and the final NaCl concentration was 400 mM. The optimal time of sonication of this solution with the Tapered Microtip attached to the Branson 350 Sonifier at 30% pulsed output, power 3 in a Nalgene centrifuge tube under argon at 4°C was 6 minutes, as judged by the pH response.

The sample was then allowed to equilibrate in the dark in the pH electrode cuvette for 10-30 minutes. The pH meter was a Beckman digital pH meter attached to a Varian Recorder. The temperature in the cuvette was maintained at 25°C by a HAAKE Circulating Water Bath. The cuvette was illuminated after sample equilibration with 58.6 mW/cm² visible light from a Bell and Howell Headliner Projector with a 300 watt GE Projector Lamp.

The initial rates of light-induced proton uptake and dark-induced proton extrusion by the bR lipid vesicles were recorded by monitoring the slope of these pH changes while using a chart speed of 20 cm/minute, and sensitivity of 1 mV/Full Scale. The extent of proton extrusion was calibrated by injecting 20 or 25 µl of 2 mM HCl into the bR lipid vesicle suspension and comparing the line height produced with that of the bR sample. Changes in the buffer capacity of the solution were also noted.
V. B. 4. pH Response of a pH Indicator Dye

The method used to measure flash-induced proton release and uptake from PM in distilled water was to monitor pH changes in solutions of PM with the pH-sensitive dye 7-hydroxycoumarin (35,36). The flash-induced kinetic changes of this indicator were observed by subtracting the change in absorbance at 365 nm of a bR sample without indicator from the change in absorbance at 365 nm of a bR sample with indicator. Initially, all buffer was removed from control and modified bR samples by diluting in 25 ml distilled water and centrifuging at 150,000 x g for 30 minutes, repeated three times. The final pellet was resuspended in distilled water and 3 ml of bR sample was adjusted to pH 7.5 with dilute HCl or NaOH with Abs(568) = 0.73. 30 ul of 10 mM 7-hydroxycoumarin, \[
\text{HC}\begin{array}{c}
\text{O} \\
\text{O}
\end{array}
\text{H}
\]
was injected into one bR sample and 30 ul distilled water was injected into an identical bR sample. After addition of the dye and water, the pH was checked again. It was essential that the initial pH be identical in the two samples.

During flash photolysis, the temperature was maintained at 20°C with a MGW Lauda RC3 Brinkman Circulating Water Bath. Samples were preequilibrated to 20°C before placing in the cuvette. Usually 20 or 40 flashes were accumulated in the PDP 11-34, as described under Flash Photolysis Apparatus. Using the computer data manipulation program, it was possible to store summed or averaged data while more kinetic traces were being accumulated. Then two stored data sets were subtracted and the difference printed out on a Varian X-Y Plotter. First the kinetic traces with and without dye at 412 nm were subtracted. Since the dye does not absorb at 412 nm this difference should have equaled zero. A nonzero difference spectrum at 412 nm indicated that
either the absorbance or pH was not equal in the two samples, or that the number of flashes accumulated was not equal. When the 412 nm difference spectrum was equal to zero, the kinetic traces at 365 nm with and without dye were subtracted. The kinetics for the 365 nm difference spectra were analyzed as the sum of two exponentials in a similar manner to the $M_{412}$ kinetics as described under Flash Photolysis.

V. B. 5. Surface Charge Changes During Photocycling

An EPR spin probe technique was used to monitor surface charge in collaboration with Dr. Alex Quintanilha. The positively charged paramagnetic amphiphile 4-(dodecyl dimethyl ammonium)-1-oxy1-2,6,6,6-tetramethyl piperidine bromide (CAT$_{12}$), which partitions between the membrane and aqueous phases, was synthesized by Dr. Rolf Mehlhorn and used as a probe of surface potential (37,38). Electron paramagnetic spectra were recorded in a Varian E-109E Spectrometer and plotted on a Varian X-Y Plotter. The lamp used to illuminate the EPR cavity was a Sylvania EHT Quartz Bromine Lamp with light intensity of 145 mW/cm$^2$ inside the cavity. No variac or filters were used.

Identical samples were prepared by first centrifuging 0.4 mg of control or chemically modified bR sample at 200,000 x g for 20 minutes. 100 µl distilled water was added to resuspend the pelleted samples, which were then transferred to small glass test tubes. To this 100 µl solution, CAT$_{12}$ spin probe was added from a 20 mM stock with a Hamilton syringe so that the final concentration of probe was in the range of 1.3-1.8 mM. A small aliquot (30 µl) was removed and placed into a glass capillary and then into the EPR cavity.
8 minute scans were first taken of the sample in the dark to determine the amount of dark aqueous signal, as represented by the high field line at g = 1.9986 in the 3 line EPR spectrum. The light-induced decrease in the amount of this aqueous signal was monitored by setting the magnetic field at g = 1.9986, increasing the gain and then switching on the light. Changes in surface potential were calculated from the Boltzman relation (38):

\[ \Delta \psi_s = \frac{RT}{2F} \ln \frac{P_1}{P_2} \]

where \( P_1 \) and \( P_2 \) stand for the partitioning of CAT at two different states. The height of the light-induced change was measured in cm and multiplied by 2, since this represents only one side of the EPR high field line. The height of the dark signal at the high field line was multiplied by the ratio of the gains \( \text{Gain in Light/Gain in Dark} \) such that:

\[ \frac{\text{Light-induced height} \times 2}{\text{Dark height} \times \text{Ratio of Gains}} = \text{calculated \%}. \]

This percentage was subtracted from 100% and substituted into the surface potential calculation as

\[ \Delta \psi_s = 26 \text{ mV} \ln \left( \frac{\text{calc. \%}}{100 \%} \right) \]

This calculated mV represented the surface charge change upon illumination. The surface charge change upon illumination of the 400 and 600 mM BD modified samples was expressed as percent of control, and compared to the M\text{412} photostationary steady state absorbances of the same samples after dilution to 3 ml with distilled water. The pH of these samples was determined to be 6.0.

V. B. 6. Light-to-Dark Adaptation Kinetics

To measure the kinetics of the transition from the light-adapted to the dark-adapted state of bR, dilute samples of bR
(0.15 mg/ml) in 10 mM phosphate buffer, pH 8.2 to 8.5, were initially completely dark-adapted by covering the samples with aluminum foil for 24 hours at room temperature or for 40 minutes at 50° C. Samples were preequilibrated to 20° C with a MGW Model T-2 Brinkman Circulating Water Bath. A sample was then placed into the temperature-equilibrated cuvette holder in the Cary 14R Recording Spectrophotometer in the dark and the absorption spectrum from 590 to 550 nm was read using a slide-wire of 0.1-0.2 sensitivity. The height at 580 nm of the dark-adapted state was used as a reference for comparing to the light-adapted state, since this wavelength was shown by Oesterhelt et al. (39) to have the largest light-dark difference.

The sample was then removed from the Cary 14R and light adapted for 30 seconds with light of 404 mW/cm², which was sufficient to completely light adapt bR as shown by no further increase in absorption. The sample was then reequilibrated in the water bath at 20° C for 1 minute and replaced into the Cary 14R. The height at 580 nm was observed by switching on the photomultiplier without readjusting the baseline from the previous measurement of dark-adapted bR. At 10 minute intervals the absorption at 580 nm was read, usually over a period of two hours. The log of the change in absorption (light minus dark) at 580 nm as a function of time was plotted versus time to obtain the half-life of this first order light-to-dark adapted stated transition.
RESULTS

I. Imidoester Modification at pH 10.0 of Lysine Residues in bR

A. Number of Lysines Modified

1. Effect of Imidoester Concentration

After chemical modification with imidoesters at pH 10.0, the questions must be asked, 1) how many total lysines are available for chemical modification with imidoesters and, 2) what fraction of the total lysines are modified by different concentrations of imidoester? The actual number of lysines modified will usually differ from the total number of lysines available to the reagent due to the statistical equilibria of the reactions involved, i.e., not all of the lysines available will be modified on every bR. Figure 21 shows the number of lysines modified by increasing concentrations of monofunctional imidoesters. This graph represents the average of four concentration curves. Besides the standard deviation at each concentration, the results presented in this section are inaccurate by ±13% as calculated in Appendix 3. The number of lysines in Figure 21 was found by multiplying the relative fluorescence from the fluorescamine determination by 6 total lysines available for reaction. This follows from the amino acid composition data of 7 total lysines (see Results II.A.1.), and the fact that one lysine has a retinal residue attached to it (40) so it will not be available to the imidoesters. The number of total lysines available for modification can be verified by plotting the data from Figure 21 in the form of a Scatchard plot (41). The x axis in a Scatchard plot is in units of v, i.e., number of lysines modified. The y axis is v/L, where L is the concentration of imidoester (ligand). The x-intercept is the total number of lysines available, N.
Figure 21

Extent of Amidination by Monofunctional Imidates

Number of Lysines Modified

MA, ○
EA, △
MB, □

Imidoester Concentration in mM

1.0  1.0  10.0  100

5.0  4.0  3.0  2.0  1.0
As shown in Figure 22, \( N = 6 \) for monofunctional imidoester modification of lysines in bR. The Scatchard plot may be used only if it is certain that the reaction has reached equilibrium; then, the slope is the negative inverse of the equilibrium constant of dissociation. In the case of the monofunctional imidoester modification at pH 10.0, equilibrium is rapidly achieved because the half-time for the reaction is short, and hydrolysis of the reagent is 30 times slower than the reaction (see Appendix 2.A.). In addition, Appendix 8 shows that by all of the criteria examined, the number of treatments necessary for complete modification with monofunctional imidates was only one.

The value of total lysines available (\( N \)) determined from the Scatchard plot can be further verified with a Hill plot (see Figure 23). Deviations from linearity in the Hill plot indicate an incorrect choice for the total number of lysines available (41). As shown in Figure 23, 6 was the correct number of \( N \), since only 6, not 5 or 7 total lysines, yielded a linear Hill plot. Additionally, the slope of the Hill plot for \( N = 6 \) was 0.96, which indicates non-cooperative binding of imidates to lysines. If an initial incorrect choice of 5 or 7 is made to convert fluorescence to number of lysines modified, then linearity is not seen in the Hill plot with \( N = 4, 5, 6 \) or 7.

Besides the value of \( N \), the Scatchard plot in Figure 22 yields other important information. The Scatchard plot has two straight line components, which indicate two classes of lysines reacting with different dissociation equilibrium constants (41). The x-intercepts of the components yield the number of lysines with particular equilibrium constants. In Figure 22 one lysine had \( K_D = 3.0 \times 10^{-4} \text{ M} \), and 5 lysines (\( N-1 \)) had \( K_D = 1.5 \times 10^{-2} \text{ M} \). This important result indicates that the binding of imidoesters to one lysine was about 50 times higher than to
Figure 22

Scatchard Plot of Monofunctional Imidoester Modification of bR

\[ \frac{V}{L} \]

Slope = \( -\frac{1}{K_D} \), \( K_D = 3.0 \times 10^{-4} \text{ M} \)

\( K_D = 1.5 \times 10^{-2} \text{ M} \)
Figure 23

Hill Plot to Test the Value of N From the Scatchard Plot

Of Monofunctional Imidate Modification of bR

\[ \ln \left( \frac{N}{V} - 1 \right) \]

- □, N = 7
- ○, N = 6
- △, N = 5
the other 5 lysines. Thus at any particular monofunctional imidate ([MI]) concentration, one lysine will be preferentially modified. This result may be quantitated as follows:

For one lysine, an equilibrium of MI binding will exist such that

\[ K_{D1} = \frac{(\text{LYS}_1 - \text{LYS}_1^*)([\text{MI}])}{\text{LYS}_1^*} \]

where

- \( K_{D1} = 3.0 \times 10^{-4} \text{ M} \),
- \( \text{LYS}_1 = \text{Number of unmodified lysines of class 1 (with } K_{D1}) \),
- \( \text{LYS}_1^* = \text{Number of modified lysines of class 1} \)

For 5 other lysines, an equilibrium of MI binding will exist such that

\[ K_{D5} = \frac{(\text{LYS}_5 - \text{LYS}_5^*)([\text{MI}])}{\text{LYS}_5^*} \]

where

- \( K_{D5} = 1.5 \times 10^{-2} \text{ M} \),
- \( \text{LYS}_5 = \text{Number of unmodified lysines of class 5 (with } K_{D5}) \),
- \( \text{LYS}_5^* = \text{Number of modified lysines of class 5} \)

At a given concentration of MI, the total number of modified lysines will be \( \text{LYS}_1^* + \text{LYS}_5^* \), where

- \( \text{LYS}_1^* = \frac{(1 - \text{LYS}_1^*)([\text{MI}])}{K_{D1}} \) and
- \( \text{LYS}_5^* = \frac{(5 - \text{LYS}_5^*)([\text{MI}])}{K_{D5}} \)

For example, at 20 mM MI, the one lysine with a lower dissociation equilibrium constant will be 98.4% modified and the 5 lysines with a higher dissociation equilibrium constant will be 57.2% modified,
so the total lysines modified will be:

\[
0.98 \text{ (class 1 lysine)} + 2.86 \text{ (class 5 lysine)}
\]

3.84 total lysines modified

This agrees quite well with the initial plot of lysines modified vs. [MI] in Figure 21. In addition, a calculation of this type predicts that the one lysine of class 1 will be almost completely modified while the other 5 lysines of class 5 will be only about half modified statistically. The interesting questions that arise from this analysis are, what could shift the equilibrium constant of only one lysine, and which lysine could this be? These questions and the significance of this result will be answered in Discussion I.A.I.

Contrary to the case of the monofunctional imidoesters, the bifunctional imidoester reagents probably do not reach equilibrium within 30 minutes of reaction at pH 10.0 (42-44). Indeed, Appendix 8 showed that an average of 2 repeated treatments with these reagents was required in order to complete the modification. The difference in reactivity of the bifunctional reagents compared to the monofunctional reagents is reasonable, due to the sterically more difficult requirement of linking two lysines with one reagent. As a result, hydrolysis of the bifunctional reagents may become competitive with the reaction half-time. Thus a Scatchard plot should not be drawn for these reagents. Instead, Figures 24 and 25 show the number of lysines modified vs. imidoester concentration, assuming that 6 is the correct value of N, which is based on the case of the monofunctional imidoesters. Given this assumption it is shown by Figure 25 that DMA, the 8.3 Å cross-linker, was able to maximally modify 75 % of the total 6 lysines. Figure 24 shows that DMS, the 11.3 Å cross-linker, was able to modify about 70 %
Figure 24

Extent of Amidination by DMS

Number of Lysines Modified

DMS Concentration in mM
Figure 25

Extent of Amidination by DMA

Number of Lysines Modified

DMA Concentration in mM
of the 6 total available lysines before major deterioration of the chromophore structure occurred (represented by a break in the graph) (see also Results I.E.1.). More lysines were available to DMS after denaturation of the protein structure, but as with the monofunctional reagents, all 6 of the total available lysines were not completely modified with either cross-linking imidoester reagent at any concentration.

Throughout the rest of this thesis, experimental results will be correlated either with the number of lysines modified, based on the fluorescamine calculation using 6 total lysines, or 7 total lysines in the case of bleached bR, or correlated with the fraction of total lysines modified. This fraction is a more correct representation for the extent of modification, since all 6 of the lysines will be modified to some degree at any concentration of imidoester.

I. A. 2. Effect of Temperature

One reason for amidinating bR at various temperatures was to explore the effect of temperature on the number of lysines modified by the cross-linking reagents. Since it is known that the lipids of bR do not undergo a phase transition in the range of 0 to 60° C (45,46), the observed biphasic temperature dependence of photocycling kinetics (47,48) may be due to a change in the bR protein's molecular structure. At higher temperatures, where the bR protein may be less rigid, the extent of amidination, especially by the longer chain cross-linker, is expected to increase.

Amidination was carried out at pH 10.0 as described in Materials and Methods II.A.2. at only one concentration, 10 mM of both DMS and DMA, at 5° C, 22° C, 40° C, 50° C and 60° C. The number of lysines
modified in this experiment are shown in Table 4. The results in Table 4 represent the average of 2 temperature experiments in duplicate; the fluorescence test may be inaccurate by ±13 % as calculated in Appendix 3.

As shown, the shorter cross-linker, DMA (8.3 Å), modified the greatest number of lysines at 40°C, and increasing the temperature above this did not render additional lysines available to DMA. DMS (11.3 Å), on the other hand, showed an increase in the number of lysines modified up to 50°C, while 60°C did not render any more lysines available to DMS. These results could be explained by an unfolding of the protein structure that is complete by 50°C (see Discussion I.A.2.).

I. A. 3. Effect of Modifying bR in Bleached PM

In order to ascertain if any more lysines could be made available to imidoesters in bR with retinal removed by reacting with hydroxylamine in strong light, the fluorescamine test was performed on bR after different combinations of bleaching and treating with monofunctional imidoesters. In evaluating the number of lysines modified, bleached samples were always compared to a standard curve constructed from bleached bR. The relative fluorescence obtained from the fluorescamine test with the bleached samples was multiplied by 7 lysines, not 6, since retinal should no longer be attached to one lysine during the fluorescamine test. The retinal oxide will most likely be dissociated from the SDS-treated protein in the fluorescence test since SDS is a well-known protein and lipid solubilizer (198). Thus it is thought that retinal oxide will not interfere with this test. The results may be inaccurate by ±13 % due to the error in the fluorescamine test.
Table 4
Effect of Temperature on the Extent of Amidination by DMS and DMA

<table>
<thead>
<tr>
<th>Sample</th>
<th>Number of Lysines Modified</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM DMS</td>
<td></td>
</tr>
<tr>
<td>5°C</td>
<td>0.5 ± 0.5</td>
</tr>
<tr>
<td>22°C</td>
<td>2.8 ± 0.7</td>
</tr>
<tr>
<td>40°C</td>
<td>3.4 ± 0.2</td>
</tr>
<tr>
<td>50°C</td>
<td>4.5 ± 0.1</td>
</tr>
<tr>
<td>60°C</td>
<td>4.7 ± 0.3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sample</th>
<th>Number of Lysines Modified</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM DMA</td>
<td></td>
</tr>
<tr>
<td>5°C</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>22°C</td>
<td>3.3 ± 0.3</td>
</tr>
<tr>
<td>40°C</td>
<td>4.4 ± 0.2</td>
</tr>
<tr>
<td>50°C</td>
<td>4.2</td>
</tr>
<tr>
<td>60°C</td>
<td>5.0 ± 0.4</td>
</tr>
</tbody>
</table>
as calculated in Appendix 3.

As shown in Table 5 in Experiments # 1 and # 2, bleaching and then treating with a monofunctional imidoester could not increase the number of lysines modified beyond those modified by a monofunctional imidoester in Experiment # 3. Two treatments with EA were necessary in Experiment # 3 to modify the number of lysines ordinarily modified by 20 mM EA in one treatment (see Appendix 8), for reasons that are not clear. Subsequent bleaching of this sample and then retreating with EA could not further increase the number of modified lysines. These experiments showed that no additional lysines became available to the monofunctional imidoester reagents after bleaching with hydroxylamine. This indicates that even though retinal had reacted with hydroxylamine, the retinaloxime formed is capable of preventing imidoesters from reacting with the retinal-containing lysine (see Discussion II.A.2.).

I. B. Photocycling Activity

1. Effect of Number of Lysines Modified

Once the extent of amidination modification is known, the next step is to correlate the number of lysines modified divided by the total number of lysines, or fraction of total lysines modified, with bR's photoactivity. bR has several measurable photoactivities: photocycling, proton pumping in liposomes, proton release and uptake, surface charge changes, and photopotential generation in bilayer lipid membranes. Photocycling activity is one of the simplest to measure, since bR in isolated PM with no other added components in distilled water or buffer is readily photolyzed by a short laser flash. The kinetics of formation and decay of the absorbance of one of the
Table 5
Effect of Bleaching bR on Extent of Amidination

<table>
<thead>
<tr>
<th>Sample</th>
<th>Number of Lysines Modified</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment # 1</td>
<td></td>
</tr>
<tr>
<td>Bleached, Then Treated</td>
<td></td>
</tr>
<tr>
<td>With 5 mM MA</td>
<td>0.9</td>
</tr>
<tr>
<td>Bleached, Then Treated</td>
<td></td>
</tr>
<tr>
<td>With 10 mM MA</td>
<td>0.3</td>
</tr>
<tr>
<td>Bleached, Then Treated</td>
<td></td>
</tr>
<tr>
<td>With 20 mM MA</td>
<td>4.2</td>
</tr>
<tr>
<td>Experiment # 2</td>
<td></td>
</tr>
<tr>
<td>Bleached, Then Treated</td>
<td></td>
</tr>
<tr>
<td>With 20 mM EA</td>
<td>4.3 ± 0.3</td>
</tr>
<tr>
<td>Experiment # 3</td>
<td></td>
</tr>
<tr>
<td>Treated with 20 mM EA</td>
<td>3.9 ± 0.2</td>
</tr>
<tr>
<td>Retreated with 20 mM EA</td>
<td>4.5 ± 0.3</td>
</tr>
<tr>
<td>Then Bleached, and Retreated</td>
<td></td>
</tr>
<tr>
<td>With 20 mM EA</td>
<td>4.5 ± 0.2</td>
</tr>
</tbody>
</table>
(for number sequence only)
longest-lived photointermediates, $M_{412}$, can be easily monitored as described in Materials and Methods V.B.1.

The kinetics of the rise of $M_{412}$ absorbance are given in Table 6. There was very little change in the time needed for $M$ formation except for the DMS sample with the highest fraction of lysines modified, which also showed considerable protein denaturation (see Results I.E.1.). In this sample, the formation of $M_{412}$ was significantly faster, perhaps due to greater accessibility of the aqueous phase to the Schiff base in the denatured protein. The rise of $M_{412}$ always appeared as a single exponential phase, even after imidoester modification.

The decay kinetics of $M_{412}$ were analyzed as the sum of two exponentials as described in Materials and Methods V.B.1.b. As shown in Figure 26, the decay of $M_{412}$ of control bR and imidoester modified bR always appeared to be the sum of two exponentials. After modification with monofunctional imidates even at the highest fraction of total lysines modified, the activity was very little changed from the control (see Figure 26). This graph represents the average of three concentration curves, using MA, MB and EA with an average of 2 flashes at 2 pH's at each concentration. Thus, monofunctional imidoester modification did not alter photocycling kinetics markedly.

As also seen in Figure 26, the light-induced $M_{412}$ photostationary steady state followed the trend seen in both phases of the $M_{412}$ decay. This measurement is another indication of changes in photocycling activity upon chemical modification as seen from the following derivation:

In its simplest form the photocycle of bR is:

$$
\begin{align*}
&bR_{568} \xrightarrow{k_1} K_{590} \xrightarrow{k_2} L_{550} \xrightarrow{k_3} M_{412} \xrightarrow{k_4} \\
&bR_{568} \xrightarrow{} L_{550} \xrightarrow{} K_{590} \xrightarrow{} bR_{568}
\end{align*}
$$
Table 6
Effect of Imidoester Modification on the Rise Kinetics of M<sub>412</sub>
At 22° C

<table>
<thead>
<tr>
<th>Imidoester Modified bR Sample</th>
<th>Rise t&lt;sub&gt;1/2&lt;/sub&gt;</th>
<th>% Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>usecs</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>50.3 ± 2.5</td>
<td>100</td>
</tr>
<tr>
<td>EA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.80</td>
<td>50.1</td>
<td>99.6</td>
</tr>
<tr>
<td>DMA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.45</td>
<td>50.9</td>
<td>102</td>
</tr>
<tr>
<td>0.63</td>
<td>53.6</td>
<td>107</td>
</tr>
<tr>
<td>0.67</td>
<td>54.6</td>
<td>109</td>
</tr>
<tr>
<td>0.75</td>
<td>49.8</td>
<td>99</td>
</tr>
<tr>
<td>DMS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.48</td>
<td>48.1</td>
<td>95.6</td>
</tr>
<tr>
<td>0.57</td>
<td>51.7</td>
<td>103</td>
</tr>
<tr>
<td>0.78</td>
<td>34.4</td>
<td>68.4</td>
</tr>
</tbody>
</table>
Figure 26

412 nm Decay Kinetics of bR Modified by
Monofunctional Imidoesters

412 nm Decay $t_{1/2}$
First Phase as Percent Control First Phase, □
Second Phase as Percent Control Second Phase, ○

412 nm Photostationary Steady State Absorbance (412 nm/568 nm Molar Ratio as Percent Control), △

Fraction of Total Lysines (6) Modified
Under steady state conditions:

\[
\frac{d[M_{412}]}{dt} = k_3[L] - k_4[M] = 0, \text{ and } [M] = \frac{k_3[L]}{k_4}
\]

\[
\frac{d[L]}{dt} = k_2[K] - k_3[L] = 0, \text{ and } [L] = \frac{k_2[K]}{k_3}
\]

So \( M \) may be expressed as \([M] = \frac{k_2[K]}{k_4}\)

Thus it is seen that the concentration of \( M_{412} \) in the photostationary steady state should be inversely related to the rate of decay of \( M \). However, it is also dependent on the product of \( k_2 \), the rate of decay of the first intermediate, and \([K]\), how much \( K \) is formed from \( bR \).

Since changes in the \( M_{412} \) absorption followed so closely changes in the \( M_{412} \) decay kinetics in the imidoester-treated \( bR \) sample, it is likely that \([K]\) and \( k_2 \) did not change from the control.

Figures 27 and 28 show the effect of the bifunctional imidoester modifications on photocycling kinetics. Each of these graphs represents the average of two concentration curves with an average of 2 flashes at 2 pH's at each concentration. The two cross-linkers of different chain lengths inhibited the \( M_{412} \) decay about equally with the exception of high concentrations of DMS for which deterioration of the chromophore structure was considerable (represented by a break in Figure 27). In comparison to the monofunctional imidates, the bifunctional imidoesters were able to significantly slow the photocycle, about 3-fold at 50% modification. The possibility that the inhibition was due to simply adding a bulky group onto lysine can be ruled out, because one of the monofunctional imidoesters, MB, has a chain length equal to the length of the cross-link of DMA, and inhibited only slightly. Thus,
Figure 27

412 nm Decay Kinetics of bR Modified by DMS

412 nm Decay $t_{1/2}$

First Phase as Percent Control First Phase,

Second Phase as Percent Control Second Phase,

412 nm Photostationary Steady State Absorbance (412 nm/568 nm Molar Ratio as Percent Control),

Fraction of Total Lysines (6) Modified
Figure 28

412 nm Decay Kinetics of bR Modified by DMA

$412 \text{ nm Decay } t_{1/2}$

First Phase as Percent Control First Phase, □

Second Phase as Percent Control Second Phase, ○

$412 \text{ nm Photostationary Steady State Absorbance (412 nm/568 nm Molar Ratio as Percent Control)}$, △

Fraction of Total Lysines (6) Modified
cross-linking lysines with either an 8.3 Å or 11.3 Å cross-linker, rather than just amidinating, was able to slow photocycling activity. The $M_{412}$ photostationary steady state absorbance of bR modified by bifunctional imidoesters was more markedly changed than M decay. Thus it is possible that the early events of the photocycle, K decay, and the quantum yield of K formation, were also changed by these modifications, as well as M decay, since $[M] = \frac{k_2[K]}{k_4}$. Alternatively, a more complex photocycle with other photointermediates and back photoreactions may be involved.

I. B. 2. Effect of Temperature

One purpose of amidinating bR at various temperatures was to correlate the extent of amidination at different temperatures with the extent of inhibition of the $M_{412}$ decay. Table 7 gives the $M_{412}$ decay kinetics measured at 22°C for bR samples modified at 22°C, 40°C and 60°C by 10 mM DMS and DMA. As shown, inhibition by DMS or DMA was similar in both phases of the decay of bR modified samples at all 3 temperatures. A similar inhibition of the decay at 22°C and 60°C was correlated with a similar extent of modification of lysines at these temperatures. The fact that inhibition by DMS was slightly greater at 40°C, where DMS only modified 0.56 fraction of total lysines compared to 0.73 by DMA is within the error of the fluorescamine test. Both DMS and DMA inhibited the $M_{412}$ decay most markedly at 60°C, where the fraction of total lysines modified was the greatest (about 0.8). The inhibition by both reagents at 60°C was similar to that caused by 0.75 fraction of total lysines modified by DMA at room temperature (see Results I.B.1.) within the standard deviation in Figure 28.
Table 7

Effect of Temperature During Amidination on $M_{412}$ Kinetics

Assay at 22° C

<table>
<thead>
<tr>
<th>Temperature</th>
<th>$M_{412}$ Decay as % Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$t_{1/2} - 1$</td>
</tr>
</tbody>
</table>

Imidoester Modified  
br Sample

Fraction of Total (6)  
Lysines Modified

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>22° C</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMS</td>
<td>0.47</td>
<td>164 ± 51</td>
</tr>
<tr>
<td>DMA</td>
<td>0.55</td>
<td>258 ± 3</td>
</tr>
<tr>
<td>40° C</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMS</td>
<td>0.56</td>
<td>285 ± 120</td>
</tr>
<tr>
<td>DMA</td>
<td>0.73</td>
<td>259 ± 96</td>
</tr>
<tr>
<td>60° C</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMS</td>
<td>0.78</td>
<td>331 ± 154</td>
</tr>
<tr>
<td>DMA</td>
<td>0.83</td>
<td>374 ± 151</td>
</tr>
</tbody>
</table>
To summarize the effect of temperature on the inhibition of the $M_{412}$ decay kinetics, it may be said that increased amidination is associated with increased inhibition of the $M_{412}$ decay. This result is consistent with the fact that there is an unfolding of the protein structure between 40° and 60° C.

I. B. 3. Effect of Modifying bR in Bleached PM

In order to determine the effect of bleaching and treating bR with a monofunctional imidoester on the photocycling kinetics, it was necessary to regenerate bR with all-trans retinal after the bleaching treatment, as described in Materials and Methods II.A.3., and then assay by flash photolysis. The $M_{412}$ kinetics were assayed two days after regeneration. These results are shown in Table 8. "% Control" values in this table are a comparison of bleached sample with bleached bR control, and unbleached sample with unbleached bR control. Included in this table is the 20 mM EA sample before bleaching for comparison.

As shown in Table 8, the $M_{412}$ rise $t_{1/2}$ of regenerated control bR was similar to unbleached control bR, which indicates that regeneration of the functional protein was successful. Bleaching bR and then treating with 20 mM EA caused this regenerated sample to have a slightly faster $M_{412}$ rise than the regenerated control bR, while a single 20 mM EA treatment of unbleached bR did not change the rise $t_{1/2}$. This indicates that reprotonation of the Schiff base was slightly facilitated by these two treatments, which may have been due to a looser protein structure after bleaching and modification, and then regeneration.
Table 8
Effect of Bleaching bR on M$_{412}$ Kinetics
At 24°C

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fraction of Total (6 or 7) Lysines Modified</th>
<th>M$<em>{412}$ Rise $t</em>{1/2}$ μsecs</th>
<th>% Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unbleached Control bR</td>
<td>0</td>
<td>38</td>
<td>100</td>
</tr>
<tr>
<td>20 mM EA Treated, Unbleached bR</td>
<td>0.65</td>
<td>37</td>
<td>97.4</td>
</tr>
<tr>
<td>Bleached and Regenerated Control bR</td>
<td>0</td>
<td>34</td>
<td>100</td>
</tr>
<tr>
<td>Bleached, 20 mM EA Treated and Regenerated bR</td>
<td>0.64</td>
<td>21</td>
<td>63</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>M$_{412}$ Decay</th>
</tr>
</thead>
<tbody>
<tr>
<td>$t_{1/2}^{-1}$ msecs % Control</td>
</tr>
<tr>
<td>-------------------------------------------</td>
</tr>
<tr>
<td>Unbleached Control bR</td>
</tr>
<tr>
<td>20 mM EA Treated, Unbleached bR</td>
</tr>
<tr>
<td>Bleached and Regenerated Control bR</td>
</tr>
<tr>
<td>Bleached, 20 mM EA Treated and Regenerated bR</td>
</tr>
</tbody>
</table>
Table 8 also shows that regenerated control bR had a decay $t_{1/2}$ similar to unbleached control bR, again indicating that regeneration of the functional protein was successful. The once-treated 20 mM EA bR sample had an $M_{412}$ decay similar to the control bR in the first phase and about 2-fold slower in the second phase, as was seen in the monofunctional imidate concentration curve (Results I.B.1.). bR that had been bleached, treated with 20 mM EA and then regenerated, had about the same inhibition relative to the regenerated control bR as the unbleached 20 mM EA treated sample had relative to the unbleached bR control. These results suggest that the lysines which are modified in bleached bR are the same as in unbleached bR, and thus have about the same effect on photocycling kinetics.

I. B. 4. D$_2$O Effect

The deuterium isotope effect has been used as evidence for the occurrence of hydrogen transfer in the rate-determining step of a reaction, and this effect has been ascribed to the difference in zero-point energies of the stretching vibrations of bonds to hydrogen and deuterium (49). The zero-point energy is equal to $1/2 \hbar v$ (where $\hbar$ is Planck's constant and $v$ is the frequency of the C-H or C-D vibration) (49). The magnitude of the D$_2$O effect may reveal information about the mechanism of the reaction. The deuterium isotope effect was examined as described in Materials and Methods V.B.l.c. by monitoring $M_{412}$ decay and $M_{412}$ photostationary steady state absorbance in H$_2$O and D$_2$O, as shown in Table 9. The $M_{412}$ kinetic results in Table 9 were obtained by computer analysis of a minimum of 10 flashes repeated at least twice. The isotope effect for the rise of $M_{412}$ was little changed in the imidester treated samples from the control. In the $M_{412}$ decay kinetics,
Table 9
Effect of D$_2$O on M$_{412}$ Kinetics
At 20° C

<table>
<thead>
<tr>
<th>bR Sample</th>
<th>Fraction of Total (7) Arginines Modified</th>
<th>M$<em>{412}$ Rise $t</em>{1/2}$ in usecs</th>
<th>$t_{1/2}$-1 in msecs</th>
<th>$t_{1/2}$-2 in msecs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>H$_2$O D$_2$O D$_2$/H$_2$O</td>
<td>H$_2$O D$_2$O D$_2$/H$_2$O</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>69 330 4.8</td>
<td>3.7 7.7 2.1</td>
<td></td>
</tr>
<tr>
<td>500 mM BD</td>
<td>0.65</td>
<td>112 620 5.5</td>
<td>3.1 384 1170 3.0</td>
<td></td>
</tr>
</tbody>
</table>

M$_{412}$/bR$_{568}$ Molar Ratio as % Control

<table>
<thead>
<tr>
<th>bR Sample</th>
<th>M$<em>{412}$/bR$</em>{568}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H$_2$O D$_2$O D$_2$/H$_2$O</td>
</tr>
<tr>
<td>Control</td>
<td>0 100 320 3.2</td>
</tr>
<tr>
<td>500 mM BD</td>
<td>0.65 2600 5720 2.2</td>
</tr>
</tbody>
</table>
the curve peeling computer program did not always analyze the $M_{412}$ decay as the sum of two exponentials, and for this reason the fast phase ($t_{1/2}-1$) was not detected in the control bR and EA modified samples. The slow phase decay ($t_{1/2}-2$) isotope effect in the EA and DMA modified bR samples was similar to the control $t_{1/2}-2$ isotope effect. The $M_{412}/bR_{568}$ molar ratio reflected the effect of $D_2O$ on the $M_{412}$ decay kinetics, and was similar in the imidoester samples as in control bR.

The magnitude of the isotope effects for the rise and decay of $M_{412}$ were smaller than a "normal" $D_2O$ effect as seen in solution (6-10) (50), and the isotope effect for the decay was smaller than for the rise. The significance of these results will be discussed (see Discussion II. B.1.b.).

I. C. pH Response of a pH Indicator Dye

Besides photocycling kinetics, bR's photoactivity can be measured with a pH indicator dye. Using this technique it has been shown that during the course of each photoreaction cycle, solutions of PM take up and release protons (51-53). When PM is in cell envelope vesicles, light causes bR molecules to first release protons on the extracellular surface and then take them up on the intracellular surface (36) with similar kinetics as in PM solutions. Thus it is thought that monitoring proton release and uptake of bR in PM in solution is indicative of the protons pumped across the PM during the photocycle.

As shown in Figure 29, photocycling kinetics of the 412 nm photocycle intermediate for control bR without dye were monitored at 365 nm where $M_{412}$ has an appreciable absorption. The 412 nm kinetics were similar if determined at 412 nm or at 365 nm in the bR samples without dye,
Figure 29

Absorbance at 365 nm
Relative Units

Kinetics of Proton Movement

Control bR

Without Dye
With Dye

Proton Uptake
Spectrum With Dye - Spectrum Without Dye
and the dye was seen to have no absorbance at 412 nm. In the sample with 7-hydroxycoumarin, the flash-induced proton release caused the dye to absorb light, so the observed absorbance decreased from the sample without dye. The maximum difference between the two samples is seen as the difference of the spectrum with dye - the spectrum without dye. The rise of the difference spectrum indicated proton release, while the decay represented proton uptake from purple membrane in distilled water. These summed traces were smoothed by computer averaging with a window of 10 points and also manually. In the case of one bR sample, proton release was also observed on a shorter time scale. The \( t_{1/2} \) determined using this time scale was similar to that determined from the time scale in Figure 29, so most of the data was analyzed using the time scale in Figure 29.

Table 10 compares the kinetics of proton movement with \( M_{412} \) kinetics for the bR samples analyzed. As shown, in all cases the rise of \( M_{412} \) was faster than proton release, by a factor of 6 or more. The use of pH indicator dyes as accurate monitors of proton movements will be further discussed (Discussion II.B.1.a.).

The \( t_{1/2} \)'s associated with proton uptake followed more closely the kinetics of the \( M_{412} \) decay, within a factor of 2, since they are much slower than the response time of the dye. The factor that proton uptake lagged behind the \( M_{412} \) decay differed with the type of imidoester used and was not related to the use of a bifunctional or monofunctional reagent. Thus, this study showed that proton movement, and especially proton uptake, paralleled the kinetics of the photocycle. For example, 10 mM DMS was about 3-fold slower in both phases of \( M_{412} \) decay than the control, and its proton uptake \( t_{1/2} \)'s were similarly about 3-fold slower than control. Whether the proton that is released to the aqueous phase
Table 10
Comparison of Effect of Imidoester Modification on Proton Movements and $M_{412}$ Kinetics

<table>
<thead>
<tr>
<th>bR Sample</th>
<th>$M_{412}$ Kinetics</th>
<th>Proton Movements</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rise $t_{1/2}$</td>
<td>Release $t_{1/2}$</td>
</tr>
<tr>
<td></td>
<td>Decay $t_{1/2}^{-1}$</td>
<td>Uptake $t_{1/2}^{-1}$</td>
</tr>
<tr>
<td></td>
<td>Decay $t_{1/2}^{-2}$</td>
<td>Uptake $t_{1/2}^{-1}$</td>
</tr>
</tbody>
</table>

| Control   | 71 μsecs,       | 460 μsecs,     |
| 0         | 2.5 msecs       | 5 msecs        |
|           | 15 msecs        | 24 msecs       |

| 10 mM DMS | 70 μsecs,       | 1.5 msecs,     |
| 0.57      | 7.7 msecs       | 14 msecs       |
|           | 48.5 msecs      | 68 msecs       |

| 5 mM DMA  | 75 μsecs,       | 510 μsecs,     |
| 0.63      | 6.9 msecs       | 11.5 msecs     |
|           | 53 msecs        | 55 msecs       |

| 200 mM EA | 70 μsecs,       | 1.5 msecs,     |
| 0.80      | 4.6 msecs       | 5.6 msecs      |
|           | 17.4 msecs      | 30.2 msecs     |
measured in this experiment is the proton that also protonates the
Schiff base is not understood at present, nor does this experiment
prove this. The number of protons released was not quantitated in this
experiment. In the Discussion section II.B.1.a., the relation of protons
released to protons pumped will be further examined.

I. D. Oligomerization of bR

1. Effect of Number of Lysines Modified

After quantitating the number of lysines modified
by the imidoesters and correlating the chemical changes with changes in
photoactivity, it was of interest to quantitate the amount of inter-
molecular cross-linking caused by the bifunctional, and possibly, mono-
functional imidates. This was done as described in Materials and Methods
II.C.3. by observing bR polymer (oligomer) formation on SDS gels.

An example of the gel pattern obtained of bR after reacting with
20 mM MA, 10 mM DMA or 10 mM DMS is shown in Figure 30. This repro-
duction of a photograph shows that even 20 mM MA was capable of a small
amount of intermolecular cross-linking. This pattern also reveals that
both 10 mM DMA and 10 mM DMS produced higher polymers of bR. It should
be noted that in the case of 10 mM DMS, there was sometimes a dark band
of Coomassie blue stain at the top of the gel. This indicates that under
these conditions of modification, bR was highly polymerized and during
electrophoresis not all of the protein entered the gel. In the gels of
the DMA and MA modifications, it appeared as if all of the protein entered
the gels.

The Coomassie blue bands on the gels were quantitated by first
scanning the gel as described in Materials and Methods II.C.3. Figure 31
shows a typical gel scan containing polymers of bR as high as a pentamer.
Figure 30
Imidoester Gel Patterns

Control
20 mM MA
10 mM DMA
10 mM DMS
Figure 31

Typical Gel Scan

Monomer  Dimer  Trimer  Tetramer  Pentamer

10 mM DMA-Modified bR
Polymers higher than pentamers were difficult to distinguish clearly and so were called "higher polymers". The area of the peaks of polymers up to pentamers were quantitated by triangulation as described. Figure 32 shows a bar graph presentation of the gel scan results for the average of two DMS concentration curves. 2 and 3 lysines modified increased the amount of tetramers and pentamers, and at 3 and more lysines modified higher polymers were formed. After deterioration of the chromophore protein structure (shown by a break in the graph), higher polymers of bR were more prominent.

Figure 33 is a bar graph representation of the gel pattern obtained by DMA modification. At all concentrations of DMA, all the protein appeared to enter the gel. Only at higher concentrations of DMA, where 4 lysines were modified, were polymers as high as pentamers formed. The oligomer pattern obtained after DMA modification contrasts sharply with that following DMS modification. At 4 lysines modified, for example, DMS had about 55% oligomers and DMA had only 30% oligomers, indicating that intermolecular cross-linking was easier for DMS. Intramolecular cross-linking by both reagents may have been comparable to the amount of intermolecular cross-linking, but the study of this by gel electrophoresis necessitates proteolytic digestion of bR, which was not carried out in this experiment.

Figure 34 shows a similar bar graph representation of the gel pattern obtained after MA modification. All of the protein appeared to enter the gel at all concentrations of MA. Only at 3.5 lysines modified were trimers seen. Since there was a maximum of about 10% intermolecular cross-linking seen, there was probably also a small amount of intramolecular cross-linking. However, intramolecular cross-linking was not specifically studied in this experiment.
Oligomerization of bR by DMS Monitored by Gel Electrophoresis

Percent Total Protein Area in Gel

- $x_1$ = Monomer
- $x_2$ = Dimer
- $x_3$ = Trimer
- $x_4$ = Tetramer
- $x_5$ = Pentamer
- H.P. = Higher Polymers

Number of Lysines Modified (+ 0.7 Lysines Modified)
Figure 33

Oligomerization of Bacteriorhodopsin by DMA Monitored by Gel Electrophoresis

Percent Total Protein
Area in Gel

- 100
- 80
- 60
- 40
- 20

0 1 1.5 2 4
Number of Lysines Modified (+0.7 Lysines Modified)

x1 = Monomer
x2 = Dimer
x3 = Trimer
x4 = Tetramer
x5 = Pentamer
Figure 34

Oligomerization of Bacteriorhodopsin by MA Monitored by Gel Electrophoresis

Percent Total Protein

Area in Gel $x_1$

Number of Lysines Modified (+0.4 Lysines Modified)

$x_1 = \text{Monomer}$

$x_2 = \text{Dimer}$

$x_3 = \text{Trimer}$
I. D. 2. Effect of Temperature

It was of interest to quantitate the amount of intermolecular cross-linking by bifunctional amidination at different temperatures. Figure 35 shows a bar graph representation of the quantitation of gel electrophoresis scans obtained of bR amidinated at several temperatures. As shown, the pattern of polymer formation by DMS was similar at temperatures of 40°C and below, but at 50°C and 60°C, DMS caused a marked formation of "higher polymers". The quantity of higher polymers formed was calculated using an internal standard of cytochrome C as described in Materials and Methods II.C.3. This formation of higher polymers was correlated with a higher than normal fraction of total lysines modified by DMS indicating a structural change of the bR protein between 40°C and 50°C.

If a structural change was the cause of formation of higher polymers by DMS, it did not affect intermolecular cross-linking by DMA. As shown in Figure 35, the oligomer pattern produced by DMA was similar at all temperatures of amidination. This suggests that the primary role of DMA is to intramolecularly cross-link. Since DMA is a shorter cross-linker, it may be able to penetrate easily to internal lysines and modify these and external lysines simultaneously. Intermolecular cross-linking by this reagent may occur after internal lysines have been modified. Thus no new internal lysines would be expected to be modified by DMA by intermolecular cross-linking due to a loosening of the protein structure at higher temperatures.

I. D. 3. Effect of Modifying bR in Bleached PM

It was of interest to examine the extent of
Figure 35

Effect of Temperature During Amidination on Oligomerization of bR

100 Percent Total Protein Applied to Gel

\[ x_1 = \text{Monomer} \]
\[ x_2 = \text{Dimer} \]
\[ x_3 = \text{Trimer} \]
\[ x_4 = \text{Tetramer} \]
\[ x_5 = \text{Pentamer} \]
\[ \text{H.P.} = \text{Higher Polymers} \]

Temperature During Amidination

(Fraction of Total (6) Lysines Modified)
Figure 36

Oligomerization of Bleached bR by MA Monitored by Gel Electrophoresis

Percent Total Protein Area in Gel

Number of Lysines Modified

x1 = Monomer
x2 = Dimer
x3 = Trimer
cross-linking in bleached bR to compare to cross-linking in control bR. It will be recalled that the monofunctional imidate, MA, caused a small amount of intermolecular cross-linking (about 10% see Results I.D.1.). 20 mM MA treatment of bR in bleached PM also caused only a small amount of cross-linking (see Figure 36). The interesting aspect of this figure is that at all extents of MA modification, the trimer was more predominant than the dimer. This suggests that the bR molecules within the trimer in the bR crystalline lattice have moved closer together, perhaps due to a weakening of the lattice structure. This indicates that retinal may have an important structural role in maintaining the crystalline lattice, as well as functional role in photocycling activity (see Discussion I.B.2.).

I. E. Absorption Spectroscopy

1. Effect of Number of Lysines Modified

The effect of increasing lysine modification by imidoesters on the absorption spectra of bR samples is given in Table 11. Since there was no shift in the wavelength maximum of the 568 nm chromophore at any concentration of imidoester, the chromophore environment and especially the excited state of retinal may not have been substantially changed. This result will be fully discussed in Discussion II.A.8.a. As shown in Table 11, there was a small decrease in the 568 nm chromophore following imidoester modification with DMA, monofunctional imidates and low concentrations of DMS. This small decrease in absorbance yield may indicate that some of the chromophores can no longer absorb light efficiently; i.e., that the reagent has caused a slight deterioration of the chromophore structures so that the quantum efficiency of light.
Table 11

Effect of Imidoester Modification on bR's 568 nm Chromophore

<table>
<thead>
<tr>
<th>Imidoester Concentration</th>
<th>568 nm Chromophore</th>
<th>Lowry Protein (% Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>94, 85, 86</td>
<td>91</td>
</tr>
<tr>
<td>0.1 mM DMS</td>
<td>100</td>
<td>91</td>
</tr>
<tr>
<td>0.5 &quot;</td>
<td>96</td>
<td>91</td>
</tr>
<tr>
<td>1.0 &quot;</td>
<td>87</td>
<td>91</td>
</tr>
<tr>
<td>5.0 &quot;</td>
<td>98</td>
<td>91</td>
</tr>
<tr>
<td>10.0 &quot;</td>
<td>97, 96, 92, 94</td>
<td>91</td>
</tr>
<tr>
<td>25.0 &quot;</td>
<td>100, 98</td>
<td>91</td>
</tr>
<tr>
<td>50.0 &quot;</td>
<td>100, 98</td>
<td>91</td>
</tr>
<tr>
<td>100.0 &quot;</td>
<td>100, 98</td>
<td>91</td>
</tr>
<tr>
<td>200.0 &quot;</td>
<td>100, 98</td>
<td>91</td>
</tr>
</tbody>
</table>

| Control                  | 0                  | 100                       |
| 0.1 mM DMA               | 100                | 100                       |
| 0.5 "                    | 96                 | 91                       |
| 1.0 "                    | 87                 | 91                       |
| 5.0 "                    | 98                 | 91                       |
| 10.0 "                   | 97, 96, 92, 94     | 91                       |
| 200.0 "                  | 100, 98            | 91                       |

| Control                  | 0                  | 100                       |
| 0.4 mM MA                | 100                | 100, 94                   |
| 1.6 mM MA                | 99, 98             | 100, 94                   |
| 2.0 mM EA                | 96                 | 100, 94                   |
| 5.0 mM EA                | 94                 | 100, 95                   |
| 8.0 mM EA                | 100, 95            | 100, 95                   |
| 10.0 mM EA, MB           | 100, 98            | 100, 95                   |
| 20.0 mM EA, MB           | 97, 100, 100       | 100, 95                   |
| 25.0 mM EA               | 100                | 97, 100, 100              |
| 25.0 mM MA               | 91, 99             | 97, 100, 100              |
| 50.0 mM EA               | 100                | 97, 100, 100              |
| 200.0 mM EA              | 80                 | 97, 100, 100              |

| Control                  | 0                  | 100                       |
| 0.5 mM DMS               | 100                | 100, 94                   |
| 1.0 "                    | 96                 | 91                       |
| 5.0 "                    | 98                 | 91                       |
| 10.0 "                   | 97, 96, 92, 94     | 91                       |
| 25.0 "                   | 100, 98            | 91                       |
| 50.0 "                   | 100, 98            | 91                       |
| 100.0 "                  | 100, 98            | 91                       |
| 200.0 "                  | 100, 98            | 91                       |
absorption decreased slightly in some of the bR molecules. At 25 mM DMS, however, considerable deterioration of the chromophore structure started to occur, which was more marked at higher concentrations. One reason that higher concentrations of DMS but not DMA caused bleaching may be that the amount of hydrolysis of DMA was less than that of DMS. This could occur as previously suggested if both ends of the DMA molecule had reacted by intramolecular cross-linking and to a limited extent by intermolecular cross-linking, thus preventing hydrolysis of the functional groups. DMS, on the other hand, may have a greater number of unreacted functional groups exposed to the aqueous phase, which will cause more hydrolysis. The products of hydrolysis include ammonia and the side chain alcohol which can have deleterious effects on the lipid-protein crystalline array of bR.

I. E. 2. Effect of pH

Figure 37 shows a pH titration of a bR sample with one lysine modified by DMS. As shown, at low pH's a peak at 600 nm was more prominent in the sample that had one lysine modified by DMS. This peak, the so-called "acid form" of bR (54), is thought to represent the change in protein structure upon protonation of the aspartate and glutamate amino acid side chains (55). Since it is formed more easily in the bR sample with one lysine modified by DMS, the involvement of a lysine, either directly or indirectly, in charge interactions with a carboxyl is implied. After DMS modification, the carboxyl group may be more accessible to acid titration. The significance of this result will be discussed (see Discussion II.A.8.a.).
Figure 37

pH Titration of Purple Membrane Visible Spectra

Wavelength in nm

500 550 600 650

| | | | |

pH

1.90
1.98
2.20
2.66
2.90
5.42
7.63
8.58

Control

1.85
2.0
2.50
2.93
3.93
5.95
7.50
8.40
9.90
10.3

1 Lysine Modified by DMS
I. E. 3. Effect of Modifying bR in Bleached PM

Absorption spectroscopy of bR's 568 nm chromophore is one indication of the presence of retinal in the bR protein structure, and "bleaching" bR with hydroxylamine is expected to remove this absorbance. Bleached PM was examined spectroscopically and found to have no absorption at 568 nm, but a peak at 366 nm was present indicating the presence of retinaloxime that had not been removed by the centrifugation procedures (10). Using a calculation from (10), it appeared as though none of the retinaloxime was removed by the distilled water washes as described in Materials and Methods II.A.3.

In addition, absorption spectroscopy allows measurement of regeneration of the chromophore after addition of all-trans retinal to the apomembrane. The percent regeneration is calculated by comparing this ratio to the same ratio in unbleached control bR. Table 12 gives the percent of regeneration one hour and one week after addition of all-trans retinal. These results may be inaccurate by ± 5 % due to the Lowry protein error calculated in Appendix 3. As shown, after one week both control and 20 mM EA treated samples were nearly completely regenerated. The fact that they were not regenerated after only one hour may be due to the steric difficulty of binding retinal back into its site in the protein. Specificity of the retinal binding site during regeneration will be discussed further in Discussion II.A.3.

I. F. Tryptophan Fluorescence Spectroscopy

Figures 38 and 39 show the tryptophan fluorescence excitation and emission spectra for a DMS concentration curve. The fact that only one broad band was seen in the fluorescence emission spectrum
**Table 12**

Regeneration of bR's 568 nm Chromophore with All-Trans Retinal

<table>
<thead>
<tr>
<th>Sample - Fraction of Total (7) Lysines Modified</th>
<th>% Regeneration</th>
<th>$\frac{568 \text{ nm Chromophore}}{\text{Lowry Protein}}$ as % Control</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>One Hour After Regeneration</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bleached and Regenerated Control bR - 0</td>
<td>82</td>
<td></td>
</tr>
<tr>
<td>Bleached, 20 mM EA Treated and Regenerated bR - 0.61</td>
<td>72</td>
<td></td>
</tr>
<tr>
<td><strong>One Week After Regeneration</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bleached and Regenerated Control bR - 0</td>
<td>94</td>
<td></td>
</tr>
<tr>
<td>Bleached, 20 mM EA Treated and Regenerated bR - 0.61</td>
<td>94</td>
<td></td>
</tr>
</tbody>
</table>
Figure 38
Fluorescence Excitation Spectra of DMS-Modified bR

Fluorescence Emission = 315 nm

DMS Concentration in mM

270 280 290 300
Excitation Wavelength in nm
Figure 39

Fluorescence Emission Spectra of DMS-Modified bR

Excitation Wavelength = 287 nm

DMS Concentration in mM

Emission Wavelength in nm

Control

300 310 320 330
with no dependence of $\lambda_{\text{MAX}}$ on the wavelength of excitation has several interpretations which will be discussed (see Discussion II.A.8.a.).

Of particular interest is the position of the wavelength maximum of excitation and emission. The positions of the wavelength maxima and decreases in fluorescence quantum yields for DMS and the other imidoesters are summarized in Table 13. As shown, in both the excitation and emission spectra of DMS, at 50 mM and higher a marked red shift occurred in the wavelength maxima, with the red shift more dramatic in the emission spectra. At lower concentrations of DMS, and all concentrations of DMA and EA, the red shift was smaller. The red shift indicates that there is a smaller energy difference between the ground and excited states. A perturbation in the environment of the ground and excited state energies is a function of the 1) polarizability, 2) polarity and/or 3) mobility of the local environment (56). The possible tryptophans involved will be discussed (see Discussion II.A.8.a.).

A different explanation for a red shift is that selective quenching of one or two tryptophans emphasizes the contribution of others (57,58). The slight decrease (about 5 %) in the fluorescence quantum yield could be selective quenching of tryptophans in a hydrophobic environment, which would result in a red shift.

The explanations for quenching of the fluorescence quantum yield are also numerous:

1) red shifts in the emission and excitation wavelength maxima,
2) formation of a ground state complex,
3) formation of a transient excited state collisional complex which does not change the energy but shortens the lifetime of the excited state,
4) formation of a long-range resonance energy transfer acceptor,
Table 13

Effect of Imidoester Modification on bR's Tryptophan Fluorescence

<table>
<thead>
<tr>
<th>Imidoester Concentration</th>
<th>Fraction of Total (6) Lysines Modified</th>
<th>Excitation λ_MAX (nm)</th>
<th>Emission λ_MAX (nm)</th>
<th>Fluorescence Yield (% Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>287</td>
<td>315</td>
<td>100</td>
</tr>
<tr>
<td>5 mM DMS</td>
<td>0.48</td>
<td>287</td>
<td>318</td>
<td>93</td>
</tr>
<tr>
<td>10 &quot;</td>
<td>0.57</td>
<td>287</td>
<td>318</td>
<td>93,100</td>
</tr>
<tr>
<td>50 &quot;</td>
<td>0.78</td>
<td>287</td>
<td>325.4</td>
<td>103,107</td>
</tr>
<tr>
<td>200 &quot;</td>
<td>0.85</td>
<td>291</td>
<td>328</td>
<td>111,113</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>287</td>
<td>315</td>
<td>100</td>
</tr>
<tr>
<td>5 mM DMA</td>
<td>0.63</td>
<td>287</td>
<td>317</td>
<td>98</td>
</tr>
<tr>
<td>10 &quot;</td>
<td>0.67</td>
<td>288.4</td>
<td>317</td>
<td>93</td>
</tr>
<tr>
<td>50 &quot;</td>
<td>0.75</td>
<td>288.4</td>
<td>318.3</td>
<td>88</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>287</td>
<td>315</td>
<td>100</td>
</tr>
<tr>
<td>5 mM EA</td>
<td>0.30</td>
<td>289</td>
<td>315</td>
<td>80</td>
</tr>
<tr>
<td>10 &quot;</td>
<td>0.45</td>
<td>289</td>
<td>315</td>
<td>90</td>
</tr>
<tr>
<td>25 &quot;</td>
<td>0.68</td>
<td>288</td>
<td>316.6</td>
<td>91</td>
</tr>
<tr>
<td>50 &quot;</td>
<td>0.79</td>
<td>288</td>
<td>316.6</td>
<td>90</td>
</tr>
<tr>
<td>200 &quot;</td>
<td>0.80</td>
<td>288</td>
<td>316.6</td>
<td>86</td>
</tr>
</tbody>
</table>
5) denaturation of the protein, causing internal quenching groups or solvent to be brought closer to tryptophan,
6) protonation near the fluorescing tryptophan,
7) increase in polarity near the fluorescing tryptophan (80).

The significance of fluorescence quenching will be further discussed (see Discussion II.A.8.a.).

I. G. Resonance Raman Spectroscopy

1. Effect of Number of Lysines Modified

Resonance Raman spectroscopy gives detailed information about the interaction between the chromophore, retinal, and the protein moiety, bacterio-opsin, by monitoring the Raman scattering from vibrations coupled to an electronic transition when the excitation wavelength is within or near the absorption band of the chromophore. Figure 40 shows the resonance Raman spectra of 20 mM EA modified bR with 0.63 fraction of total lysines modified and control bR taken by Gregory Perreault under steady illumination with constant stirring. Part B. shows a survey of the wavenumbers from 800 to 1800 cm\(^{-1}\) (scan time = 10 seconds). Part A. was scanned over a narrower wavenumber region in order to delineate more clearly the bands at 1642 cm\(^{-1}\) and 1622 cm\(^{-1}\), which have been assigned to the protonated and deprotonated forms of the Schiff base C=N vibration, respectively (61). In addition, this region contains bands at 1531 cm\(^{-1}\) and 1566 cm\(^{-1}\), which represent the C=C ethylenic stretching frequencies of the bR\(_{568}\) and M\(_{412}\) chromophore structures, respectively. Both sets of data represent the unsmoothed sum of 3 Raman scans under computer control. The particulate nature of the sample, which remained considerable even with constant stirring, caused the noise in the spectra.
Figure 40

Resonance Raman Spectra of 20 mM EA Modified bR

Using 458 nm Probe Laser

With Steady Illumination

A.

B.

Wavenumber in cm⁻¹

Control bR

20 mM EA

1400 1500 1600 1700

1178 cm⁻¹ 1202 cm⁻¹ 1010 cm⁻¹

1531 cm⁻¹ 1566 cm⁻¹ 1622 cm⁻¹ 1642 cm⁻¹
As shown in Figure 40, none of the peaks shifted frequency in the 20 mM EA modified sample compared to the control bR. The frequencies of the lines in a resonance Raman spectrum give the energies of the vibrational modes in the ground state of the chromophoric group. This was expected since there was no shift in \( \lambda_{\text{MAX}} \) of the 568 nm chromophore, and since it has been found empirically that \( \nu(C=C) \) depends linearly on \( \lambda_{\text{MAX}} \) of the optical absorption band such that a red shift of \( \lambda_{\text{MAX}} \) is accompanied by a downfield shift of \( \nu(C=C) \) (62). It should be noted that a shift in \( \nu(C=C) \) is a ground state effect while the excited state plays a dominant role for the shift of \( \lambda_{\text{MAX}} \).

The most marked changes in the 20 mM EA sample were in the peak heights associated with the \( M_{412} \) and \( bR_{568} \) states. As shown in Table 14 the 1566 cm\(^{-1}\) and 1622 cm\(^{-1}\) bands increased slightly, and the 1531 cm\(^{-1}\) and 1642 cm\(^{-1}\) bands decreased slightly relative to the 1010 cm\(^{-1}\) normalization band. The peak at 1010 cm\(^{-1}\), which is composed of C-CH\(_3\) stretching vibrations, mainly at the C(9) and C(13) positions, is used as an internal standard of concentration, since this peak is nearly invariant with different forms of the chromophore (63). The intensities of these lines give information on the excited electronic states of the chromophore, since the intensities depend not only on the nature of the ground state vibrations, but also on the geometrical distortion of the molecule in the excited electronic state (64). Neither the protonated Schiff base state (\( bR_{568} \)) nor the deprotonated Schiff base form (\( M_{412} \)) should be specifically resonance enhanced, since the 458 nm probe laser was set at the isosbestic point between \( bR_{568} \) and \( M_{412} \) (Gregory Perreault, personal communication). Since the light intensity at the sample was very high (equivalent to \( 14 \times 10^6 \, \text{mW/cm}^2 \)), the photocycle was probably saturated. I.e., as soon as \( M_{412} \) was formed it was photoconverted back.
Table 14

Effect of Imidoester Modification of bR on Resonance Raman Spectroscopy

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fraction of Total (6) Lysines Modified</th>
<th>Peak Height Ratios As Percent Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1642 cm(^{-1})</td>
<td>1622 cm(^{-1})</td>
</tr>
<tr>
<td>Control bR - 0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>20 mM EA - 0.63</td>
<td>96</td>
<td>104</td>
</tr>
<tr>
<td>(Stationary Sample)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 mM DMA - 0.67</td>
<td>106</td>
<td>-</td>
</tr>
<tr>
<td>(Flowed Sample)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fingerprint Region

<table>
<thead>
<tr>
<th></th>
<th>1202 cm(^{-1})</th>
<th>1178 cm(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control bR - 0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>20 mM EA - 0.63</td>
<td>97</td>
<td>93</td>
</tr>
<tr>
<td>(Stationary Sample)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 mM DMA - 0.67</td>
<td>105</td>
<td>112</td>
</tr>
<tr>
<td>(Flowed Sample)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Control bR - 0

20 mM EA - 0.63 (Stationary Sample)

10 mM DMA - 0.67 (Flowed Sample)
to the bR$_{568}$ form. Such a high photon flux density is necessary since the quantum efficiency for transforming an incoming photon into a scattered Raman photon is 6 orders of magnitude smaller than the quantum efficiency of light absorption by bR (65) and $3 \times 10^5$ times smaller than the primary photochemical event which has a quantum yield of $\approx 0.3$ (66, 67). Thus the increase in the bands associated with the deprotonated Schiff base, and decrease in the bands associated with the protonated Schiff base could be due to a shift in equilibrium in the photostationary state between these two forms caused by a change in the quantum efficiencies involved, or by inhibition of the back photoreaction $M_412 \rightarrow bR_{568}$.

In the fingerprint region, there appeared to be a slight decrease in the heights of the bands at 1200 cm$^{-1}$ and 1178 cm$^{-1}$. The band at 1200 cm$^{-1}$ can be described as C-C stretch/C-CH$_3$ rock. This band is nearly invariant with the isomeric state of retinal since its normal mode is mainly concentrated at the C(9) and C(13) positions of the isoprenoid chain and is therefore not so dependent on conformational changes (63). Indeed, Braiman and Mathies (30) have shown that both the bR$_{LA}$ spectrum and the $M_412$ spectrum have a peak at 1200 cm$^{-1}$ that is about 1.3 times as intense as the 1010 cm$^{-1}$ band. The 1178 cm$^{-1}$ band, which is a C-C stretch, is thought to be more sensitive to isomeric changes in bR. This band has been shown to be associated with the all-trans configuration of retinal in bR$_{LA}$ (30). After 20 mM EA modification, this band decreased slightly, and the band next to it at 1180 cm$^{-1}$ increased slightly, so their heights were almost equal. The 1180 cm$^{-1}$ band has been shown to be associated with the $M_412$ form (30). Thus these two changes reflected the changes seen above in the bands associated with the protonated and deprotonated Schiff
base chromophores. In addition, the peak at 1174 cm\(^{-1}\) was more prominent in the 20 mM EA spectrum. It has been shown by Stockburger et al. (63) that the peak at 1174 cm\(^{-1}\) is due to M', which is a photo-product of M that contains all-trans retinal. M' can be visualized by illuminating the spectrum of M with light of 440 nm at -196\(^{\circ}\) C (68); it is converted to bR\(_{568}\) upon warming to -90\(^{\circ}\) C. The significance of this result and its interpretation in the light of some new biochemical evidence (69) will be presented in Discussion II.A.1.d.

Table 14 also summarizes the peak height changes from the low photoalteration Raman spectrum in Figure 41 of 10 mM DMA modified bR compared to a bR control low photoalteration spectrum. These spectra were obtained by Mark Braiman by flowing the bR sample passed the laser probe beam at 300 cm/sec as described in Materials and Methods V.A.3. and also in (30), and should thus represent the peaks associated with the bR\(_{568}\) form. The longer lived intermediates, M and O, will not be represented in these spectra. As shown, there were no shifts in the frequencies of the peaks. The normalized height at 1531 cm\(^{-1}\) was increased in the 10 mM DMA sample above the control. This may indicate a distortion of the C=C ethylenic stretch of the bR\(_{568}\) chromophore. The other peaks associated with the bR\(_{568}\) chromophore, the 1642 cm\(^{-1}\), 1597 cm\(^{-1}\) and 1582 cm\(^{-1}\) peaks were also slightly increased. The 1178 cm\(^{-1}\) band, which is thought to arise from the all-trans retinal isomer in the bR\(_{568}\) chromophore, was also increased. Thus all of the peaks associated with the bR\(_{568}\) chromophore were increased, perhaps due to a distortion of the chromophore environment.
Figure 41
Low Photoalteration Resonance Raman Spectrum of DMA Modified bR
Using 514 nm Probe Laser
(Flowed Samples)
I. G. 2. Effect of Modifying bR in Bleached PM

Since resonance Raman spectroscopy gives much more detailed information about the interaction between the chromophore and the protein moiety than absorption spectroscopy, it is well suited to studying the chromophore structures of bleached and regenerated bR. Figure 42 shows the resonance Raman spectra of regenerated control and 40 mM MA treated bR with 0.60 fraction of total lysines modified compared to unbleached control bR. These samples were examined by Mark Braiman with the capillary technique under steady illumination as described in Materials and Methods V.A.3. The probe laser was at 514 nm which resonance enhanced those bands associated with the 568 nm chromophore. The peaks of interest are indicated in the figure.

As shown, the peaks did not shift frequency in either regenerated sample compared to unbleached control bR. The most marked changes seen in both regenerated samples compared to control bR were in the peak heights of the bands of interest. The heights of these bands are quantitated in Table 15 and are presented as percent of the unbleached control bR after normalization to the 1010 cm\(^{-1}\) band. The most striking result was that the intensities of all the bands in the regenerated samples, with the exception of the 1530 cm\(^{-1}\) band, were increased above the unbleached control bR. This suggests that the regenerated chromophore environment may be somewhat distorted, perhaps due to the presence of unremoved retinaloxime, which was observed but not quantitated in the absorption spectra (see Results I.E.3.). The 1530 cm\(^{-1}\) band, which is identified as the C=C ethylenic stretch of the 568 nm chromophore, may have experienced less distortion of its C=C vibrations than the environment near the Schiff base.
Figure 42
Resonance Raman Spectra of Regenerated bR
Using 514 nm Probe Laser
With Steady Illumination

Unbleached Control bR

Bleached and Regenerated bR

Bleached, 40 mM MA Treated and Regenerated bR
Table 15

Effect of Imidoester Modification of Bleached bR on Resonance Raman Spectroscopy

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fraction of Total (7) Lysines Modified</th>
<th>Peak Height Ratios as Percent Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1642 cm⁻¹</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1010 cm⁻¹</td>
</tr>
<tr>
<td>Unbleached Control bR</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Bleached and Regenerated bR - 0</td>
<td>176</td>
<td>281</td>
</tr>
<tr>
<td>Bleached, 40 mM MA Treated and Regenerated bR - 0.60</td>
<td>178</td>
<td>193</td>
</tr>
<tr>
<td>Fingerprint Region</td>
<td></td>
<td>1202 cm⁻¹</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1010 cm⁻¹</td>
</tr>
<tr>
<td>Unbleached Control bR</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Bleached and Regenerated bR - 0</td>
<td>129</td>
<td>156</td>
</tr>
<tr>
<td>Bleached, 40 mM MA Treated and Regenerated bR - 0.60</td>
<td>177</td>
<td>174</td>
</tr>
</tbody>
</table>
The 1642 cm\(^{-1}\) band which represents the C=N vibration of the protonated Schiff base, had a similar height in the regenerated control and MA treated samples. Also similar were the heights of the 1567 cm\(^{-1}\) bands in both samples. The 1567 cm\(^{-1}\) band has been identified with the C=C stretch of the \(\beta\)-ionone ring (70) of the deprotonated Schiff base chromophore. Since the 1567 cm\(^{-1}\) bands were of almost equal height in the regenerated samples, it was expected that the 1622 cm\(^{-1}\) and 1530 cm\(^{-1}\) bands would also have similar heights. Although the 1530 cm\(^{-1}\) peak heights were equal, the bands at 1622 cm\(^{-1}\), which are caused by the C=N stretches of the deprotonated Schiff base, did not have equal heights in both samples. This result may indicate a slightly different distortion near the Schiff base than in the rest of the chromophore environment. Alternatively, a recent investigation (70) suggests that the 1622 cm\(^{-1}\) (1620 cm\(^{-1}\)) band is sensitive to C-H bends on the isoprenoid chain as well as the protonation state of the Schiff base.

In the fingerprint region, the peaks at 1178 cm\(^{-1}\) and 1200 cm\(^{-1}\) were slightly higher in the bleached and treated regenerated sample than in the regenerated control bR. It is not easily understood why the 1200 cm\(^{-1}\) peak height was so different in the treated and control regenerated samples, since this peak is thought not to change too much with a change from all-trans to 13-cis retinal. It is possible that this difference indicates a distortion of the chromophore such that it is neither all-trans nor 13-cis in the regenerated samples. The fact that the 1178 cm\(^{-1}\) band was higher and shifted slightly towards 1180 cm\(^{-1}\), may be due to the increased presence of \(M_{412}\), since the 1180 cm\(^{-1}\) band has been shown to be associated with \(M_{412}\) (30). An increased photo-stationary amount of M is due to a 2-fold slower M decay in the treated and regenerated sample (see Results I.B.3.).
II. 2,3-Butanedione (BD) Modification at pH 8.2 of Arginine Residues in bR

A. Number of Arginines Modified

1. Amino Acid Analysis

After chemical modification with BD at pH 8.2, the questions must be asked, 1) how many total arginines are available for chemical modification with BD, and 2) what fraction of the total arginines are modified by BD? The actual number of arginines modified will usually differ from the total number of arginines available to the reagent due to the statistical equilibria of the reactions involved, i.e., not all of the arginines available will be modified on every bR molecule.

The most accurate results of the number of arginines modified by varying concentrations of BD were those obtained by amino acid analysis. Figure 43 shows a typical chromatogram of two amino acid analyses. Part A shows the assignment of peaks in the chromatogram of the 400 mM BD modified bR sample. The small peak between valine and methionine was a buffer change. As indicated, the identity of the small peak between NH₄⁺ and arginine is unknown, and was also present in control bR. This peak is in the position where tryptophan normally elutes, but tryptophans are usually degraded during the acid hydrolysis and should not appear on this chromatogram. Part B shows an enlarged view of the scan of the positively charged amino acids in both the control and the 400 mM BD modified bR sample. Clearly, the area of the arginine peak relative to lysine was smaller in the BD modified sample as is quantitated in Table 16.

Table 16 shows the amino acid analyses of several concentrations in the BD concentration curve. Conversion of nanomoles to numbers of amino
Figure 43

Typical Amino Acid Analysis Chromatogram

A. 400 mM BD-Modified bR

B. Control bR

LEU

NH₄

THR

ASP

GLY

ALA

ILE

MET

GLU

VAL

TYR

PHE

LYS

NH₄

ARG

400 mM BD-Modified bR
Table 16

Amino Acid Analysis of BD-Modified bR

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Control</th>
<th>100</th>
<th>200</th>
<th>300</th>
<th>400</th>
<th>Average + Std. Dev. (10 Samples)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic Acid</td>
<td>13.49</td>
<td>12.72</td>
<td>12.74</td>
<td>12.97</td>
<td>13.32</td>
<td>13.1 ± .34</td>
</tr>
<tr>
<td>Threonine</td>
<td>18.61</td>
<td>18.52</td>
<td>18.46</td>
<td>18.56</td>
<td>18.54</td>
<td>18.5 ± .13</td>
</tr>
<tr>
<td>Serine</td>
<td>12.49</td>
<td>12.41</td>
<td>12.46</td>
<td>12.79</td>
<td>13.03</td>
<td>12.6 ± .29</td>
</tr>
<tr>
<td>Proline</td>
<td>10.80</td>
<td>11.32</td>
<td>11.31</td>
<td>11.41</td>
<td>11.31</td>
<td>11.2 ± .24</td>
</tr>
<tr>
<td>Glycine</td>
<td>25.16</td>
<td>26.18</td>
<td>26.41</td>
<td>26.22</td>
<td>26.82</td>
<td>26.2 ± .65</td>
</tr>
<tr>
<td>Alanine</td>
<td>28.74</td>
<td>29.53</td>
<td>29.58</td>
<td>29.72</td>
<td>29.79</td>
<td>29.5 ± .41</td>
</tr>
<tr>
<td>Methionine</td>
<td>8.55</td>
<td>8.87</td>
<td>8.93</td>
<td>9.07</td>
<td>8.75</td>
<td>8.88 ± .21</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>12.66</td>
<td>13.42</td>
<td>13.52</td>
<td>13.73</td>
<td>13.31</td>
<td>13.3 ± .41</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>10.35</td>
<td>10.63</td>
<td>10.72</td>
<td>10.45</td>
<td>10.27</td>
<td>10.5 ± .21</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>12.58</td>
<td>12.70</td>
<td>12.85</td>
<td>12.72</td>
<td>12.92</td>
<td>12.8 ± .21</td>
</tr>
<tr>
<td>Histidine</td>
<td>0.27</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.05 ± .01</td>
</tr>
<tr>
<td>Lysine</td>
<td>6.67</td>
<td>6.86</td>
<td>6.90</td>
<td>6.88</td>
<td>6.54</td>
<td>6.80 ± .60</td>
</tr>
<tr>
<td>Arginine</td>
<td>8.88 ± 0</td>
<td>6.67 ± .028</td>
<td>6.05 ± .13</td>
<td>5.16 ± .16</td>
<td>5.09 ± .085</td>
<td></td>
</tr>
</tbody>
</table>

Number of Arginines Modified:

- Control: 0
- 100 mM BD: 2.21 ± .028
- 200 mM BD: 2.83 ± .13
- 300 mM BD: 3.72 ± .16
- 400 mM BD: 3.79 ± .085
acid was performed by normalizing to the same number of amino acids as Khorana et al. (24) and Ovchinnikov et al. (23), excluding tryptophan, which does not appear, leucine and valine. Leucine's peak was sometimes too high to be accurately determined, and valine's peak was sometimes part of the buffer change peak. The numbers in the columns represent the average of analyses on duplicate hydrolyses of that sample. In the last column, the average and standard deviation for each amino acid represent the average and standard deviation of 10 analyses. The average standard deviation for all the amino acids was 0.31. The arginine values show the average and standard deviation of duplicate analyses at each BD concentration. Subtracting the number of arginines present from the control value, 8.88, determined the number of arginines modified at each concentration of BD.

A second method used to convert nanomoles to numbers of amino acids was to find the smallest sum of the standard deviations from the nearest whole number for all of the amino acids analyzed for each sample. Different converting factors were used, with lysine equal to between 6.5 and 7.0. This method yielded an average minimum standard deviation from whole numbers when the number of lysines equaled 6.8, which gave the rounded-off results shown in Table 17 (This Study, 1980). Either method of converting nanomoles to numbers of amino acids was judged satisfactory, since there was little difference between the results obtained by these two methods. Table 17 also shows the difficulty in quantitating chemical modifications of bR. No two of the bR compositions in this table are alike, which may be due to the type of amino acid analyzer or to the H. halobium strain studied, two crucial details which are omitted from several of these studies. In the latter regard, the extremely high frequency of mutation of the
Table 17
Comparison of bR Amino Acid Analyses

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td>Aspartic Acid</td>
<td>13</td>
<td>16</td>
<td>15</td>
<td>13</td>
<td>12</td>
<td>12</td>
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<tr>
<td>Threonine</td>
<td>18</td>
<td>18</td>
<td>18</td>
<td>18</td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td>Serine</td>
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<td>14</td>
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<td>14</td>
<td>14</td>
<td>13</td>
</tr>
<tr>
<td>Glutamic Acid</td>
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<td>17</td>
<td>16</td>
<td>14</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td>Proline</td>
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<td>11</td>
<td>11</td>
<td>11</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>Glycine</td>
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<td>24</td>
<td>26</td>
<td>25</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>Alanine</td>
<td>29</td>
<td>28</td>
<td>28</td>
<td>29</td>
<td>30</td>
<td>29</td>
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<tr>
<td>Valine</td>
<td>20</td>
<td>19</td>
<td>20</td>
<td>20</td>
<td>21</td>
<td>21</td>
</tr>
<tr>
<td>Methionine</td>
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<td>8</td>
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</tr>
<tr>
<td>Isoleucine</td>
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<td>12</td>
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</tr>
<tr>
<td>Leucine</td>
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<td>30</td>
<td>30</td>
<td>33</td>
<td>34</td>
<td>36</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>11</td>
<td>10</td>
<td>11</td>
<td>10</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>13</td>
<td>13</td>
<td>13</td>
<td>12</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td>Histidine</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Lysine</td>
<td>7</td>
<td>8</td>
<td>7</td>
<td>6</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Arginine</td>
<td>9</td>
<td>9</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td>240</td>
<td>238</td>
<td>239</td>
<td>233</td>
<td>240</td>
<td>240</td>
</tr>
</tbody>
</table>

*Including Pyroglutamic Acid
bacterio-opsin protein in H. halobium, $10^{-4}$ (71), may be the cause of the variability seen in this table. It has been found (71) that mutants of H. halobium which have lost the capability of forming bacterio-opsin carry insertions in the plasmid pH1, which are distributed over a rather large region of the plasmid. Thus the results obtained in this section are assumed to be correct for my strain (nominally S9) of bR.

The results shown in Table 16 are plotted in Figure 44 vs. BD concentration. The total number of arginines available to BD was obtained from a Scatchard plot (41) of the curve in Figure 45. The x axis in the Scatchard plot in Figure 45 is $v$, the number of arginines modified. The y axis is $v/[BD]$. The x-intercept is the total number of arginines available to BD modification, $N$. The Scatchard plot may be used only if it is certain that the reaction has reached equilibrium. The slope is the negative inverse of the equilibrium constant of dissociation. In the case of BD modification, a reaction time of three hours at pH 8.2, has been shown to be sufficient for equilibrium to be achieved; the reaction of BD with free arginine is 96 % complete in 15 minutes (15).

The value of total arginines available, $N$, can be further verified with a Hill plot (see Figure 46). Deviations from linearity in the Hill plot indicate an incorrect choice for the total number of arginines available, $N$. As shown in Figure 46, 7 was the correct choice for $N$, since only 7, not 4, 5, 6 or 8, yielded a linear Hill plot. Additionally, the slope of the Hill plot for $N = 7$ was 0.96, which indicates non-cooperativity of binding of BD to arginines.

Besides the value of $N$, the Scatchard plot in Figure 45 yields other important information (41). The Scatchard plot has one straight
Figure 44

Number of Arginines Modified by BD

Number of Arginines
Modified

BD Concentration in mM

6
5
4
3
2
1

100 200 300 400 500

BD Concentration in mM
Scatchard Plot of BD Modification of bR

Figure 45

\[
\text{Slope} = \frac{1}{K_D}, \quad K_D = 0.268
\]

\[
\text{Intercept} = \frac{N}{K_D}, \quad K_D = 0.269
\]

\[
\text{Intercept} = N = 7
\]
Figure 46

Hill Plot to Test the Value of N From the Scatchard Plot

\[ \ln \left( \frac{N}{V} - 1 \right) \]

\[ \ln [BD] \]

\( \triangle, N = 4 \)
\( \square, N = 5 \)
\( \diamond, N = 6 \)
\( \bigcirc, N = 7 \)
\( \bigcirc, N = 8 \)
line component, which indicates that all 7 of the arginines that bind BD are of the same class with the same dissociation equilibrium constant, 0.268 M. The binding of BD to these 7 arginines may be expressed as:

\[
\text{Number of Arginines Modified} = \text{ARG}^* = \frac{(7-\text{ARG}^*)([\text{BD}])}{K_D}, \quad K_D = 0.268 \text{ M}
\]

When BD concentrations in the range 100-400 mM are fit to this equation, the ARG* values obtained agree quite well with the initial plot of number of arginines modified vs. [BD] in Figure 44. Thus this analysis reveals that 2 of the 9 arginines in my strain of bR were completely inaccessible to BD, and that the remaining 7 arginines all reached the same reaction equilibrium with BD.

II. A. 2. Ninhydrin Fluorescence Test

For purposes of comparison to the results obtained by amino acid analysis, the results of the ninhydrin fluorescence test are given in Table 18. The standard curve in the ninhydrin fluorescence test was similar to that produced by the fluorescamine assay (see Materials and Methods II.C.1.). The results in Table 18 represent the average of 4 samples at each concentration of BD, which were duplicates in two ninhydrin fluorescence assays. Thus it appeared that the full number of arginines modified, 4.2 at 400 mM BD as seen by amino acid analysis, was not shown by the ninhydrin fluorescence test, and that the test was less sensitive at low concentrations of BD. In addition, the average standard deviation, 0.52, was higher than in amino acid analysis.
Table 18
Comparison of Ninhydrin Fluorescence Test and Amino Acid Analysis of BD Modified bR

<table>
<thead>
<tr>
<th>BD Concentration in mM</th>
<th>Number of Arginines Modified</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ninhydrin Fluorescence Test</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
</tr>
<tr>
<td>50</td>
<td>$2.1 \pm 0.5$</td>
</tr>
<tr>
<td>100</td>
<td>$2.1 \pm 0.6$</td>
</tr>
<tr>
<td>200</td>
<td>$1.4 \pm 0.6$</td>
</tr>
<tr>
<td>300</td>
<td>-</td>
</tr>
<tr>
<td>400</td>
<td>$3.2 \pm 0.4$</td>
</tr>
</tbody>
</table>
II. B. Photocycling Activity

1. Effect of Number of Arginines Modified

Once the extent of arginine modification is known, the next step is to correlate the chemical modification with bR's photoactivity. Several of bR's photoactivities were measured after BD modification: photocycling, proton pumping in liposomes, proton release and uptake, and surface charge changes. As with the lysine modification, photocycling activity was the first indication of a change in bR's photoactivity. The kinetics of the formation and decay of the absorbance of one of the longest-lived photointermediates, $M_{412}$, can be easily monitored as described in Materials and Methods V.B.1.

The kinetics of the rise of the $M_{412}$ absorbance are given in Table 19. By 300 mM BD modification, where over half of the total available arginines have reacted with BD, there was a small but noticeable slowing of the rise of $M_{412}$ (1.7 times slower than control). However, higher concentrations of BD did not increase this inhibition. At lower concentrations of BD, the $M_{412}$ rise was similar to the control. The rise of $M_{412}$ always appeared as a single exponential.

In comparison to the relatively small inhibition seen in the rise kinetics, there was quite a marked inhibition seen in the decay of $M_{412}$ after BD modification. As shown in Figure 47, the inhibition, defined as

$$I(x) = \frac{t_{1/2}^{BD \text{ modified } bR}}{t_{1/2}^{control \text{ bR}}} \times 100,$$

where $x = \text{fraction of modified arginines}$, did not increase linearly with $x$. If only one arginine were important for the inhibition, then $I(x)$ would be a linear function of $x$. (See Appendix 14 for additional probability calculations.) Even if two
Table 19

M₄₁₂ Rise Kinetics of BD Modified bR
At 20.5°C

<table>
<thead>
<tr>
<th>bR Sample</th>
<th>Fraction of Total (7) Arginines Modified</th>
<th>Rise t₁/₂ μsecs</th>
<th>% Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>63.1 ± 2.3</td>
<td>100</td>
</tr>
<tr>
<td>2 mM BD</td>
<td>-</td>
<td>61.7 ± 9.9</td>
<td>98 ± 15</td>
</tr>
<tr>
<td>10 &quot;</td>
<td>0.029</td>
<td>64.7 ± 1.9</td>
<td>103 ± 3</td>
</tr>
<tr>
<td>20 &quot;</td>
<td>0.057</td>
<td>68.4 ± 2.8</td>
<td>108 ± 4</td>
</tr>
<tr>
<td>300 &quot;</td>
<td>0.53</td>
<td>110 ± 5.7</td>
<td>174 ± 9</td>
</tr>
<tr>
<td>400 &quot;</td>
<td>0.60</td>
<td>98.3 ± 0.4</td>
<td>156 ± 0.6</td>
</tr>
<tr>
<td>500 &quot;</td>
<td>0.65</td>
<td>92.7 ± 3.5</td>
<td>147 ± 5.5</td>
</tr>
<tr>
<td>600 &quot;</td>
<td>0.69</td>
<td>101.3 ± 6.6</td>
<td>161 ± 10.5</td>
</tr>
</tbody>
</table>
Figure 47

Inhibition of $M_{412}$ Decay Kinetics by BD Modification of bR

$M_{412}$ Decay $t_{1/2}$

First Phase as Percent Control First Phase,

Second Phase as Percent Control Second Phase,

$M_{412}$ Photostationary Steady State Absorbance ($M_{412}/bR_{568}$ Molar Ratio as % Control),

Fraction of Total Arginines (7) Modified (+.03)
or more arginines were important, as long as they had an independent
effect on the inhibition, then \( I(x) \) would be a linear function,
\( 100 + a_1x \). The fact that \( I(x) \) was not a linear function of \( x \) in Figure
47, suggests that the arginines were not independent determinants of the
inhibition. For example, if it is necessary to modify a particular pair
of arginines in order to inhibit the photocycle, then \( I_2(x) \) will be
given by a quadratic function, \( 100 + a_2x^2 \). To test this, the solid
lines in Figure 47 were drawn to fit \( I_2(x) = 100 + a_2x^2 \), where
\( a_2 = 6552 \) for the first phase of \( M_{412} \) decay, and \( a_2 = 9610 \) for the
second phase of \( M_{412} \) decay (top and bottom lines in Figure 47). Since
the fit of these lines to the data is fairly close, this suggests that
a pair of arginines inhibits the photocycle. Another example of a
cooperative inhibition pattern is if any one of 3 pairs of arginines
inhibits the photocycle. In this case \( I_3(x) = 100 + a_3(3x^2 - 2x^3) \),
but this function did not fit the data as well. Another example of
a cooperative inhibition pattern is if any one of 6 pairs of arginines
inhibits the photocycle. In this case, \( I_6(x) = 100 + a_4(6x^2 - 8x^3 + 3x^4) \).
This function also did not fit the data as well as \( I_2(x) \). The fit
of the functions to the data is seen more clearly if the data is
graphed as a function of \( x \) vs. \( I(x) - 100 \), since only the correct
function will yield a straight line that intersects zero. As shown in
Figure 48, when the first phase of the \( M_{412} \) decay (\( I(x) - 100 \)) is plotted
vs. each function, only \( x^2 \) yields a straight line, indicating that it
is the correct choice. This result can be checked by plotting both
phases of the decay data as the square root of \( I(x) - 100 \), since only
the square root of \( a_2x^2 - 100 \) will yield straight lines. As shown in
Figure 49, this plot yields two straight lines which intersect zero.
Figure 48

First Phase of $M_{412}$ Decay Graphed to Yield a Straight Line

I(x) - 100

KEY to Functions of x

- $\bigcirc = x^3$
- $\bigtriangleup = x^2$
- $\blacksquare = 3x^2 - 2x^3$
- $\blacklozenge = 6x^2 - 8x^3 + 3x^4$

Function of x (Fraction of Arginines Modified) - See Key
Figure 49

$M_{412}$ Decay Graphed as Square Root of $I(x) - 100$

$\sqrt{I(x) - 100}$

= First Phase

= Second Phase

$x$, Fraction of Total Arginines (7) Modified (+ .03)
Needless to say, one could examine any number of cases of cooperative inhibition of the photocycle. In general, one could fit a polynomial

\[ I_p(x) = 100 + a_1x + a_2x^2 + a_3x^3 + a_4x^4 + a_5x^5 \]

for the data to determine the best values of \( a_i \), \( i = 1, \ldots, 5 \). However, there are too few data points and too much noise to warrant using this procedure. Therefore only simple cases, such as \( I_2(x) \), \( I_3(x) \) and \( I_6(x) \), have been tried. The best fit by far is \( I_2(x) \) which corresponds to the case of a single pair of important arginines. This important result, that 2 arginines out of the 7 able to be modified are important for photoactivity, leads one to speculate about which arginines these may be. This will be treated in Discussion II.A.6.

As shown in Figure 47, the \( M_{412} \) photostationary steady state absorbance followed a trend similar to both phases of \( M_{412} \) decay. As shown in Results I.B.1., \([M]\) is related to the simplest photocycle by \( [M] = \frac{k_2[K]}{k_4} \), where \( k_2 \) is the decay of the first photointermediate, \( [K] \) is the concentration of the first photointermediate, and \( k_4 \) is the decay of \( M_{412} \). \([M]\) can be fit to a plot of \( 100 + a_2x^2 \) (middle solid line in Figure 47), using \( a_2 = 7571 \), just as both phases of \( M_{412} \) decay could be fit to a plot of \( a_2x^2 \). This suggests that the product \( k_2[K] \) may not have changed significantly from the control, but rather the change in \([M]\) was due to the change in \( M_{412} \) decay.

II. B. 2. Effect of pH

A pH titration of photocycling activity can reveal the pK of the \( M_{412} \) decay and thus which amino acid residues are
important for this activity. In addition, the pH dependence can reveal if an amino acid residue participates directly in proton donation to the Schiff base. If there is direct involvement, the change in $pK$ of $M_{412}$ decay will be directly correlated with the change in $pK$ of the modified amino acid. Figures 50 and 51 show the pH dependence of bR control and 400 mM BD modified bR, respectively. The pH was determined immediately after measuring the photoactivity as described in Materials and Methods V.B.1.a. As shown in Figure 50, when the $M_{412}$ decay was analyzed as the sum of two exponentials, the first phase was fast and appeared to be independent of pH over the entire pH range studied. This suggests that one step of reprotonation of the Schiff base nitrogen is not accessible to the aqueous phase. The other, slower part of the Schiff base reprotonation is accessible to the aqueous phase.

After 400 mM BD modification, both phases were markedly slower starting about pH 11.8 and the $pK$ of both phases was above 12. In this case, both phases of reprotonation of the Schiff base experienced the bulk pH. This could indicate either than BD modification has caused an "opening up" of the protein structure so that the aqueous phase is now able to penetrate to both environments of Schiff base, or that the aqueous phase now has time to penetrate to the Schiff base, since after BD modification both phases are slower. In the 400 mM BD modified bR sample, where 0.60 fraction of total arginines have added a negative charge to the positively charged guanidinium ion, the $pK$ of the modified guanidinium group is also expected to be raised. This result will be further discussed in Discussion II.B.2.a.
Figure 50

pH Dependence of 412 nm Decay Kinetics of Control bR
Figure 51

pH Dependence of 412 nm Decay Kinetics of 400 mM BD-Modified bR
II. B. 3. Effect of Surface Charge

One interesting aspect of BD modification is the relation between adding a negative charge to arginine's positive charge and the overall light-induced surface charge. Also, if there has been a change in the overall light-induced surface charge, how is this related to the $M_{412}$ kinetic changes?

Figure 52, Part A, shows the 3-lined EPR signal obtained using the spin probe CAT$_{12}$ to measure surface potential changes of 400 mM BD modified bR in the dark. The spin probe CAT$_{12}$ was used to measure the surface potential of PM (72,73), since it partitions between the membranous and aqueous phases, primarily according to charge and hydrophobicity of the membrane. It is thought the CAT$_{12}$ measures the PM surface charge primarily, since control studies with the uncharged spin probe 2N9 (2,2-dimethyl-5,5-methylheptyl-N-oxazolidinyloxy) showed that there was very little change in the hydrophobicity of the PM during illumination (74). CAT$_{12}$ probably partitions into the constrained lipid environment of the PM since the hyperfine splitting of the EPR signal of the bound component is = 60 gauss at room temperature (74). However, it is possible that the charge experienced by the probe is a delocalized one, resulting not only from the lipid head groups but also from the amino acid residues that are exposed at the surface of the PM.

When PM was illuminated as described in Materials and Methods V.B.5., the aqueous signal of CAT$_{12}$ decreased as CAT$_{12}$ increased its association with the more negatively charged membrane. The results of this experiment are quantitated in Table 20. The light-induced negative surface charge changes in the 400 mM and 600 mM BD modified samples
Figure 52

bR Surface Charge Monitored With CAT$_{12}$

A. 400 mM BD-Modified bR in the Dark

Gain = 2.5 x 10$^4$

B. 400 mM BD-Modified bR
Upon Illumination

Gain = 1.6 x 10$^5$

hv

hv

hv Off

hv Off
Table 20

Effect of BD Modification on Light-Induced Surface Charge Changes

<table>
<thead>
<tr>
<th>bR Sample</th>
<th>Fraction of Total (7)</th>
<th>mV</th>
<th>M&lt;sub&gt;412&lt;/sub&gt;/bR&lt;sub&gt;568&lt;/sub&gt; Molar Ratio</th>
<th>M&lt;sub&gt;412&lt;/sub&gt;/bR&lt;sub&gt;568&lt;/sub&gt; Molar Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>-1.78</td>
<td>0.13</td>
<td>-9.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+0.6</td>
<td>+.05</td>
<td>+3.9</td>
</tr>
<tr>
<td>400 and 600 mM BD</td>
<td>0.65</td>
<td>-4.80</td>
<td>0.43</td>
<td>-11.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+1.3</td>
<td>+.05</td>
<td>+ 4.2</td>
</tr>
</tbody>
</table>
were averaged, since these concentrations of BD modified 0.60 and 0.69 fraction of total arginines, respectively. The results represent the average of two illuminations in the CAT\textsubscript{12} experiment at each concentration of BD. Table 20 shows that the negative surface charge measured by CAT\textsubscript{12} was larger in the BD modified samples. However, since the M\textsubscript{412}/bR\textsubscript{568} molar ratio was also larger, due to the inhibition of M\textsubscript{412} decay by BD (see Results II.B.1.), the increase in negative surface charge per M\textsubscript{412} absorbance was only slightly enhanced (-11.41/-9.7 = 1.18). This result indicates that during photocycling a negative surface potential accompanies the state of M\textsubscript{412}, and when M\textsubscript{412} increases, the negative surface charge also increases. The significance of this result, and its implications for the mechanism of photocycling will be discussed in Discussion II.B.2.c.

II. B. 4. Effect of D\textsubscript{2}O

The deuterium isotope effect has been used as evidence for the occurrence of hydrogen transfer in the rate-determining step of a reaction, and this effect has been ascribed to the difference in zero-point energies of the stretching vibrations of bonds to hydrogen and deuterium (49). The magnitude of the D\textsubscript{2}O effect may reveal information about the mechanism of the reaction. The deuterium isotope effect was examined as described in Materials and Methods V.B.1.c. by monitoring M\textsubscript{412} decay and M\textsubscript{412} photostationary steady state absorbance in H\textsubscript{2}O and D\textsubscript{2}O, as shown in Table 21. The M\textsubscript{412} kinetics in Table 21 were obtained by computer analysis of a minimum of 10 flashes repeated at least twice. The isotope effect for the rise of M\textsubscript{412} was little changed in the BD treated sample from the control. In the M\textsubscript{412} decay kinetics, the curve...
Table 21

Effect of D$_2$O on M$_{412}$ Kinetics
At 20°C

<table>
<thead>
<tr>
<th>Imidoester Modified bR Sample -</th>
<th>M$<em>{412}$ Rise $t</em>{1/2}$ in μsecs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H$_2$O</td>
</tr>
<tr>
<td>Control - 0</td>
<td>69</td>
</tr>
<tr>
<td>EA - 0.80</td>
<td>67</td>
</tr>
<tr>
<td>DMA - 0.67</td>
<td>72</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Imidoester Modified bR Sample -</th>
<th>M$<em>{412}$ Decay $t</em>{1/2}$ in μsecs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H$_2$O</td>
</tr>
<tr>
<td>Control - 0</td>
<td>-</td>
</tr>
<tr>
<td>EA - 0.80</td>
<td>-</td>
</tr>
<tr>
<td>DMA - 0.67</td>
<td>5.9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Imidoester Modified bR Sample -</th>
<th>M$<em>{412}$/bR$</em>{568}$ Molar Ratio as Percent Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H$_2$O</td>
</tr>
<tr>
<td>Control - 0</td>
<td>100</td>
</tr>
<tr>
<td>EA - 0.80</td>
<td>250</td>
</tr>
<tr>
<td>DMA - 0.67</td>
<td>960</td>
</tr>
</tbody>
</table>
peeling computer program did not always analyze the $M_{442}$ decay as the sum of two exponentials, and for this reason the fast phase ($t_{1/2}^{-1}$) was not detected in the control bR sample. The isotope effects of both phases of the BD modified sample were similar to the slow phase isotope effect of control bR. The $M_{442}/bR_{568}$ molar ratio reflected the effect of D$_2$O on the $M_{442}$ decay kinetics, and was similar in the BD treated sample to control bR.

As shown in Table 21, the magnitude of the isotope effect for the rise and decay of $M_{442}$ were smaller than a "normal" isotope effect of 6-10 (50), and the isotope effect for the decay was smaller than for the rise. The significance of these results will be discussed (see Discussion II.B.2.b.).

II. C. pH Activity

1. pH Response of a pH Indicator Dye

In addition to photocycling activity, bR's photoactivity can be measured with a pH indicator dye. As was explained in Results I.C., this technique monitors proton release and uptake which are thought to be indicative of protons pumped across the PM during the photocycle (36). Table 22 shows the kinetics of proton release and uptake compared to $M_{442}$ kinetics of control and BD modified bR. These results are the average of 20 flashes taken in the absence of buffer at $20^\circ$ C, pH 7.5.

As shown, in both samples proton release was more than 6-fold slower than the rise of $M_{442}$. The use of pH indicator dyes as accurate monitors of proton movements will be further discussed (see Discussion II.B.1.a.). The 400 mM BD modified sample had an $M_{442}$ rise $t_{1/2}$ only slightly slower than the control rise $t_{1/2}$, and a proton release $t_{1/2}$
Table 22

Comparison of Effect of BD Modification on Proton Movements and $M_{412}$ Kinetics

<table>
<thead>
<tr>
<th>bR Sample</th>
<th>Fraction of Total (7) Arginines Modified</th>
<th>$M_{412}$ Kinetics</th>
<th>Proton Movements</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rise $t_{1/2}$,</td>
<td>Release $t_{1/2}$,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>71 µsecs,</td>
<td>460 µsecs,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.5 msecs</td>
<td>5.0 msecs</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15 msecs</td>
<td>24.0 msecs</td>
</tr>
<tr>
<td>400 mM BD</td>
<td>0.60</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>82 µsecs,</td>
<td>569 µsecs,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7.7 msecs</td>
<td>11.5 msecs</td>
</tr>
<tr>
<td></td>
<td></td>
<td>34.6 msecs</td>
<td>51.3 msecs</td>
</tr>
</tbody>
</table>
similarly slower than the control's. Thus the proton release kinetics reflected the $M_{412}$ rise kinetics with about a 6.5-fold lag in both samples.

The $M_{412}$ decay kinetics of BD modified bR were only slightly slower than the control due to reaction reversal, since all of the borate buffer had to be removed for this experiment by 3 washing procedures as described (see Materials and Methods V.B.4.). Of the inhibition that remained, proton uptake kinetics in the BD modified sample were about 2-fold slower than the control, and so were $M_{412}$ decay kinetics. This study, as in the imidoester experiments (see Results I.C.), showed that proton movements paralleled photocycling kinetics. It suggested that protons are pumped simultaneously with the photocycle of bR and that when the photocycle is slowed, proton pumping is also slowed. The role of arginine in proton pumping will be further discussed (see Discussion II.B.2.).

II. C. 2. Light-Induced pH Changes of bR Lipid Vesicles Monitored by pH Electrode

Several investigators have measured the photocactivity of bR by using various techniques to incorporate bR into lipid vesicles, and then monitoring the light-induced pH changes of the aqueous solution. This method yields information on protons actually pumped across the PM after a chemical modification. When pH changes were measured of solutions containing bR lipid vesicles with BD modified bR incorporated as described in Materials and Methods V.B.3., the initial rate of both the light-induced alkalinization and the dark-induced acidification were slowed as shown in Figure 53. These results are the average of five sets of BD concentration curves; some standard deviations do not appear.
Figure 53
Effect of BD Modification on Proton Translocation by bR-Lipid Vesicles

Initial $t_{1/2}$ of Proton Translocation as Percent Control

○, Alkalization in Light
□, Acidification in Dark

Fraction of Total (7) Arginines Modified
because they were smaller than the symbols or because there was only one sample at that BD concentration. The kinetics were treated as single exponential monophasic decays to detect changes in the initial rates of proton translocation, although in a few cases biphasic kinetics were seen. The results are presented as percent control since the actual values of the $t_{1/2}$'s of the initial rates varied considerably. The range of $t_{1/2}$'s of alkalinization for different controls was 0.8 to 20 seconds and the range of $t_{1/2}$'s of acidification was 0.6 to 25 seconds. These rates are comparable to what others have seen (75,76), and changed depending on the condition of the lipid vesicles. Thus each set of BD treated bR lipid vesicles in this study was compared to its own control and the percent control calculated.

As shown, at 0.27 fraction of total arginines modified and higher, the alkalinization was slowed about 7-fold and the acidification was slowed about 4-fold. However, the ratio

\[
\frac{\text{Initial } t_{1/2} \text{ of acidification}}{\text{Initial } t_{1/2} \text{ of alkalinization}}
\]

did not deviate markedly from the control ratio. As a result, the extent of proton translocation, $H^+/bR$ nanomole ratio, represented as percent control in Table 23, also did not deviate markedly from the control in the BD modified bR lipid vesicles. The actual extents varied from 0.2 to 3.5 $H^+/bR$ nanomole ratio which is in the range of what others have seen (75,76). The significance of these changes will be discussed in Discussion II.B.2.a.

Changes in the buffer capacity were noted by observing the change in the line height of the HCl standard. Although the buffer capacity changed by as much as 5-fold from one set of lipid vesicles to the next,
<table>
<thead>
<tr>
<th>bR Sample</th>
<th>Fraction of Total (7) Arginines Modified</th>
<th>Initial $t_{1/2}$ of Acidification</th>
<th>Initial $t_{1/2}$ of Alkalinization</th>
<th>Extent - $H^+/bR$ Nanomolar Ratio as % Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>1.98 ± 0.61</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>2 mM BD</td>
<td>0.007</td>
<td>2.23 ± 1.0</td>
<td></td>
<td>87.5 ± 62</td>
</tr>
<tr>
<td>10 &quot;</td>
<td>0.036</td>
<td>2.17 ± 1.0</td>
<td></td>
<td>85.2</td>
</tr>
<tr>
<td>20 &quot;</td>
<td>0.069</td>
<td>2.65</td>
<td></td>
<td>71 ± 41</td>
</tr>
<tr>
<td>50 &quot;</td>
<td>0.16</td>
<td>1.57</td>
<td></td>
<td>118</td>
</tr>
<tr>
<td>100 &quot;</td>
<td>0.27</td>
<td>1.75</td>
<td></td>
<td>106</td>
</tr>
<tr>
<td>200 &quot;</td>
<td>0.43</td>
<td>1.75</td>
<td></td>
<td>114</td>
</tr>
<tr>
<td>300 &quot;</td>
<td>0.53</td>
<td>1.39 ± 0.4</td>
<td></td>
<td>108 ± 65</td>
</tr>
<tr>
<td>400 &quot;</td>
<td>0.60</td>
<td>2.3 ± 1.5</td>
<td></td>
<td>102 ± 66</td>
</tr>
<tr>
<td>500 &quot;</td>
<td>0.65</td>
<td>1.7 ± 0.3</td>
<td></td>
<td>78 ± 55</td>
</tr>
</tbody>
</table>
the change in buffer capacity within one set of lipid vesicles was usually smaller than 2-fold, and no trend with increasing BD concentration emerged. Since in addition there was no correlation in the control lipid vesicles between buffer capacity and rates of acidification or alkalization, changes in buffer capacity were noted but not used when calculating initial $t_{1/2}$'s of proton translocation. In a few sets of lipid vesicles, the buffer capacity decreased slightly in the BD modified samples, whereas in other sets, it remained nearly constant.

One might first ask, how is dark-induced acidification, which should not be involved with bR's photoactivity, slowed after BD modification? Since dark-induced acidification is just passive proton permeability, it was of interest to know if the rates of this permeability were comparable to the proton permeability in synthetic lipid vesicles seen by other investigators. Using the equation for permeability from Nichols and Deamer (77):

$$ J_{\text{net}} = \frac{P_{\text{net}} \cdot \Delta \left[ H^+ \right]}{[H^+]_o - [H^+]_i} $$

where $P_{\text{net}}$ = permeability coefficient for protons

$$ J_{\text{net}} = \text{net proton flux}, $$

$$ [H^+]_o = \text{proton concentration outside} $$

$$ [H^+]_i = \text{proton concentration inside} $$

and

$$ J_{\text{net}} = \frac{d \left[ H^+ \right]_o}{dt} \frac{B \cdot V}{A} $$

where $B_o$ = buffer capacity

$$ V_o = \text{outside volume} $$

$$ A = \text{total area of lipids} $$

and estimating the inner volume of the lipid vesicles from the known quantity of lipids added, a proton permeability of the same magnitude as that of Nichols and Deamer (77) was calculated (about $5 \times 10^{-4}$ cm/sec).
This indicates that the dark-induced acidification seen in this study may have been just proton diffusion down its concentration gradient through the lipids. One possible reason for the slowing of dark-induced acidification concerns the protein-lipid interaction. After BD modification, the PM will carry a greater net negative charge on its surfaces, due to the addition of the negatively charged BD-borate adduct to the positive guanidinium group of arginines. It is possible that the negative charge will affect the nearby soybean phospholipid headgroups. It has been shown that sodium diffusion through dipalmitoyl phosphatidyl choline vesicles is highly dependent on the physical state of the lipids, especially near the phase transition of the lipids (78). BD modification might cause the soybean lipids to become more fluid which would decrease the proton permeability if it were near the phase transition of the soybean lipids (see Figure 1.D in Ref. 78). Another possibility is that flow of protons through the protein might be decreased if BD has caused a permanent conformational change of the protein.

Secondly, why is the initial rate of light-induced alkalinization slowed? The most obvious answer is that since the $M_{412}$ decay kinetics were slowed, the proton uptake was also slowed. However, the shape of the inhibition curve of proton translocation seen in Figure 53 is quite different from the shape of the inhibition curve of $M_{412}$ decay seen in Figure 47. In addition, the total degree of inhibition was much smaller than the inhibition of the $M_{412}$ decay. Since the initial rates were measured, the light-induced alkalinization should not be affected by the back leakage of protons. It is possible that the large degree of inhibition seen at the millisecond time scale by flash photolysis is not discernible at the time scale of seconds seen by the pH electrode. Another complication is that the orientation of bR in these lipid
vesicles was not known. It is thought by some investigators (79) that the highly negatively-charged carboxyl terminus of bR is responsible for orienting PM in lipid vesicles during sonication. In order to test the orientation one must carry out proteolytic digestion studies of bR sonicated into lipid vesicles. Thus changes in proton pumping in bR lipid vesicles were regarded as qualitative, not quantitative, effects of BD modification.

II. D. Light-to-Dark Adaptation Kinetics

Besides photocycling activity, where retinal undergoes a conformational change, bR can also interconvert between the light-adapted and the dark-adapted forms. The light-adapted form is produced by exposing bR to intense light for 30-60 seconds (see Materials and Methods V.A.1.). If left in the dark, this form will convert to the dark-adapted form with a $t_{1/2}$ of 116 minutes (80) at 30° C, pH 10.0. It is thought that the isomer composition of retinal in the dark-adapted form is one-half 13-cis and one-half all-trans retinal, and 100 % all-trans retinal in the light-adapted form (81). Since the retinal isomer of the photocycle intermediate $M_{412}$ is thought to be 13-cis retinal (30), it is possible that the photocycle conformational change is related in some way to conversion to the dark-adapted form. Thus the kinetics of light-to-dark adaptation were monitored as described in Materials and Methods V.B.6., after BD modification.

In Part A. in Figure 54 is shown the dark and light-adapted spectra of 200 mM BD modified bR. There was a 10 nm shift of the peak maximum from 558 to 568 nm and 11.4 % increase in intensity upon light adaptation. These changes are seen more clearly in Part B. of Figure 54 where only the peaks of each spectrum were monitored at high sensitivity, using
Figure 54

Visible Spectroscopy of Dark and Light-Adapted bR

Absorbance Units

A. 200 mM BD-Modified bR

Absorbance

Wavelength in nm

B. 200 mM BD-Modified bR

Control bR
Table 24
Effect of BD Modification on Light-to-Dark Adaptation Kinetics

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fraction of Total (7) Arginines Modified</th>
<th>$t_{1/2}$ of Light-to-Dark Adaptation</th>
<th>% Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>105</td>
<td>100</td>
</tr>
<tr>
<td>10 mM BD</td>
<td>0.036</td>
<td>115</td>
<td>110</td>
</tr>
<tr>
<td>50 &quot;</td>
<td>0.16</td>
<td>118</td>
<td>112</td>
</tr>
<tr>
<td>100 &quot;</td>
<td>0.27</td>
<td>108</td>
<td>103</td>
</tr>
<tr>
<td>200 &quot;</td>
<td>0.43</td>
<td>102</td>
<td>97</td>
</tr>
<tr>
<td>300 &quot;</td>
<td>0.53</td>
<td>98</td>
<td>93</td>
</tr>
<tr>
<td>400 &quot;</td>
<td>0.60</td>
<td>107</td>
<td>102</td>
</tr>
<tr>
<td>500 &quot;</td>
<td>0.65</td>
<td>110</td>
<td>105</td>
</tr>
</tbody>
</table>
the Cary 14R. As shown, the maximum wavelength of control and 200 mM BD modified bR changed similarly with light adaptation. The shapes of the spectra vary slightly due to small differences in concentration. The greatest difference between the light and the dark-adapted states occurred at 580 nm in both samples.

When the kinetics of dark adaptation were monitored as described at pH 8.2-8.5 at 20° C, the results shown in Table 24 were obtained. As shown, the kinetics of dark adaptation were similar to those of Ohno et al. (80), and there was very little change in the t_1/2 of light-to-dark adaptation at any concentration of BD. Thus even though photocycling activity was greatly inhibited (see Results II.B.1.), the conversion of the light-adapted state to the dark-adapted state was unchanged. This suggests that these processes are unrelated, although they both contain the 13-cis retinal isomer.

II. E. Spectroscopy

1. Absorption

The effects of increasing concentrations of BD on bR's 568 nm chromophore are shown in Figure 55 with BD in situ, and are quantitated in Table 25 with BD removed. In Figure 55, all of the samples contained the same amount of bR stock solution with BD reagent in situ, as described in Materials and Methods III.A.1.a., and BD reagent of the same concentration as each sample in the reference cuvette. Since there was no shift in the wavelength maximum of the 568 nm chromophore at any concentration of BD, the chromophore environment and especially the excited state of retinal, may not have been substantially changed by BD. However, two equal and opposite forces to shift λ_MAX may have been operating as will be discussed in Discussion II.A.8.a. and II.A.8.b., and II.B.3.a.
Figure 55

Effect of BD Concentration In Situ on bR's 568 nm Chromophore

Absorbance Units

Control, 50, 100
200
300, 500
400

BD Concentration in mM

Wavelength in Nanometers

560  580  600
Table 25

Effect of BD Modification on bR's 568 nm Chromophore

<table>
<thead>
<tr>
<th>bR Sample</th>
<th>Fraction of Total (7) Arginines Modified</th>
<th>568 nm Chromophore Lowry Protein (% Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>10 mM BD</td>
<td>0.036</td>
<td>98 ± 2</td>
</tr>
<tr>
<td>50 &quot;</td>
<td>0.16</td>
<td>98 ± 2</td>
</tr>
<tr>
<td>100 &quot;</td>
<td>0.27</td>
<td>95 ± 7</td>
</tr>
<tr>
<td>200 &quot;</td>
<td>0.43</td>
<td>97 ± 2</td>
</tr>
<tr>
<td>300 &quot;</td>
<td>0.53</td>
<td>94 ± 7</td>
</tr>
<tr>
<td>400 &quot;</td>
<td>0.60</td>
<td>99 ± 3</td>
</tr>
<tr>
<td>500 &quot;</td>
<td>0.65</td>
<td>97 ± 3</td>
</tr>
<tr>
<td>600 &quot;</td>
<td>0.69</td>
<td>100</td>
</tr>
</tbody>
</table>
Table 25 shows the average deterioration of the 568 nm chromophore for four concentration curves with the BD reagent removed, (see Appendix 10 for a complete description of the quantitation of BD reagent removal). The results in Table 25 may be inaccurate by \( \pm 5\% \) due to the Lowry protein error. As shown, deterioration of the 568 nm chromophore structure by this reagent was very slight. This small decrease in absorbance yield may indicate that some of the chromophores could no longer absorb light efficiently, i.e., that the reagent caused a slight deterioration of some of the chromophore structures so that the quantum efficiency of light absorption decreased. Experiments that might explain this small decrease in the 568 nm chromophore absorbance, such as quantum efficiency measurements and fraction cycling determinations were not undertaken.

II. E. 2. Tryptophan Fluorescence

Tryptophan fluorescence emission and excitation spectra after BD modification showed one broad band that was slightly red shifted. The fact that only one broad band was seen in the fluorescence emission spectrum with no dependence of \( \lambda_{\text{MAX}} \) on the wavelength of excitation has several interpretations that will be discussed in Discussion II.A.8.a.

Of particular interest is the position of the wavelength maxima in the excitation and emission spectra and decreases in fluorescence quantum yield of bR's tryptophan fluorescence after BD modification of arginines. This information is summarized in Table 26 from four concentration curves of BD modification. The quantum yield results may be inaccurate by \( \pm 5\% \) due to the Lowry protein error calculated in Appendix 3.
Table 26
Effect of BD Modification on bR's Tryptophan Fluorescence

<table>
<thead>
<tr>
<th>bR Sample</th>
<th>Fraction of Total (7) Arginines Modified</th>
<th>Excitation $\lambda_{\text{MAX}}$ in nm</th>
<th>Emission $\lambda_{\text{MAX}}$ in nm</th>
<th>Fluorescence Yield (% Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>287</td>
<td>315</td>
<td>100</td>
</tr>
<tr>
<td>10 nM</td>
<td>0.036</td>
<td>290</td>
<td>315</td>
<td>$93 \pm 5$</td>
</tr>
<tr>
<td>20</td>
<td>0.057</td>
<td>290.5</td>
<td>316</td>
<td>$99 \pm 11$</td>
</tr>
<tr>
<td>50</td>
<td>0.16</td>
<td>-</td>
<td>-</td>
<td>$91 \pm 11$</td>
</tr>
<tr>
<td>100</td>
<td>0.27</td>
<td>-</td>
<td>-</td>
<td>85</td>
</tr>
<tr>
<td>200</td>
<td>0.43</td>
<td>-</td>
<td>-</td>
<td>$93 \pm 10$</td>
</tr>
<tr>
<td>300</td>
<td>0.53</td>
<td>290.5</td>
<td>316.5</td>
<td>$98 \pm 5$</td>
</tr>
<tr>
<td>400</td>
<td>0.60</td>
<td>292.5</td>
<td>317</td>
<td>$96 \pm 4$</td>
</tr>
<tr>
<td>500</td>
<td>0.65</td>
<td>293</td>
<td>317.4</td>
<td>$95 \pm 6$</td>
</tr>
<tr>
<td>600</td>
<td>0.69</td>
<td>294.5</td>
<td>317.4</td>
<td>$97 \pm 5$</td>
</tr>
</tbody>
</table>
As shown there was a slight red shift in the emission $\lambda_{\text{MAX}}$ at higher concentrations of BD modification, and a more marked red shift in the excitation $\lambda_{\text{MAX}}$. As explained in Results I.F., the red shift indicates that there is a smaller energy difference between the ground and the excited states of tryptophan. A perturbation in the environment of the ground and excited state energies is a function of the 1) polarizability, 2) polarity and/or 3) mobility of the local environment. Another explanation for a red shift is that selective quenching of one or two tryptophans emphasizes the contribution of the others (57,58). The slight decrease (about 5 %) in the fluorescence quantum yield could be selective quenching of tryptophans in a hydrophobic environment, which would result in a red shift. The importance of each of these effects will be discussed in Discussion II.A.8.b.

Table 26 also shows a small decrease in the quantum yield of tryptophan fluorescence with excitation at 287 nm and emission at 315 nm. This decrease may simply be due to the fact that the $\lambda_{\text{MAX}}$ of excitation and emission have red-shifted. However, tryptophan fluorescence quenching could be due to a number of other factors as was related in Results I.F. The relative importance of each of these factors will be discussed in more detail in Discussion II.A.8.b.

II. E. 3. Resonance Raman

As described in Results I.G.1., resonance Raman spectroscopy gives detailed information about the interaction between the chromophore and the protein moiety by monitoring the Raman scattering from vibrations coupled to an electronic transition when the excitation
Figure 56

Resonance Raman Spectra of 200 mM BD Modified bR

Using 458 nm Probe Laser

With Steady Illumination

A.

Control bR

200 mM BD Modified bR

B.

Wavenumber in cm\(^{-1}\)

1400 1500 1600 1700

1010 cm\(^{-1}\) 1202 cm\(^{-1}\) 1178 cm\(^{-1}\)

1566 cm\(^{-1}\) 1582 cm\(^{-1}\) 1597 cm\(^{-1}\)

1622 cm\(^{-1}\) 1642 cm\(^{-1}\)
wavelength is within or near the absorption band of the chromophore.

Figure 56 shows the resonance Raman spectra taken by Greg Perreault of control bR and 200 mM BD modified bR with 0.43 fraction of total arginines modified. Part B. shows a survey of the wavenumbers from 800 to 1800 cm\(^{-1}\). The data of both the control and 200 mM BD modified bR sample is the sum of 3 runs (scan time = 10 seconds) under constant illumination with the probe laser at 458 nm. These data were not smoothed. Part A. was scanned over a narrower wavenumber region in order to delineate more clearly the bands at 1642 cm\(^{-1}\) and 1622 cm\(^{-1}\), which have been assigned to the protonated and deprotonated forms of the Schiff base C=N vibration, respectively (61). In addition this region contains bands at 1531 cm\(^{-1}\) and 1566 cm\(^{-1}\), which represent the C=C ethylenic stretching frequencies of the bR\(_{568}\) chromophore and M\(_{412}\) chromophore structures, respectively.

As shown, none of the peaks of interest shifted frequency in the 200 mM BD modified sample compared to control bR. In order to quantitate these results, the peak height ratios of the peaks of interest were compared in the BD modified and control bR samples as shown in Table 27. The peak height at 1010 cm\(^{-1}\) was used as an internal measure of concentration, since this C-CH\(_3\) stretching vibration is nearly invariant with different forms of the chromophore (63).

The most dramatic changes in intensity were in the C=C ethylenic stretches. While the 1566 cm\(^{-1}\) band increased 39 % in the BD modified sample relative to the control, the 1531 cm\(^{-1}\) band decreased 39 %. Neither band should be specifically resonance enhanced, since the 458 nm probe laser was set at the isosbestic point between bR\(_{568}\) and M\(_{412}\). Since the photon flux density at the sample was very high, saturation of the photocycle was most likely achieved; i.e., as soon as M\(_{412}\) was formed
Table 27

**Effect of BD Modification of bR on Resonance Raman Spectroscopy**

<table>
<thead>
<tr>
<th>bR Sample</th>
<th>Fraction of Total (7) Arginines Modified</th>
<th>Peak Height Ratios As Percent Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1642 cm(^{-1})</td>
</tr>
<tr>
<td>Control bR -</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>200 mM BD</td>
<td>Modified bR - 0.43</td>
<td>71</td>
</tr>
<tr>
<td>Fingerprint Region</td>
<td></td>
<td>1202 cm(^{-1})</td>
</tr>
<tr>
<td>Control bR -</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>200 mM BD</td>
<td>Modified bR - 0.43</td>
<td>96</td>
</tr>
</tbody>
</table>
it was photoconverted back to the $bR_{568}$ form. Thus the increase in the band associated with the deprotonated Schiff base (1566 cm$^{-1}$) and decrease in the band associated with the protonated Schiff base (1531 cm$^{-1}$) could be due to a shift in equilibrium in the photostationary state between these two forms caused by a change in the quantum efficiencies involved, or by inhibition of the back photoreaction, $M^\rightarrow bR_{568}$. The 1642 cm$^{-1}$ band, which is also indicative of the protonated Schiff base, decreased about 30% which agrees with the above changes. However, the corresponding 1622 cm$^{-1}$ band did not increase 30% above the control. A recent investigation (70) has implicated this band in vibrations of the C-H bends of the isoprenoid chain as well as the Schiff base, which may have caused this discrepancy. In addition, the band at 1597 cm$^{-1}$ decreased, but the band at 1582 cm$^{-1}$ did not decrease. These last two bands are reduced or absent in the $M_{412}$ spectrum, and thus are expected to decrease in the BD modified spectrum. This inconsistency is not easy to explain, but it may be that the tallest peaks, the C=C ethylenic stretches, are the most reliable indicators of the amounts of deprotonated and protonated Schiff base forms after BD modification.

In the fingerprint region the 1178 cm$^{-1}$ band, which can be described as a C-C stretch vibration, appeared to increase after BD modification. It may also have shifted to 1180 cm$^{-1}$, but this difference was not easily discernible. The 1178 cm$^{-1}$ and 1180 cm$^{-1}$ bands are sensitive to the isomeric form of retinal, and an increased amount of $M$ in the photostationary steady state is expected to cause this increase at 1180 cm$^{-1}$. The 1200 cm$^{-1}$ band was nearly invariant which was also expected.
Thus, resonance Raman showed a general increase in the peaks associated with M$_{412}^+$, which may indicate that the back photoreaction M$^bR$ was inhibited as well as the thermal decay of M.

II. E. 4. Retinal Fluorescence

At higher frequencies than those used for resonance Raman spectroscopy, the Raman spectrometer can be used to measure a fluorescence emission from bR. Although the exact molecular source of the fluorescence is still controversial (see below), this fluorescence arises from an excited state of retinal (or perhaps from two different excited states of retinal at different light intensities), and thus is a useful internal probe of protein structure at the chromophore site.

Figures 57 and 58 show the steady state fluorescence as a function of frequency of a tuneable dye laser obtained by Greg Perreault. The spectra are height normalized to the peak produced by a frequency of 16,400 cm$^{-1}$ since this was the most intense spectrum. The least intense spectrum was that produced by 15,000 cm$^{-1}$ light, which was 1/8 as intense as the highest peak. Height normalization showed the trend towards blue shifting of the high energy edge of the spectra of 200 mM BD modified bR compared to the control. This blue shift was seen at a photon flux of 6.5 x 10$^{15}$ photons/cm$^2$/sec but not at a photon flux of 3 x 10$^{14}$ photons/cm$^2$/sec.

The cause of the blue shift follows from an understanding of the fluorescence emitter(s). Lewis et al. (82) found that the degree of polarization of this fluorescence remains constant throughout the observed emission band within the time scale of any one measurement at 77° K (-196° C) suggesting that the fluorescence emanates from the
Figure 57

Emission Spectra as a Function of Frequency of Excitation Light
Figure 58

Emission Spectra as a Function of Frequency of Excitation Light

Normalized Intensity

Frequency of Excitation Light in
Wavenumber (cm⁻¹)

16,400 16,200 16,000 15,800 15,600 15,400 15,200 15,000

16,000 15,000 14,000 13,000 12,000

Wavenumber in cm⁻¹
excited state of a single species. In a subsequent study, however, they showed a lowering of quantum efficiency at photon fluxes higher than $10^{15}$ photons/cm$^2$/sec. At photon fluxes below $10^{15}$ photons/cm$^2$/sec, the quantum efficiency increased due to the contribution of a species with an emission maximum at 670 nm at temperatures near 77° K. Thus this work suggested the presence of two fluorescing species, with a light-intensity dependent contribution to the fluorescence. This work was confirmed by the work of Kriebel et al. (83) who found two separate fluorescence maxima if they illuminated at low light intensity (714 nm at $1.4 \times 10^{14}$ photons/cm$^2$/sec) or high light intensity (735 nm at $8.2 \times 10^{18}$ photons/cm$^2$/sec) at room temperature.

The first report of the lifetime of the fluorescence produced by low intensity light was by Alfano et al. (84). These authors measured the lifetime at 90° K (-183° C) to be about 40 picoseconds and estimated it to be $<3$ picoseconds at room temperature. The photon flux was $6 \times 10^{14}$ photons/cm$^2$/sec. A measurement of the lifetime of the fluorescence at room temperature was by Hirsch et al. (85), who measured it to be 15 picoseconds. The photon flux at the sample was $3.5 \times 10^{31}$ photons/sec but the area was not given.

The lifetime measurements suggest that the "low-intensity" fluorescence forms either before or concommitantly with the first photoproduct, K, which has been shown to form in 11 picoseconds at room temperature by Kaufmann et al. (86). In another study, Ippen et al. (87) have shown the formation time of K to be 1.0 picosecond. Even though the excitation wavelength maximum of the fluorescence is similar to the absorption $\lambda_{MAX}$ of K (590 or 610 nm), most investigators do not think that K fluoresces since the fluorescence yield is extremely temperature dependent whereas the yield of K from bR is temperature independent from
Several investigators have attempted to identify spectrophotometrically another photointermediate that might be the fluorescence emitter. One requirement of any photoproduct or excited state of retinal that produces the fluorescence is that it have a temperature dependent lifetime, like the fluorescence. One such study was by Kaufmann et al., who showed that a transient bleaching at 580 nm is formed in 6 picoseconds (90), that occurs simultaneously with an increase in absorption at 620-630 nm. The 620 nm absorption is far less prominent at room temperature than at -205°C. The 580 nm transient bleaching decayed in about 15 picoseconds and this decay time was not sensitive to temperature. This study suggests that the low temperature absorbance at 620-630 nm may be the origin of the fluorescence.

Similarly, Gillbro has attributed the fluorescence to an intermediate called P-bR with an excitation $\lambda_{\text{MAX}}$ of 597 nm. A build-up of this intermediate can be produced at 77°C (-196°C) using subsequent flashes of low intensity light ($1.4 \times 10^{14}$ photons/cm$^2$/sec) (91). The fluorescence can be attributed entirely to this intermediate, and at 77°C (-196°C) P-bR is stable for 1 hour. At 90°C (-183°C) it decays on a time scale of minutes and so does the fluorescence. These investigations lead one to believe that there are two temperature-sensitive photoproducts of bR, produced at high and low light intensity, each with their own fluorescence with different $\lambda_{\text{MAX}}$'s.

To summarize the literature for purposes of analysis, the fluorescence of bR may be said to originate from one, or two, excited state(s) of the bR retinal chromophore. One may then ask, what is known about blue-shifts of retinal fluorescence? One detailed study was carried out by Becker et al. (92), who found that the excitation and emission
wavelengths and changes in quantum yield of retinal fluorescence are highly sensitive to the H-bonding character of the solvent, since the excited state that is formed is solvent-dependent. They found that a state of principally \(^1(\pi, \pi^*)\) character is the lowest excited state formed in a H-bonded retinal, and that an equilibrium mixture of non-H-bonded and H-bonded retinal exists in the presence of hydroxylic solvents. Empirically this converts to a red shift in excitation and emission wavelengths as the retinal goes from a hydrophobic solvent to a H-bonding solvent. Thus the blue shift seen in the 200 mM BD modified sample could mean that the environment of the fluorescence-producing retinal has become more hydrophobic. It is interesting to note that the blue shift was only seen at photon fluxes greater than \(10^{15}\) photons/cm\(^2\)/sec indicating that only the "high intensity" fluorescence experienced a more hydrophobic environment after BD modification. The significance of two fluorescing species, and the implications for the mechanism of photocycling will be discussed in Discussion II.A.8.b.

II. E. S. Circular Dichroism

Circular dichroism provides a means of studying the three dimensional configurational and conformational asymmetry of optically active molecules in solution. Optically active molecules have the property of rotating the plane of polarization of incident, plane polarized light \((93)\). Since retinal is not intrinsically optically active, the optical activity seen in the bR molecule is due to the apoprotein interacting with retinal. The bR CD spectra obtained by Dr. Bonnie Wallace are shown in Figure 59. Since the concentrations were not exactly the same, the spectrum of the control was height
Figure 59

Molar Ellipticity $[\theta] \times 10^{-4}$ deg cm$^2$/dmole

Circular Dichroism of 400 mM BD-Modified bR

Control bR

400 mM BD-Modified bR

Wavelength in nm
normalized to the same peak at 525 nm. The samples were not specifically light-adapted as described in Materials and Methods V.A.1., but the experiments were performed in room light.

The positive and negative bands in the visible region are thought to arise from the retinal chromophore. Two different mechanisms have been suggested to account for the induced optical activity of the bound retinal of rhodopsin: 1) the chromophore becomes spatially distorted due to binding to the protein (94), 2) there is dipole-dipole resonance coupling of the retinal transitions with the optically active transitions of the apoprotein aromatic amino acid residues in the apoprotein amide bond (95). The first mechanism is the favored one since the oscillatory strengths of the transitions of retinal are extremely weak, evidenced by the 317 nm band, and thus are not expected to couple to the dipole resonance of the aromatic amino acid groups. Thus the induced optical activity is more likely to be due to a constraint on retinal by the asymmetric apoprotein (10).

In the visible region, there is some disagreement in the literature regarding the cause of the positive and negative bands. The cross-over of the bands is near 568 nm, but the positive and negative CD peaks do not have counterparts in the absorption spectrum. In dark-adapted bR, the positive peak is at 525 nm and the negative peak is at 595 nm with cross-over at 563 nm. Upon illumination the peaks shift to 535 nm and 602 nm, respectively, with cross-over at 574 nm. The positive band increases 25% and the negative band increases 35%. In a similar manner, the absorption bands of the dark-adapted bR undergo an intensity increase and red shift upon illumination (96). These results suggest exciton interaction between the retinal ($\pi-\pi^*$)($\text{NV}_2^*$) transition moments of different bR molecules (97). Another possibility is that there are
two nearly degenerate transitions with opposite rotatory strengths as suggested by Hudson for certain conjugated polyenes similar to retinal (98). Alternatively, there may be two different retinal binding sites on each bR apoprotein with only one site occupied on the average (10) since the retinal:protein ratio is 1:1 (96). Figure 59 shows that both samples had a maximum for the positive peak at 525 nm as in the dark-adapted form. This is not unexpected since neither sample was specifically light-adapted. The rest of the control spectrum in the visible range appears to be normal for the dark-adapted form. The 400 mM BD modified sample, however, appears to have a distorted dark-adapted form. The cross-over and negative band are characteristic of the wavelengths of the light-adapted form. However, as shown in Results II.D., the rate of light-to-dark adaptation was not changed from the control for this sample, and the dark-adapted absorption spectrum was normal, with a peak at 558 nm. Thus one explanation for this result is that in the BD modified sample the exciton-exciton interaction has been changed due to a change in distance between bR chromophores. Some investigators believe that the negative band results directly from exciton-exciton interaction between bR chromophores in a trimer, since loosening the crystalline structure of the PM with dimethylsulfoxide/water mixtures leads to the disappearance of this negative band (99).

The next peak of interest in the CD spectrum is the peak at 317 nm. This CD band results from transitions which are magnetic-dipole allowed but electric-dipole forbidden since only absorption bands with weak oscillatory strengths are found in this region (100). The behavior of this band is thought to be a sensitive indicator of the rotational
freedom of the retinal molecule relative to the apoprotein molecules. J. Cassim has found (unpublished results) that perturbations which result in the loss of the rigidity of the retinal-apoprotein bond also cause a sharp decrease in the intensity of this CD band. Thus modification of arginines at the surface of the bR molecule could cause a less constrained environment near retinal, thus decreasing this peak in the 400 mM BD modified sample as shown in Figure 59.

The next peak of interest is at 290 nm, which has been attributed to tryptophan. There was quite a difference in the peak maximum and shape of the BD modified spectrum compared to the control, indicating that several tryptophans experienced a different environment.

The largest change in this spectrum occurred at 260 nm. As shown in Figure 59, there was a marked decrease of the 260 nm peak height in the BD modified sample. It is thought that this peak does not have a contribution from retinal, but rather represents the secondary and/or tertiary structure of bR, resulting from the π-π* transitions of the aromatic amino acid chains (101). A large decrease in this band as shown could signify a delocalized conformational change of the protein caused by BD modification.

III. Double Chemical Modifications

A. EA and BD Followed by Iodination

1. Number of Amino Acids Modified

Before chemical modification with iodine, two bR samples were modified with 20 mM EA or 200 mM BD. The 20 mM EA sample had 0.63 fraction of total lysines modified and the 200 mM BD sample had 0.43 fraction of total arginines modified. After chemical modification
with iodine at pH 7.2, the questions must be asked, 1) how many total tyrosine sites are available for chemical modification with iodine, and 2) what fraction of the total available tyrosine sites are modified by different times of iodination?

Table 28 shows the number of tyrosine sites iodinated, determined in two ways as described in Materials and Methods IV.A. X-ray fluorescence was carried out by Dr. Stanley Seltzer and spectrometric titration was carried out by Dr. Peter Scherrer on a bR sample that had not been pre-modified with EA or BD. The number of tyrosines modified in the bR samples that had been pre-modified with EA or BD was determined indirectly by comparing the corresponding decrease in the 568 nm chromophore in the doubly-chemically modified samples with the bR sample that had been iodinated only, as an indication of the number of tyrosines modified. Although this is not an exact method to determine the extent of iodination, the 568 nm chromophore decrease may be used, since neither 20 mM EA nor 200 mM BD modified bR significantly decreased the chromophore absorption (see Results I.E.1. and II.E.1.). The chromophore decreases are compared in Results III.A.3.a.

The apparent discrepancy between these two methods to determine the extent of iodination may be explained by the inability of the spectro-photometric titration to reveal all of the tyrosines iodinated. However, spectroscopy did indicate that diiodinated tyrosine was being formed at longer times of iodination. This is expected since formation of the mono-iodinated tyrosine is rate-limiting at neutral pH (102) and the reaction was carried out at pH 7.2. At pH's higher than 8.0, formation of the diiodinated tyrosine is rate-limiting. As shown, iodination of both the 20 mM EA and 200 mM BD modified bR samples proceeded at a faster rate than iodination of bR control. This might indicate a loosening of
Table 28
Extent of Iodination of Tyrosines

<table>
<thead>
<tr>
<th>Time of Iodination</th>
<th>Mole I/Mole br</th>
<th>X-ray Fluorescence</th>
<th>Spectrophotometric Titration</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 Minutes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$I^{-}$</td>
<td>$5.1 \pm 3.3$</td>
<td>1 MIT*</td>
<td></td>
</tr>
<tr>
<td>$EA/I^{-}$</td>
<td>$11.3 \pm 2.1$</td>
<td>2 MIT</td>
<td></td>
</tr>
<tr>
<td>$BD/I^{-}$</td>
<td>$11.3 \pm 2.1$</td>
<td>2 MIT</td>
<td></td>
</tr>
<tr>
<td>60 Minutes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$I^{-}$</td>
<td>$11.3 \pm 2.1$</td>
<td>2 MIT</td>
<td></td>
</tr>
<tr>
<td>$EA/I^{-}$</td>
<td>$17.0$</td>
<td>2 MIT/1 DIT*</td>
<td></td>
</tr>
<tr>
<td>$BD/I^{-}$</td>
<td>$17.0$</td>
<td>2 MIT/1 DIT</td>
<td></td>
</tr>
<tr>
<td>120 Minutes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$I^{-}$</td>
<td>$17.0$</td>
<td>2 MIT/1 DIT</td>
<td></td>
</tr>
<tr>
<td>$EA/I^{-}$</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>$BD/I^{-}$</td>
<td>$15.0 \pm 1.0$</td>
<td>3 MIT/1 DIT</td>
<td></td>
</tr>
<tr>
<td>180 Minutes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$I^{-}$</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>$EA/I^{-}$</td>
<td>$15.0 \pm 1.0$</td>
<td>3 MIT/1 DIT</td>
<td></td>
</tr>
<tr>
<td>$BD/I^{-}$</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>240 Minutes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$I^{-}$</td>
<td>$15.0 \pm 1.0$</td>
<td>3 MIT/1 DIT</td>
<td></td>
</tr>
<tr>
<td>$EA/I^{-}$</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>$BD/I^{-}$</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

*MIT = Moniodinated Tyrosine

*DIT = Diiiodinated Tyrosine
the protein structure after EA or BD modification, evidenced by the slight red shift seen in the tryptophan fluorescence spectra (see Results I.F. and II.E.2.), causing the iodination of buried tyrosines to proceed more efficiently. The fact that iodination appeared to stop at 17 tyrosine sites modified, may indicate that the pH on the interior of the protein was higher than the aqueous phase pH. A basic internal pH would prohibit diiodinated tyrosines from forming and thus the full 22 sites would not be modified. Thus it appeared that 17 of the 22 tyrosines sites were available for modification with iodine, but only 4 of these could be visualized by spectrophotometric titration. Due to this discrepancy, the results in the following sections will be presented as time of iodination, rather than fraction of total tyrosines modified.

III.  A.  2. Photocycling Activity

After double chemical modification with EA/I\(^-\) or BD/I\(^-\) it was of interest to correlate the double chemical modifications with photocycling activity of bR. Changes in the kinetics of the rise of the M\(_{412}\) photointermediate are given in Table 29. Included for comparison are the kinetics of the rise of M\(_{412}\) of an iodinated bR sample without prior chemical modification.

As shown, iodination alone gradually caused an increase in the rate of deprotonation of the Schiff base, until this was 3 times faster at 180 minutes of iodination. Scherrer et al. (27) have implicated tyrosine as a primary acceptor of the Schiff base proton; after iodination it will more likely be in the deprotonated state and thus will more likely accept a proton. In the case of the doubly modified samples
Table 29

Effect of EA/I\(^{-}\) and BD/I\(^{-}\) on \(M_{412}\) Rise Kinetics

At 22\(^{\circ}\) C

<table>
<thead>
<tr>
<th>bR Sample</th>
<th>Time of Iodination</th>
<th>(M_{412}) Rise (t_{1/2}) in (\mu)secs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 Minutes</td>
<td>49</td>
</tr>
<tr>
<td></td>
<td>30 &quot;</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td>60 &quot;</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>120 &quot;</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>180 &quot;</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td></td>
<td>55</td>
</tr>
<tr>
<td></td>
<td></td>
<td>28</td>
</tr>
<tr>
<td></td>
<td></td>
<td>58</td>
</tr>
<tr>
<td></td>
<td></td>
<td>45</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>80</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>75</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>46</td>
</tr>
</tbody>
</table>
the rise was seen to become faster after only 30 minutes of iodination. This indicates that the tyrosine that accepts a proton from the Schiff base is iodinated much more readily in the doubly modified samples, probably due to an "opening up" of the protein structure before iodination. In both doubly modified samples, the rise kinetics then were slowed and then became faster towards 180 minutes of iodination. It is possible that in the premodified bR samples, a looser protein structure alters the geometry at the Schiff base. At 180 minutes of iodination the fast rise kinetics were restored, indicating domination of the iodination modification.

The $M_{412}$ decay kinetics were slowed in both phases as shown in Table 30, after iodination. The $M_{412}$ decay kinetics of $I^-$ modified bR calculated by Dr. Peter Scherrer are included for comparison although his method of analysis did not always yield biphasic kinetics. The zero time kinetics were typical of 20 mM EA and 200 mM BD modified samples. As shown, the double chemical modification, EA/$I^-$, was more effective at inhibiting the $M_{412}$ decay than $I^-$ alone, and the most effective was the BD/$I^-$ double modification. These results are graphed in Figure 60 as % control (zero time value) for each set of samples. The difference in the shape of the curves in Figure 60 may be due to the subsequent modification of different tyrosines, or to more complex factors created by a doubly modified sample. Of all the chemical modifications in this thesis, the doubly modified BD/$I^-$ sample at 180 minutes of iodination had the slowest $M_{412}$ decay. The significance of these results in terms of the mechanism of photocycling of bR will be discussed in Discussion II.B.3.b.
Table 30

Effect of EA/I\textsuperscript{−} and BD/I\textsuperscript{−} on M\textsubscript{412} Decay Kinetics

<table>
<thead>
<tr>
<th>Time of Iodination</th>
<th>M\textsubscript{412} Decay t\textsubscript{1/2} in msecs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>I\textsuperscript{−}</td>
</tr>
<tr>
<td></td>
<td>t\textsubscript{1/2}</td>
</tr>
<tr>
<td>0 Minutes</td>
<td>2</td>
</tr>
<tr>
<td>30 &quot;</td>
<td>8</td>
</tr>
<tr>
<td>60 &quot;</td>
<td>10</td>
</tr>
<tr>
<td>120 &quot;</td>
<td>18</td>
</tr>
<tr>
<td>180 &quot;</td>
<td>40</td>
</tr>
</tbody>
</table>
Figure 60

412 nm Decay Kinetics of bR Modified by EA/I⁻ and BD/I⁻

412 nm Decay $t_{1/2}$

First Phase as Percent
Zero Time First Phase, □, ●

Second Phase as Percent
Zero Time Second Phase, ○, ●

Time of Iodination in Minutes

[Graph showing decay kinetics with different phases and times]
III. A. 3. Spectroscopy

a. Absorption

After correlating the double chemical modifications with changes in photocycling activity, it was of interest to note changes in the bR$_{568}$ chromophore environment. Figures 61 and 62 show the absorption spectra of both sets of bR samples, EA/I$^-$ and BD/I$^-$, with the quenched iodination reagents in situ. Initially iodination of either the EA or BD modified bR samples produced a gradual blue shift. Such a blue shift might result from placing negative charges near the chain of retinal (103) or near the Schiff base (104-106) or perhaps from an increased bulk dipole resulting from an increased polarizability of the aromatic amino acids (107,108). This suggests that one or more tyrosines are in close proximity to the retinal chromophore. Both figures show a single isosbestic point indicating the conversion of one species to another species with increasing times of iodination. The fact that the isosbestic point was at 520 nm in the EA/I$^-$ modification, and at 513 nm in the BD/I$^-$ modification indicates that the two interconverting species are not identical in the two double modifications. The maximum of the new peak in both sets of samples is near 360 nm. The chromophore at 360 nm may indicate a different binding site of retinal, since either CTAB or NaBH$_4$ produces a chromophore in this region (109).

Table 31 quantitates the decreases in absorbance of the 568 nm chromophore of the doubly modified samples compared to the I$^-$ modified bR sample. As shown, the decrease of the 568 nm chromophore proceeded more rapidly in the doubly modified EA/I$^-$ samples that in the I$^-$ modified samples, and most rapidly in the BD/I$^-$ samples. The absorbance at
Figure 61

Effect of Iodination Following 20 mM EA Modification on bR's Visible Absorption Spectrum

Absorbance Units

Time of Iodination

0 minutes
30 minutes
60 minutes
120 minutes
180 minutes
24 hours

Wavelength in nm
Figure 62
Effect of Iodination Following 200 mM BD Modification on bR's Visible Absorption Spectrum

Iodination Reagents In Situ

Absorbance Units

Time of Iodination
- 0 minutes
- 30 minutes
- 60 minutes
- 120 minutes
- 180 minutes
- 24 hours

Wavelength in nm
Table 31

Effect of EA/I⁻ and BD/I⁻ on bR's 568 nm Chromophore

<table>
<thead>
<tr>
<th>bR Sample</th>
<th>568 nm Chromophore (%) Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I⁻</td>
</tr>
<tr>
<td>Time of Iodination</td>
<td></td>
</tr>
<tr>
<td>0 Minutes</td>
<td>100</td>
</tr>
<tr>
<td>30 &quot;</td>
<td>100</td>
</tr>
<tr>
<td>60 &quot;</td>
<td>98</td>
</tr>
<tr>
<td>120 &quot;</td>
<td>92</td>
</tr>
<tr>
<td>180 &quot;</td>
<td>78</td>
</tr>
<tr>
<td>240 &quot;</td>
<td>66</td>
</tr>
</tbody>
</table>
180 minutes of iodination in the BD/I⁻ samples was smaller than the absorbance of the I⁻ sample at 240 minutes of iodination. This suggests that prior EA or BD modification allowed greater accessibility to the iodinating reagents within 4 hours.

III. A. 3. b. Tryptophan Fluorescence

The quantum yield of tryptophan fluorescence was measured on the 3 times washed, doubly modified samples by comparing the 287 nm excitation/315 nm emission fluorescence of each sample with a control at the same Lowry protein concentration. These results may be inaccurate by ± 5% due to the Lowry protein error. The results given in Table 32 showed a marked decrease in the yield of tryptophan fluorescence with only 30 minutes of iodination following EA or BD treatment. The total decrease in the yield of tryptophan fluorescence was greatest for the BD/I⁻ modified samples.

Since the excitation and emission spectra were not taken of the doubly modified samples, it is not known if the fluorescence quenching was accompanied by a blue or red shift. Fluorescence quenching indicates that the tryptophan environments might be changed by:

1) increase in polarity near the tryptophans,
2) protonation near the fluorescing tryptophans,
3) denaturation of the protein, causing internal quenching groups or solvent to be brought closer to tryptophans (59).

Denaturation of the protein was evidenced by the marked decrease in the 568 nm chromophore. These may be the primary reasons for the decrease in the fluorescence yield, but other causes, such as those mentioned in Results I.F., may also be contributing.
Table 32

Effect of EA/I⁻ and BD/I⁻ on Tryptophan Fluorescence

<table>
<thead>
<tr>
<th>Time of Iodination</th>
<th>287 nm/315 nm Fluorescence</th>
<th>Lowry Protein</th>
<th>(%) Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 Minutes</td>
<td>100</td>
<td>100</td>
<td>(Control)</td>
</tr>
<tr>
<td>30 &quot;</td>
<td>75</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td>60 &quot;</td>
<td>75</td>
<td>59</td>
<td></td>
</tr>
<tr>
<td>120 &quot;</td>
<td>61</td>
<td>44</td>
<td></td>
</tr>
<tr>
<td>180 &quot;</td>
<td>36</td>
<td>32</td>
<td></td>
</tr>
</tbody>
</table>
III. B. EA Followed by EDC, and EDC Followed by EA

1. Number of Amino Acids Modified

The number of carboxyls modified by 50 mM EDC, which was the only concentration of EDC that was used in this experiment, was determined as described in Materials and Methods IV.B. by Jeffrey Herz, by quantitating the number of additional glycines in the amino acid analysis of the EDC modified bR. For bR modified by EDC alone, this number was 12 out of a possible 20 carboxyls, or 60% of the total carboxyls modified. This number may have changed with prior modification with EA, but further amino acid analysis was not undertaken in this study.

The number of lysines modified by increasing concentrations of EA was determined by the fluorescamine method as described in Materials and Methods II.C.1., using 6 as the total available lysines to convert fluorescence. These results are presented in Table 33 and may be inaccurate by \(\pm 13\%\) as calculated in Appendix 3. The number of lysines modified by increasing concentrations of EA alone, and by 50 mM EDC are included for comparison. As shown, 50 mM EDC alone modified 1.2 lysines, and prior or post 50 mM EDC modification modified 1.2 additional lysines above the lysines modified by EA at low concentrations of EA. EDC can modify lysines by cross-linking a carboxyl group to lysine, as described in Materials and Methods IV.B. In the EDC/10 mM EA modified sample, this difference decreased to 0.6 lysines, and in the EDC/50 mM EA or 50 mM EA/EDC sample, there was no difference in the number of lysines modified between this sample and the 50 mM EA sample. This data suggests that competition for the single lysine that is modified by EDC occurs by modification with EA. The significance of this result in terms of the
Table 33

Number of Lysines Modified by EA/EDC and EDC/EA Double Modifications

<table>
<thead>
<tr>
<th>bR Sample</th>
<th>Number of Lysines Modified</th>
<th>bR Sample</th>
<th>Number of Lysines Modified</th>
<th>bR Sample</th>
<th>Number of Lysines Modified</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>Control</td>
<td>0</td>
<td>Control</td>
<td>0</td>
</tr>
<tr>
<td>2 mM EA</td>
<td>$1.1 \pm 0.1$</td>
<td>2 mM EA/EDC</td>
<td>$2.3 \pm 0.1$</td>
<td>EDC/2 mM EA</td>
<td>$2.3 \pm 0.2$</td>
</tr>
<tr>
<td>5 mM EA</td>
<td>$1.8$</td>
<td>5 mM EA/EDC</td>
<td>$2.9 \pm 0.3$</td>
<td>EDC/5 mM EA</td>
<td>$2.9 \pm 0.1$</td>
</tr>
<tr>
<td>10 mM EA</td>
<td>$2.7 \pm 0.1$</td>
<td>10 mM EA/EDC</td>
<td>$-$</td>
<td>EDC/10 mM EA</td>
<td>$3.3 \pm 0.2$</td>
</tr>
<tr>
<td>25 mM EA</td>
<td>$4.1 \pm 0.1$</td>
<td>25 mM EA/EDC</td>
<td>$4.4 \pm 0.5$</td>
<td>EDC/25 mM EA</td>
<td>$-$</td>
</tr>
<tr>
<td>50 mM EA</td>
<td>$4.8 \pm 0.1$</td>
<td>50 mM EA/EDC</td>
<td>$4.7 \pm 0.1$</td>
<td>EDC/50 mM EA</td>
<td>$4.8 \pm 0.1$</td>
</tr>
<tr>
<td>200 mM EA</td>
<td>$4.8 \pm 0.1$</td>
<td>200 mM EA/EDC</td>
<td>$4.9 \pm 0.4$</td>
<td>EDC/200 mM EA</td>
<td>$-$</td>
</tr>
<tr>
<td>50 mM EDC</td>
<td>$1.2 \pm 0.4$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
structure and function of bR will be presented in the Discussion.

III. B. 2. Photocycling Activity

After the number of carboxyl and lysine residues modified were determined for the EA/EDC double modifications, the next step was to correlate the chemical changes with changes in photocycling kinetics. Flash photolysis and $M_{412}$ photostationary steady state spectroscopy were used to determine changes in photocycling activity. These results are shown in Tables 34 and 35. As shown, EDC modification alone was able to slow the second phase of $M_{412}$ decay 12 times over the control. Premodification with EA was able to reverse the EDC-induced inhibition. Since premodification with EA was also able to inhibit formation of the carboxyl-lysine cross-link by EDC, this suggests that EDC inhibition is caused by the cross-link between a lysine and carboxyl group. The second phase $t_{1/2}$ of the 200 mM EA/EDC sample was still 3.5 times slower than control, however, indicating that complete reversal had not occurred. Perhaps the cause of incomplete reversal was that even extensive EA modification will only modify 57% of 5 of the 6 available lysines in bR (see Results I.A.1.), so that the lysine-carboxyl cross-link will still be formed in some bR molecules.

When the reverse modification was carried out, EA modification after EDC modification caused a slight reversal of the EDC inhibition (see also Table 35). The reasons for the slight reversal of the inhibition are not exactly clear, since it is thought that the cross-link between the carboxyl and lysine will not reverse once it is formed. This result may have been due to complex factors caused by the double modification.
### Table 34

Effect of EA/EDC and EDC/EA Double Modifications on $M_{412}$ Decay Kinetics

<table>
<thead>
<tr>
<th>bR Sample-</th>
<th>Fraction of Total (6) Lysines Modified</th>
<th>$M_{412}$ Decay in msecs $t_{1/2}^{-1}$</th>
<th>bR Sample-</th>
<th>Fraction of Total (6) Lysines Modified</th>
<th>$M_{412}$ Decay in msecs $t_{1/2}^{-2}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>4.3</td>
<td>14.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>200 mM EA</td>
<td>0.80</td>
<td>4.3</td>
<td>16.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EDC</td>
<td>0.20</td>
<td>25</td>
<td>165</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 mM EA/EDC</td>
<td>0.38</td>
<td>12</td>
<td>145</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 mM EA/EDC</td>
<td>0.48</td>
<td>12</td>
<td>138</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 mM EA/EDC</td>
<td>-</td>
<td>13</td>
<td>73</td>
<td>EDC/10 mM EA</td>
<td>0.55</td>
</tr>
<tr>
<td>50 mM EA/EDC</td>
<td>0.78</td>
<td>6</td>
<td>85</td>
<td>EDC/50 mM EA</td>
<td>0.80</td>
</tr>
<tr>
<td>200 mM EA/EDC</td>
<td>0.82</td>
<td>9.3</td>
<td>51</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 35

Effect of EDC/EA Double Modification on $\frac{M_{412}}{bR_{568}}$ Molar Ratio

<table>
<thead>
<tr>
<th>bR Sample</th>
<th>Fraction of Total (6) Lysines Modified</th>
<th>$\frac{M_{412}}{bR_{568}}$ Molar Ratio (% Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>200 mM EA</td>
<td>0.80</td>
<td>150</td>
</tr>
<tr>
<td>EDC</td>
<td>0.20</td>
<td>1225</td>
</tr>
<tr>
<td>EDC/2 mM EA</td>
<td>0.38</td>
<td>837</td>
</tr>
<tr>
<td>EDC/5 mM EA</td>
<td>0.48</td>
<td>828</td>
</tr>
<tr>
<td>EDC/10 mM EA</td>
<td>0.55</td>
<td>923</td>
</tr>
<tr>
<td>EDC/50 mM EA</td>
<td>0.80</td>
<td>793</td>
</tr>
</tbody>
</table>
III. B. 3. Spectroscopy

a. Absorption

After treating with the double chemical modifiers EA and EDC, it was of interest to correlate the chemical changes with deterioration of the 568 nm chromophore. Neither EA nor EDC caused the $\lambda_{MAX}$ to shift from 568 nm. The loss of the peak height of the 568 nm chromophore is seen in Table 36, with the 200 mM EA and 50 mM EDC samples included for comparison. As shown, for most concentrations of EA, the decrease in the 568 nm peak height was small, about 15% below the control. This decrease is attributed to the combined effect of EA and EDC, since neither reagent alone caused the 568 nm chromophore to decrease more than 5%. This suggests that modification of lysines and carboxyls perturbs the chromophore environment, but since the order of the modifications was not important, this change should be unrelated to the one lysine that cross-linked with a carboxyl. Rather the double modifications probably affect the chromophore structure by modifying lysines and carboxyls close to the surface of bR.

III. B. 3. b. Tryptophan Fluorescence

Only one broad band was seen in the UV fluorescence emission spectra with no dependence of $\lambda_{MAX}$ on the wavelength of excitation; the origin of this fluorescence will be discussed in Discussion II.A.8.a.

Table 37 summarizes the positions of the $\lambda_{MAX}$ of excitation and emission after double chemical modifications. A red shift in the emission spectra was evident, especially for the EA/EDC double modification. This red shift was more marked than that seen with EA or EDC alone, indicating
Table 36
Effect of EA/EDC and EDC/EA Double Modifications on bR's 568 nm Chromophore

<table>
<thead>
<tr>
<th>bR Sample</th>
<th>Fraction of Total (6) Lysines Modified</th>
<th>568 nm Chromophore</th>
<th>bR Sample</th>
<th>Fraction of Total (6) Lysines Modified</th>
<th>568 nm Chromophore</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Lowry Protein (%)</td>
<td></td>
<td></td>
<td>Lowry Protein (%)</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>100</td>
<td>EDC/2 mM EA</td>
<td>0.38</td>
<td>85</td>
</tr>
<tr>
<td>200 mM EA</td>
<td>0.80</td>
<td>97</td>
<td>EDC/5 mM EA</td>
<td>0.48</td>
<td>86</td>
</tr>
<tr>
<td>EDC</td>
<td>0.20</td>
<td>95</td>
<td>EDC/10 mM EA</td>
<td>0.55</td>
<td>87</td>
</tr>
<tr>
<td>2 mM EA/EDC</td>
<td>0.38</td>
<td>84</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5 mM EA/EDC</td>
<td>0.48</td>
<td>88</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10 mM EA/EDC</td>
<td>-</td>
<td>88</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>25 mM EA/EDC</td>
<td>0.73</td>
<td>88</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>50 mM EA/EDC</td>
<td>0.78</td>
<td>85</td>
<td>EDC/50 mM EA</td>
<td>0.80</td>
<td>81</td>
</tr>
<tr>
<td>200 mM EA/EDC</td>
<td>0.82</td>
<td>73</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Table 37

**Effect of EDC and EA Double Modifications on bR's Tryptophan Fluorescence**

<table>
<thead>
<tr>
<th>bR Sample</th>
<th>Fraction of Total (6) Lysines Modified</th>
<th>Excitation $\lambda_{\text{MAX}}$ (nm)</th>
<th>Emission $\lambda_{\text{MAX}}$ (nm)</th>
<th>Fluorescence Yield (% Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>287</td>
<td>315</td>
<td>100</td>
</tr>
<tr>
<td>200 mM EA</td>
<td>0.80</td>
<td>288</td>
<td>317</td>
<td>86</td>
</tr>
<tr>
<td>EDC</td>
<td>0.20</td>
<td>289</td>
<td>318</td>
<td>98</td>
</tr>
<tr>
<td>2 mM EA/EDC</td>
<td>0.38</td>
<td>289</td>
<td>325</td>
<td>-</td>
</tr>
<tr>
<td>5 mM EA/EDC</td>
<td>0.48</td>
<td>289</td>
<td>322</td>
<td>-</td>
</tr>
<tr>
<td>10 mM EA/EDC</td>
<td>-</td>
<td>287</td>
<td>322</td>
<td>-</td>
</tr>
<tr>
<td>50 mM EA/EDC</td>
<td>0.78</td>
<td>289</td>
<td>322</td>
<td>-</td>
</tr>
<tr>
<td>EDC/2 mM EA</td>
<td>0.38</td>
<td>288</td>
<td>318</td>
<td>100</td>
</tr>
<tr>
<td>EDC/5 mM EA</td>
<td>0.48</td>
<td>289</td>
<td>318</td>
<td>89</td>
</tr>
<tr>
<td>EDC/10 mM EA</td>
<td>0.55</td>
<td>289</td>
<td>319</td>
<td>63</td>
</tr>
<tr>
<td>EDC/50 mM EA</td>
<td>0.80</td>
<td>287</td>
<td>319</td>
<td>67</td>
</tr>
</tbody>
</table>
that the internal protein structure probed by tryptophan fluorescence has been altered by the double modifications. Red shifts of the tryptophan fluorescence were explained in Results I.F., and may indicate changes in the polarizability, polarity or mobility of the tryptophan environments, or specific quenching of tryptophans in a hydrophobic environment. The fluorescence yield measured with 287 nm excitation/315 nm emission decreased quite markedly in the EDC/EA modified samples, although this was not studied in the EA/EDC samples. A decrease in the quantum yield could be due to:

1) denaturation of the protein, evidenced by the 15 % decrease in the 568 nm chromophore structure,

2) protonation near the fluorescing tryptophans,

3) formation of a transient excited state collisional complex (50), or to the other reasons stated in Results I.F.

III. C. BD Followed by EDC

1. Number of Amino Acids Modified

The number of arginines modified in the 500 mM BD/50 mM EDC double modification was 0.65 fraction of total arginines available. Amino acid analysis to determine the number of carboxyls modified by EDC in this double modification was not specifically carried out. The number of carboxyls modified by 50 mM EDC in bR that had not been premodified with BD was determined to be 0.60 fraction of total carboxyls by Jeffrey Hertz, but this may have changed following BD modification (see below). The number of lysines modified after double modification with BD/EDC was investigated by the fluorescamine test. This test revealed 3.8 + 0.03 lysines modified, or
3 more than modified by EDC alone, within the error of ± 13% calculated in Appendix 3. BD modification alone did not modify any lysines as shown in Appendix 12. This important result indicated that at least 3 carboxyl groups became available to EDC after BD modification. This could occur if 3 carboxyls that had been previously ionically bound to 3 arginines were freed by the BD modification, since it has been shown that ionic interactions in a protein compete with chemical modification (110). This is evidence for at least 3 salt bridges between arginines and carboxyl groups, each in the vicinity of a lysine that can cross-link to the carboxyl via a nucleophilic attack. It is possible that there are additional arginine-carboxyl salt bridges that are not also near a lysine. The structural significance of this result will be discussed.

III. C. 2. Photocycling Activity

After determining the chemical changes caused by the double modification, it was of interest to correlate these changes with photocycling activity. The results of flash photolysis and photo-stationary steady state spectroscopy are shown in Table 38, with 50 mM EA and 500 mM BD included for comparison. The photocycling kinetics of the 500 mM BD modified sample were only about 18-fold inhibited in this sample, not 30-fold as expected for 500 mM BD modification. The reasons for this are not clear, but since this sample was also used for the BD/EDC modification, subsequent changes caused by the double modification could still be seen. As shown, the BD/EDC double modification inhibited the M_{412} decay in a manner similar to the BD modification alone, although the first phase of M decay was slower in the BD/EDC double modification,
### Table 38

**Effect of BD/EDC Double Modification on Photocycling Activity**

<table>
<thead>
<tr>
<th>bR Sample</th>
<th>Fraction of Total (20) Carboxyls Modified</th>
<th>Fraction of Total (7) Arginines Modified</th>
<th>Fraction of Total (6) Lysines Modified</th>
<th>$M_{412}^\text{Decay}$ in msecs $t_{1/2}^{-1}$</th>
<th>$t_{1/2}^{-2}$</th>
<th>$M_{412}^\text{bR568 Mole Ratio}$ % Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4.3</td>
<td>14.5</td>
<td>100</td>
</tr>
<tr>
<td>500 mM BD</td>
<td>0</td>
<td>0.65</td>
<td>0</td>
<td>25</td>
<td>270</td>
<td>1851</td>
</tr>
<tr>
<td>50 mM EDC</td>
<td>0.60</td>
<td>0</td>
<td>0.20</td>
<td>25</td>
<td>165</td>
<td>1225</td>
</tr>
<tr>
<td>500 mM BD/50 mM EDC</td>
<td>(0.85 ?)</td>
<td>0.65</td>
<td>0.63</td>
<td>35</td>
<td>232</td>
<td>2182</td>
</tr>
</tbody>
</table>
and the $M_{412}$ photostationary state was slightly increased. The significance of these results in terms of a functional model of bR's conformational changes will be discussed (see Discussion II.A.7.).

The fact that BD modification alone inhibited in this experiment about 1.7 times more than EDC modification, and in Results II.B.1., up to 2.5 times more than EDC modification, suggests that the carboxyls that ionically bond arginines that are important for activity are not modified by EDC. This agrees with the previous interpretation that EDC inhibition is due to cross-linking a carboxyl group to a lysine, and not due to modifying carboxyl groups that are ionically linked to arginines. Thus this double modification helped to elucidate which arginines are important for bR's photoactivity (see Discussion II.A.7.).

III. C. 3. Spectroscopy

a. Absorption

After the double modification with 500 mM BD and 50 mM EDC, the 568 nm absorbance decreased as shown in Table 39. These results may be inaccurate by $\pm 5\%$ due to the error in the Lowry protein test. As shown, the double modification caused more deterioration of the chromophore environment than either BD or EDC alone. Thus the chromophore and protein structure may be affected by the 3 additional cross-links formed between lysines and carboxyls after BD/EDC modification.

III. C. 3. b. Tryptophan Fluorescence

The tryptophan fluorescence excitation and emission spectra are shown in Figures 63 and 64, since for the first time a new peak was seen in both spectra. This shoulder indicates that the environments of the fluorescing tryptophans are now heterogeneous; i.e.,
Table 39

Effect of BD/EDC Double Modification on bR's 568 nm Chromophore

<table>
<thead>
<tr>
<th>bR Sample</th>
<th>Fraction of Total (20) Carboxyls Modified</th>
<th>Fraction of Total (7) Arginines Modified</th>
<th>Fraction of Total (6) Lysines Modified</th>
<th>568 nm Chromophore Lowry Protein (% Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>500 mM BD</td>
<td>0</td>
<td>0.65</td>
<td>0</td>
<td>91</td>
</tr>
<tr>
<td>50 mM EDC</td>
<td>0.60</td>
<td>0</td>
<td>0.20</td>
<td>95</td>
</tr>
<tr>
<td>500 mM BD/50 mM EDC</td>
<td>(0.85 ?)</td>
<td>0.65</td>
<td>0.63</td>
<td>81</td>
</tr>
</tbody>
</table>
Figure 63

Fluorescence Excitation Spectra of BD/EDC-Modified bR

Fluorescence Emission = 320 nm

Excitation Wavelength in nm
Figure 64

Fluorescence Emission Spectra of BD/EDC-Modified bR

Excitation Wavelength = 287 nm

Emission Wavelength in nm
Table 40

Effect of BD/EDC Double Modification on bR's Tryptophan Fluorescence

<table>
<thead>
<tr>
<th>bR Sample</th>
<th>Fraction of Total (20) Carboxyls Modified</th>
<th>Fraction of Total (7) Arginines Modified</th>
<th>Fraction of Total (6) Lysines Modified</th>
<th>287 nm/315 nm Fluorescence Lowry Protein (% Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>500 mM BD</td>
<td>0</td>
<td>0.65</td>
<td>0</td>
<td>95</td>
</tr>
<tr>
<td>50 mM EDC</td>
<td>0.60</td>
<td>0</td>
<td>0.20</td>
<td>98</td>
</tr>
<tr>
<td>500 mM BD/50 mM EDC</td>
<td>(0.85 ?)</td>
<td>0.65</td>
<td>0.63</td>
<td>92</td>
</tr>
</tbody>
</table>
that there appear to be two classes of fluorescing tryptophans after BD/EDC double modification. Of the 1 or 2 fluorescing tryptophans near the external surface (111), this may indicate that one tryptophan is now more exposed to the aqueous phase. Alternatively this could represent two different environments inside the protein. The changes in the fluorescence quantum yield are shown in Table 40. As indicated the shifts in $\lambda_{\text{MAX}}$ of excitation and emission did not cause a marked quenching of fluorescence.
DISCUSSION

I. Structural Significance of Chemical Modifications

A. Model for Secondary Structure of bR

1. Implications of Monofunctional Imidoester Modification

Results I.A.I. reveals that one lysine in bR is modified far more easily than the other 5 lysines by monofunctional imidates. The significance of this result and its interpretation have important structural implications for placing the amino acid sequence into the 7 α-helices of bR. Using this result combined with the other results presented in this thesis I have constructed a model of bR that is different from published models (23,112-114) and appears in Figure 65. Since this figure will be referred to frequently, it has been placed on a fold-out section. The justification for placing the amino acids in these positions will be explained throughout the Discussion section. The amino acid sequence is that of Khorana et al. (24).

Two alternative models to explain a greater reactivity of one lysine could be called the "exposure" model and the "pK" model. The exposure model predicts a greater reactivity of one lysine due to its location in the aqueous phase, while the other lysines are located to some extent beneath the surface of the membrane. Preferential access of the imidoesters to one lysine will cause it to be more completely modified than the other 5 lysines. The pK model predicts a lower pK for one lysine which would shift the equilibrium of the imidoester reaction, since this lysine would be preferentially deprotonated, and since it is the deprotonated state of lysine that interacts with imidoesters (see Figure 3), this lysine would react to a greater extent than the lysines that are partially protonated at pH 10.0.
Figure 65. Model of secondary structure of bR. This model was constructed using the results of the chemical modifications in this thesis. Included for quick reference is the preferred model of the tertiary structure of bR, Model A of Agard and Stroud (126), that also appears in Figure 67.
To decide between these two possibilities it is useful to know which lysine is the most reactive. Although the location of the most reactive lysine was not specifically determined in this thesis, comparison to other investigations may pinpoint the lysine involved. Harris et al. (115) chemically modified bR with another water-soluble reagent, dansyl chloride, and found that only LYS 40 became modified. Dansyl chloride contains an $\text{SO}_2\text{Cl}$ group that would not be expected to penetrate into a hydrophobic domain and thus would react only with lysines exposed to the aqueous phase. Indeed, it has been shown by Sigrist and Zahler (116), that a molecule similar to dansyl chloride, p-sulfophenylisothiocyanate, is not able to penetrate to the hydrophobic binding site of LYS 216. Thus the work of Harris et al. (115), suggests that LYS 40 is also the most reactive lysine with the water-soluble imidoesters. In addition, comparison of their work to the work of Sigrist and Zahler (116), implicates the exposure model as the correct cause of greater reactivity of LYS 40.

The pK model gets support from Lewis et al. (117) who implicated another lysine in the vicinity of the Schiff base. If the role of LYS 40 is to share in binding the proton of the Schiff base nitrogen on LYS 41, then its pK would be lowered due to the close association with the positive charge. However, if this were the case, placing the bulky dansyl group on LYS 40 would be expected to alter the chromophore and retinal binding site. Harris et al. (115) have found just the opposite; dansyl chloride modification of LYS 40 had no effect on the 568 nm chromophore and no effect on proton release kinetics. Thus this data does not favor the pK model as the cause for different reactivities of lysines with
Another possible interpretation that is less likely is that all 6 reactive lysines are totally exposed to the aqueous phase, but 5 of the lysines have a higher pK, or 1 of the lysines has a lower pK. However, alteration of a pK in the aqueous phase on a linking region between helices will not be easily controlled by a neighboring charge since amino acid residues in the aqueous phase experience multiple charge effects. LYS 40, for example, could have a lowered pK due to the proximity of ARG 225 on helix G, but ARG 225 may be ionically bound to ASP 38 which would neutralize its positive charge (see Discussion I.A.3.). For these reasons this mixed exposure/pK model is less likely than the exposure model alone.

Thus, the structural significance of the work in this thesis is to distinguish one lysine from the other 5 reactive lysines. This result, together with the exposure model interpretation of the work of Harris et al. (115), indicates that LYS 40 is totally exposed to the aqueous phase, and that the other 5 reactive lysines are buried to some degree in the hydrophobic lipid domain of the PM. The differences in the degrees that the other 5 lysines are buried cannot be determined on the basis of the Scatchard plot in Figure 22. Rather the Scatchard plot yielded the valuable information that one lysine reacted to a much greater extent than the other 5 lysines. It is possible that there were also some small differences in reactivity of the other 5 lysines that could not be distinguished by the Scatchard plot, given the standard deviation of ± 13% of the fluorescamine test.

My model differs in some respects from the published models of Engelman et al. (112), Stroud et al. (114), and Mehlhorn (113). By constraining the 5 reactive lysines other than LYS 40 to fit within the
membrane, the start and ends of helices are also defined to some extent, and this is an important result of these chemical modification studies. The only published model that has placed all 5 reactive lysines other than LYS 40 below the surface is that of Stroud, Katre and Agard (114). These authors also place LYS 40, however, below the surface, which I believe to be incorrect. As will be explained in more detail in the discussion of the double chemical modifications, the distances that the lysines are placed beneath the surface of the PM depend on which ionic linkages they can form with negatively charged groups. The model of Engelman et al. (112) does place LYS 40 in the aqueous phase, as in my model, but they also place LYS 158 (LYS 159) in the aqueous phase. According to my results, only one lysine should be totally exposed to the aqueous phase. Thus my model has one difference from each of these two models (112,114). Between them there are two differences, so my model is a compromise in this respect. Mehlhorn (113) places LYS 129 in the aqueous phase and LYS 40 buried. Thus there are two differences between my model and that of Mehlhorn, regarding placement of lysines, and Mehlhorn's model disagrees with that of Engelman et al. (113) by three differences.

My model and the results of Harris et al. (115) are inconsistent with neutron diffraction studies that have shown the β-ionone ring to be centrally located (118). Using a length of 20 Å for the lysine side chain plus retinal, and an angle of 25° between retinal and the membrane surface, a simple calculation shows that the β-ionone ring should be located only 10-11 Å beneath the surface. This discrepancy is not explicable at present, but indicates that either both chemical modification studies are incorrect (this thesis and (115)), or the neutron diffraction data is incorrect.
An alternative explanation for this apparent discrepancy has been discussed by Bayley et al. (119). If retinal were attached by an aldamine linkage to both LYS 41 and LYS 216 simultaneously, then the β-ionone ring would be located 16 Å from the cytoplasmic surface. This seems unlikely, however, since it has been shown by numerous resonance Raman studies that the retinyl-protein linkage is a Schiff base (61,120,121). Another possibility is that retinal is permanently attached to LYS 216, in which case the β-ionone ring would be located 21 Å from the cytoplasmic surface. I do not think this to be true for two reasons:

1) the site of attachment of retinal was determined by Bayley et al. (119) to be LYS 216 during intense illumination, and 2) Sigrist and Zahler (116) could chemically modify LYS 216 with little change in the 568 nm chromophore. Heavy atom labeling of LYS 40 by isomeric replacement and then imaging by electron diffraction would help to determine if LYS 40 is exposed to the aqueous phase. A heavy atom derivative of a bulky water-soluble, lysine specific reagent would be ideal for this purpose. Benisek and Richards (122) have suggested the use of picolinamidine groups to effect heavy-atom labeling of lysines.

This derivative has the structure as shown, and could be converted into a more water-soluble derivative by attachment of a polar group into the ring.

At this point a similar study using methyl acetimidate to label bR should be discussed. Moore et al. (55) have shown that in their hands only 5 lysine residues are amidinated by MA, not 6 as shown in this study. Instead of using the Scatchard plot to determine the total number of lysines modified, they used peptide mapping combined with radioactive
succinylation to determine that two lysines, LYS 41 and LYS 216 were completely unmodified by MA. One possible cause of the discrepancy between the Moore et al. (55) paper and this work could be due to the reagent used. Moore et al. (55) used MA that they had prepared from acetonitrile and stored in vacuo at -20°C. In my study MA was purchased from Aldrich Chemical Co. and used without further purification. It was stored over drying crystals at -20°C. It is possible that the reagent in this study contained hydrolysis products of MA that caused some deterioration of the protein, thus allowing accessibility of the reagent to LYS 216. Water from the air could cause the release of ammonia and methanol from MA. This would also explain the difference in the molar ratio of MA:bR needed for maximum modification. Moore et al. (55) found this ratio to be 40:1, while in this work this ratio was 2500:1. Since MA is a relatively small molecule even a slight perturbation of the protein structure could allow accessibility to LYS 216 by MA. The small decrease in the 568 nm chromophore absorbance (see Results I.E.1.) and slight red shift of the tryptophan fluorescence (see Results I.F.) are indicative of a slight protein perturbation.

I. A. 2. Implications of Bifunctional Imidoester Modification

The difference in reactivity of the bifunctional
imidoesters, DMA 8.3 Å, and DMS, 11.3 Å, also has implications for the secondary structure of bR. As shown in Results I.A.1. and more clearly in Appendix 8, DMS, even with three successive treatments can modify only 4 of the 6 reactive lysines. DMA, on the other hand, can modify
as many lysines as the monofunctional imidates (see Results I.A.1.).

This difference in reactivity is probably due to the ability of DMA to penetrate to internal lysines while the longer chain length of DMS cannot. Figure 65 suggests that LYS 216 and LYS 172 are both unavailable to DMS which would account for the reduced reactivity of DMS.

The temperature experiment (see Results I.A.2.) confirmed the interpretation that DMA can penetrate to internal lysines while DMS ordinarily cannot. At higher temperatures, DMS is able to modify the same number of lysines as DMA, suggesting an unfolding of the protein structure above 40° C. This interpretation is at variance with the finding of Jackson and Sturtevant (46) who used differential scanning calorimetry to determine that there is no detectable phase transition between 0° and 70° C in isolated PM, or in isolated PM lipids (45). Thus the protein transition that increases accessibility of DMS to lysines must be very small. The phase transitions seen by Jackson and Sturtevant (46) at 80° C and 100° C in PM were not studied in this thesis, since they are outside of the physiologically significant domain.

Other investigators have studied different temperature dependencies in bR. Chignell and Chignell (123) observed an electron spin resonance parameter vs. the temperature, and Sherman et al. (47) observed the rate of a phototransient decay vs. the reciprocal of the absolute temperature. Both groups used a change in slope as the criteria for a transition near 30° C. However, Jackson and Sturtevant (46) caution against the use of a change in slope as an indication of a phase transition, since it is sometimes impossible to distinguish between a break in slope at a definite temperature, and a broad curvature in the plot throughout the temperature range studied.
One other group of investigators has also seen a "transition" close to that reported in this thesis. Heyn et al. (99) monitored the positive band at 525 nm in the CD spectrum as a function of temperature from 29°C to 50°C. The largest decrease in this band occurred between 40°C and 45°C. Their data could be interpreted as a change in the chromophore structure resulting from an unfolding of the protein structure. When they cooled the sample back to 29°C, the original band height was observed. Since the negative band at 602 nm still remained to some extent at 50°C, this indicated that alignment of the chromophores producing exciton-exciton interaction had been relatively little altered after the "transition". This result supports the interpretation that the transition of the protein that occurs between 40°C and 45°C may be very small. However, it is well known that changes as small as 0.1 Å can produce different protein behavior. The shift in position of iron in myoglobin from high spin to low spin may be as small as 0.01 Å (124). Although I have referred to the protein change in bR as an "unfolding" of the protein, R. Lumry would prefer to use the terminology "subtle" change for changes that do not involve changes in heat capacity (125). However, I will continue to use the term unfolding, as I believe it to be descriptive of the molecular event.

To be certain in the identification of the lysines modified by DMS and DMA at room and higher temperatures, a combination of delipidation, radioactive succinylation, cyanogen bromide (or thermolysin) cleavage, peptide mapping and amino acid analysis should be carried out. This type of analysis would reveal which lysines could be cross-linked by both reagents, and which lysines are unavailable to DMS. Such an analysis would be useful, especially in evaluating oligomerization of bR by DMA, as will be presented in Discussion I.B.1.
I. A. 3. Implications of BD Modification

Results II.A.1. shows that 7 out of the 9 arginines in my strain of bR, nominally S9, were modified by BD. The sequence in Figure 65 is that of Khorana (24) and contains 7 arginines total. The two additional arginines in my strain of bR are most likely located in the aqueous phase, since it is energetically unfavorable to place the highly positively charged guanidinium group inside a hydrophobic domain. In order to place an arginine within a hydrophobic membrane phase it should be neutralized by a negatively charged group. Even though Results II.A.1. showed that there may be two new carboxyl groups that could act to neutralize these two new positive charges, it is highly unlikely that these groups would be oppositely positioned within the membrane. Thus it is not probable that the two additional arginines lie within the membrane phase. Since it is not known on which interconnecting helices the two new arginines are located, they do not appear in Figure 65. However, since these new arginines are thought to be located in the aqueous phase, and since all the arginines had equal reactivity with BD (see Scatchard plot in Figure 45), this suggests that 7 arginines in my strain of bR are in the aqueous phase (in Figure 65, this is equivalent to 5 in the aqueous phase). This is also likely from the above reasoning, that since it is difficult to neutralize the positive charge of arginines, this amino acid would prefer to remain in the aqueous phase.

Since two arginines were totally unreactive with BD it indicates that they are buried to some degree in the hydrophobic phase. From Figure 65 the arginines unreactive with BD are probably ARG 82 on helix C and ARG 175 on helix F. In addition to being buried, they
are thought (112) to participate in salt linkages to carboxyl groups, ARG 175-ASP 115 and ARG 82-GLU 204. As can be seen in Figure 65, these ion pairs can form because their component amino acids are at approximately the same height in the PM.

Thus the BD chemical modification data would place 7 (or 5 in Figure 65) arginines in the aqueous phase as shown and 2 arginines buried (ARG 82 and ARG 175). The published model closest to obeying this constraint is that of Stroud et al. (114). In their model it appears as if ARG 7, ARG 134 and ARG 164 are just at the membrane surface instead of being totally exposed to the aqueous phase. The model of Mehlhorn (113) has placed 5 arginines within the membrane phase, which I believe to be incorrect for the reasons mentioned. In the model of Engelman et al. (112), ARG 134 appears to be buried, which I believe to be incorrect. The next step in analyzing this modification would be to use radioactive 2,3-butanedione followed by delipidation and protein chemistry to identify the arginines modified. During these procedures it is essential to maintain the protein in the presence of borate buffer to prevent reversibility of the modification (see Appendix 10). In addition, an attempt to modify all the arginines by first solubilizing the protein in SDS (198) or Triton X-100 (79) should be carried out. Amino acid composition analysis would reveal the number of arginines modified.

I. A. 4. Implications of EA and EDC Double Modifications

Results III.B.1. shows that one lysine is able to cross-link to a carboxyl via the carbodiimide adduct (see Materials and Methods IV.B., Figure 15). The lysine that is cross-linked to a
carboxyl must be near the aqueous phase, since EDC is a bulky water-soluble molecule, and also in close proximity to the carboxyl group. In addition, the lysine that is cross-linked to a carboxyl by EDC does not appear to be LYS 40, since at low concentrations of EA, where only LYS 40 will be modified appreciably (about 90 %), EDC can still modify one additional lysine. The ion-pair that appears to be in the best position to form this cross-link is LYS 159-GLU 161 on helix E. Other lysyl-carboxyl cross-links near the aqueous phase could occur but are less likely from the point of view of proximity constraints and also competing charge interactions between carboxyls and arginines (see Discussion I.A.5.).

From the discussion of the monofunctional imidoester lysine modification (I.A.1.), LYS 159 will be buried to some degree in the hydrophobic domain while GLU 161 is probably located in the aqueous phase.ALA 160 may be located in the membrane phase or exposed to the aqueous phase. The substituted amide linkage between LYS 159 and GLU 161 can easily occur as shown by model building with Framework Molecular Models, provided the helix is broken before GLU 161.

The distances that lysines other than LYS 40 are buried within the membrane cannot be ascertained on the basis of the EA/EDC double modification. From Discussion I.A.1. LYS 40 is thought to be totally exposed to the aqueous phase, and the reactivity of the other lysines depends not only one the distance they are buried, but also on the local pK near their side chains. Thus LYS 159 may be buried to the same extent as LYS 30 and LYS 129, but may have a higher pK than one of these lysines, due to its ion linkage with GLU 161. If LYS 30 is able to interact with the protonated Schiff base on LYS 41, then its pK will be lowered, causing it to be slightly more reactive than LYS 159.
More information about the distances these lysines are buried resulted from the BD/EDC modification in the following Discussion section.

I. A. 5. Implications of BD/EDC Double Modification

Results III.C.1. show that after BD modification, 4 total lysines can cross-link to carboxyls, whereas without prior BD modification, only one lysine can cross-link to a carboxyl. Since it is known that chemical reactivities of amino acid residues in proteins depend on their ionic environments (110) this indicates that there are 3 carboxyls ionically bound to arginines that are freed by the BD modification. These 3 carboxyl-arginine links must also be in the vicinity of a lysine. The arginine-carboxyl ionic linkage could form two hydrogen bonds as diagrammed below, but hydrogen-bonding is not a requirement of ionic (salt) links.

Interhelical Arginine-Carboxyl Ionic Linkage

By process of elimination, the lysines in the vicinity of three carboxyl-arginine links are LYS 30, LYS 40 and LYS 129. LYS 159 is postulated to form the first bridge to GLU 161 via EDC (see Discussion I.A.4.), and LYS 172 and LYS 216 are too deep in the membrane to be accessible (see Figure 65).

At this point it is necessary to draw on a conclusion from Discussion I.B. in order to understand the contribution of the BD/EDC double modification to the 2° structure of bR. In Discussion I.B.,
Model A of Agard and Stroud (126) of the $3^\circ$ and $4^\circ$ structure of bR is chosen primarily on the basis of the DMA and MA cross-linking results. Given Model A of Agard and Stroud (126), helix G is opposite helices A and B (Figure 65). Thus the possible arginine-carboxyl salt bridges that are involved on the cytoplasmic side are ARG 225-ASP 38 and ARG 227-ASP 36. These are the preferred linkages since once the ARG 225-ASP 38 link forms, the ARG 227-ASP 36 link follows naturally, since ASP 38 is two amino acids past ASP 36, and ARG 227 is two amino acids past ARG 225. These links could be either parallel or antiparallel as in a $\beta$-pleated sheet, but I have chosen the antiparallel links since antiparallel $\beta$-pleated sheets are more common in proteins than parallel. Model building with Framework Molecular Models shows that ASP 36 and ASP 38 are constrained to be closer than usual on a peptide chain, due to the intervention of a proline between these two amino acids (see Figure 66). Thus neutralizing these two negative charges with two guanidinium groups will relieve the repulsion of their negative charges. When the ARG 225-ASP 38 salt bridge is broken by BD modification of arginines, then LYS 40 can cross-link to ASP 38 via the substituted amide linkage (see Figure 15), since LYS 40 is totally accessible to the aqueous phase (see Discussion I.A.1.).

When the ARG 227-ASP 36 salt linkage is broken then LYS 30 can cross-link to ASP 36. The possibility of forming the LYS 30-ASP 36 amide linkage was checked using Framework Molecular Models. Even if GLY 31 is still hydrogen-bonded within helix A, only a small rotation of ASP 36 is needed to form this amide linkage.

The other arginine-carboxyl salt linkage that could be involved in this bonding scheme is ARG 134-GLU 194 on the external side. When
Figure 66
Framework Molecular Model of the
ARG 227-ASP 36 and ARG 225-ASP 38 Salt Links
this bond is broken by BD modification, then LYS 129 can cross-link to GLU 194. This bonding scheme requires GLU 194 to be located several amino acid residues further into the aqueous phase than ARG 134, since the amino acid side chains on helix E are directed downwards, while those on helix F are directed upwards. If ARG 134 and GLU 194 were at the same height these ionic bonds could not form. By model building, it is seen that this bonding scheme could occur if ARG 134, GLU 194 and LYS 129 all face toward the center of helices E, F and D. GLU 74 is thought not to interfere in these ion links since it is located on an interhelical link extending towards helix B.

Thus, given this scheme of ionic linkages, ASP 36 and ASP 38 should be located in the aqueous phase so that they can link to ARG 227 and ARG 225 in the aqueous phase just above helix G. At the external side, GLU 194 should be at least 3 residues further into the aqueous phase than ARG 134, and LYS 129 and LYS 30 should be buried not more than 2 residues. These constraints were taken into account in constructing the model in Figure 65.

I. B. Model for Tertiary and Quaternary Structure of bR

1. Implications of Bifunctional Imidoester Modification

The use of SDS polyacrylamide gel electrophoresis provides important structural information about the placement of bR into the crystalline lattice of the PM, as well as an explanation for the ability of bifunctional imidesters to inhibit the photocycle as was shown in Results I.B.1. and I.D.1. Davies and Stark (127) pioneered in this field with the use of SDS gel analysis of lightly cross-linked samples to estimate the quaternary structure of soluble oligomeric enzymes.
for purposes of establishing quaternary structure, only the first cross-link between two peptide chains provides any information; subsequent cross-links provide no additional information. In this thesis, cross-linking between bR molecules in different sheets of PM is thought not to have occurred since the gel pattern of oligomerization did not vary with protein concentration in the range used in these experiments (see Appendix 7).

The bR samples in this study that were lightly cross-linked were cross-linked by DMA, the 8.3 Å cross-linker, and also by MA, the 2.3% monofunctional imidate that is able to cross-link under certain conditions (see Discussion I.B.2.). DMA may have formed primarily intramolecular cross-links but also could cross-link up to pentamers of bR. The typical distribution for the polymers formed by DMA is seen in Appendix 8: Monomers = 40%, Dimers = 20%, Trimers = 20%, Tetramers = 10%, and Pentamers = 10%. The question that arises is, which lysines in bR could be cross-linked by an 8.3 Å cross-linking reagent? To answer this question, I have considered 5 models of Agard and Stroud (126) of alternative schemes for placing the amino acid sequence (24) into the 7 α-helices seen by Henderson and Unwin (128). As shown in my model of the bR sequence (see Figure 65), intermolecular cross-linking can only be expected to involve 3 lysines located near the cytoplasmic surface (LYS 30, LYS 40 and LYS 159) and perhaps 1 lysine located near the external surface (LYS 129). The lysines near the cytoplasmic surface would not be expected to cross-link to LYS 129 near the external surface, on the same or adjacent bR molecules, since an 8.3 Å cross-link could not span the 45 Å height of the bR protein. In addition, LYS 40 will be able to cross-link to other lysines the most easily, since it is located
in the aqueous phase. LYS 172 and LYS 216 may not contribute substantially to intermolecular cross-linking since their distance from the membrane surface is greater than the other lysines' distances.

Figures 67-71 show the possible cross-links with DMA for the 5 best models of Agard and Stroud (126) who used the criterion of visibility of amino acid links between helices seen by electron diffraction. These figures were constructed by tracing the electron diffraction map of bR as shown in (129) and placing the amino acid sequence of bR into this map in 5 different ways according to (126). In all the figures the reaction radii have been determined as follows: for 2 lysines totally exposed to the aqueous phase the distance of the DMA cross-link between the α carbons will be 21 Å. The only lysine totally exposed to the aqueous phase is thought to be LYS 40 (Discussion I.A.1.). The reaction radius for LYS 40 will thus be 21/2 = 10.5 Å. The other lysines, LYS 30, LYS 129 and LYS 159 appear to be less than two residues buried within the membrane, so their reaction radius at the surface of PM will be 10.5 Å minus 2 Å (1.5 Å for each amino acid residue into the helix). Thus the reaction radius of LYS 30 and LYS 159 is shown as 8.5 Å. There are no possible cross-links formed between two LYS 129 residues on the external side of PM using a reaction radius of 8.5 Å, so this reaction radius has not been drawn in the figures for LYS 129.

Figure 67 shows the possible cross-links between the lysines by DMA in Model A of Agard and Stroud (126). A cross-link that is considered highly likely is one where there is a large area of overlap between the reaction radii of 2 lysines. In Model A, the formation of 2 cross-links is highly likely; these are LYS 40-LYS 159 on an adjacent bR molecule in the same bR trimer, and LYS 40-LYS 30 on the same bR molecule. The inter-trimer cross-link LYS 30-LYS 30 does not appear to be very
Figure 67
Reaction Distances of DMA Cross-Links in PM
Model A (126)

KEY

= 10.5 Å, Reaction Radius of LYS 40

= 8.5 Å, Reaction Radius of LYS 30 and LYS 159

Helices

Lysines
Figure 68
Reaction Distances of DMA Cross-Links in PM
Model B (126)

KEY

- $10.5 \, \text{Å}$, Reaction Radius of LYS 40
- $8.5 \, \text{Å}$, Reaction Radius of LYS 30 and LYS 159

Helices

Lysines
Figure 69

Reaction Distances of DMA Cross-Links in PM

Model C (126)

KEY

\[ \text{Radius of LYS 40} \]

\[ \text{Radius of LYS 30 and LYS 159} \]

Helices

Lysines
Figure 70

Reaction Distances of DMA Cross-Links in PM

Model D (126)

KEY

- 10.5 \(\AA\), Reaction Radius of LYS 40

- 8.5 \(\AA\), Reaction Radius of LYS 30 and LYS 159

Helices

- 129

Lysines

- 129
- 159
- 129
- 50
- 53
- 159
- 129
Figure 71

Reaction Distances of DMA Cross-Links in PM

Model E (126)

KEY

- 10.5 Å, Reaction Radius of LYS 40

- 8.5 Å, Reaction Radius of LYS 30 and LYS 159

Helices

Lysines
favorable from the point of view of a small area of overlap of the reaction radii. However, once the LYS 40-LYS 159 cross-link has occurred on 2 adjacent trimers, the LYS 30-LYS 30 cross-link will form more easily. Thus Model A could form dimers and trimers, while tetramers and pentamers are also possible. Model A is a good candidate for the correct 3° and 4° structure of bR.

In Figure 68, the possible cross-links by DMA in Model B (126) are shown. The only cross-link that is highly likely in this figure is the LYS 40-LYS 30 cross-link on one bR molecule. If this occurs, then only bR monomers will form. Another cross-link that could form is the cross-link between two LYS 159 residues on adjacent bR molecules. This cross-link would produce bR dimers. Thus Model B does not appear capable of forming pentamers by DMA and should be rejected.

Model C is shown in Figure 69. The most highly likely cross-link in this model is between LYS 40 and LYS 30 on the same bR molecule which would form only monomers of bR. However, an occasional cross-link might occur between LYS 40 and LYS 30 on an adjacent bR molecule within the arrangement of trimers that form the lattice points of the hexagonal crystalline array. This would cause some dimers and trimers of bR to form. Higher polymers could then form due to cross-linking between two LYS 159 residues on adjacent bR molecules. However, comparison of Model C to Model A shows that the area of overlap of reaction radii needed to form higher polymers and especially trimers, is much smaller in Model C.

Figure 70 shows that Model D could form trimers in two ways: LYS 40-LYS 30 or LYS 40-LYS 40 on adjacent bR molecules. However, further polymerization would be impossible. Model D should therefore be rejected.
In the final model of Agard and Stroud (126), Model E, Figure 71, the most likely cross-link is again LYS 40-LYS 30 on the same bR molecule. Thus the most likely cross-link would only lead to monomer formation. Other less likely cross-links would lead to higher polymers. These cross-links are LYS 40-LYS 30 between bR molecules in adjacent trimers. In addition, LYS 30-LYS 159 within the bR trimers might occur. If both of these less likely links were formed, then polymers as high as pentamers could be formed. However, the distribution of higher polymers from Appendix 8 shows that dimers and trimers are formed only one-half less frequently than monomers. In Model E, the area of overlap of the reaction radii for trimer formation is not as large as that in Model A, and trimer formation may not occur as frequently as in Model A. It will be recalled that only Model A has 2 highly likely cross-links, one of which produces trimers. From this comparison of models, Model A is the best candidate for the correct 3° and 4° structure of bR.

Agard and Stroud (126) present Models A and E as their two best models according to their criteria of the most visible amino acid linking regions between helices seen by electron diffraction. These are also the two best models of Engelman et al. (112), whose criteria are the connectivity of helical segments, charge neutralization and electron scattering density. The work of Katre et al. (69) also favors Models A and E as the two best models, based on the proximity of LYS 41 to LYS 216 in these models. In the analysis in this thesis, consideration of the most probable cross-links produced by the 8.3 Å cross-linker, DMA, favors Model A. Thus, in the controversy between the two most accepted models by most investigators, Model A stands out as a superior model. This model is also Model 1 of Engelman et al. (112).
The results of the temperature experiment using the 11.3 Å cross linker, DMS, while not useful in deciding between Model A and Model E, do not contradict either model. One may ask, which new cross-links are available to DMS during amidination at 60°C? Since both LYS 216 and LYS 172 are about 12 Å buried beneath the PM surface (see Figure 65), these lysines will not be able to participate in intermolecular cross-linking to other LYS 216 and LYS 172 residues on an adjoining bR molecule. However, at 60°C, the hydrogen bonds that support the α-helix may be weakened due to formation of new hydrogen bonds to water that can now penetrate into the helices (130). An increase in the random coil nature of these helices may totally expose LYS 30, LYS 129 and LYS 159 to the aqueous phase. Thus the reaction radius of these lysines will become 12 Å with the 11.3 Å cross-linker. If LYS 216 and LYS 172 are also moved closer to the surface as a result of weakening of the α-helix, their reaction radius might become 6 Å at the surface of PM. Using these new reaction radii both Model A and Model E are capable of a high extent of intermolecular cross-linking, which was observed by DMS cross-linking at 60°C (see Results I.D.2.).

Two other groups of investigators have used chemical cross-linking as a means to study the quaternary structure of bR. DMS was one of the reagents used by Dellweg and Sumper (131) to cross-link bR. In their hands DMS formed 33% Monomers, 9% Dimers, 26% Trimers, 16% Tetramers and 16% higher polymers. As was shown in Appendix 8 in this thesis, 10 mM DMS formed on the first amidination 37% Monomers, 18% Dimers, 15% Trimers, 15% Tetramers and 15% Pentamers. Thus these two studies yielded about the same results for the 11.3 Å cross-linker, except that trimers dominated dimers in their study. While DMS is not as useful as DMA in distinguishing between models due to the larger reaction radii.
involved, these results are consistent with either Model A or Model E.

Another group of investigators, Sigrist and Zahler (116), have used a heterobifunctional reagent, azidophenylisothiocyanate, which was able to cross-link specifically LYS 216 with another non-specified group. When the -N$_3$ group is activated by light it can react with many amino acid side chains; i.e., the nitrene functional end is non-specific (132). These investigators were able to form dimers with a 100-fold molar excess of this reagent and trimers and higher polymers with a 1000-fold molar excess of this reagent. Since it is known that LYS 216 is on helix G, the most likely dimer in Model A would be between bR molecules in two different trimer arrangements. If Model E were the correct model, then trimers, not dimers, would be easily formed with this reagent at 100-fold molar excess. Since dimers were formed and not trimers, the work of Sigrist and Zahler (116) also supports Model A over Model E.

I. B. 2. Implications of Monofunctional Imidoester Modification

Another way to analyze these models is to consider the case of MA, the monofunctional imidate. 20 mM MA was seen to form about 10% dimers and trimers (see Results I.D.1.). If this imidoester cross-links by the simplest scheme in Appendix 2.A., the length of the cross-link will be about 2.3 Å. Thus cross-linking with such a short link would require one of the lysines to be totally exposed (LYS 40) to the aqueous phase, and the other lysine to be located on an adjacent helix on an adjacent bR molecule within the natural trimer arrangement of PM. Only Model A fits these criteria. Thus MA modification of bR also favors Model A.
Additional information concerning the \( ^{4}\text{O} \) structure of bR in the PM is gained by considering MA modification of bR in bleached PM. Results show that the trimer is the only polymer of bR in significant amounts after 20 mM MA treatment of bleached PM (see Results I.D.3.). This suggests that the bR molecules in a trimer move closer upon bleaching, so trimers dominate dimers in the gels.

Other investigators have studied the structure of the PM in the absence of retinal. Becher and Cassim showed that when PM is bleached with hydroxylamine in strong light, all three bR molecules in a trimer bleached simultaneously (10). The crystallinity of "bleached" PM is decreased, with short-range order only extending over several unit cells (R. Henderson, private communication to P. Bauer (133)). In addition, Dellweg and Sumper (130) used a slightly different technique from MA modification of bleached membrane to achieve essentially the same result as herein. These authors modified brown membrane, which is PM before retinal is biosynthetically added, with the monofunctional reagent, propionimidate. They found that propionimidate at pH 8.0 was only able to form monomers and trimers of bR. Thus retinal's structural role may be to separate the bR molecules in a trimer, causing the formation of the crystalline array. These results of MA modification of bleached PM focus attention on the bR trimer as the primary structural and, perhaps, functional unit of PM, rather than a single bR molecule.
II. Functional Significance of Chemical Modifications

A. Model for Mechanism of Conformational Changes in bR

1. Inhibition of Photocycling by Bifunctional Imidoesters

Results I.B.1. reveals that either bifunctional imidoester, DMS 11.3 Å, or DMA, 8.3 Å, can inhibit the photocycle, while monofunctional imidoesters do not significantly slow photocycling. Thus cross-linking lysines is required for inhibition of the photocycle. Since chemical cross-linking of lysines will constrain the molecular mobility of bR, this suggests that a conformational change of the protein is needed for photocycling. This result was reported in (134) and has also been seen by other techniques by several investigators (39,111, 135,136).

I will now interpret these results as supporting a proposed mechanism of photocycling in bR which stems from a recent investigation by Katre et al. (69). These investigators find that in light-adapted bR during steady-state photocycling at 25°C with about 1% M$_{412}$ formed, NaBH$_4$ reduction causes retinal to be bound 60% to LYS 216, and 40% to LYS 41. At 0°C under steady state photocycling conditions with about 10% M$_{412}$ formed, retinal is bound 100% to LYS 216. This important study (69) suggests that during photocycling, retinal moves from LYS 41 to LYS 216, although these authors do not commit themselves to this interpretation. The Schiff base reduction in bR is unfortunately complicated by the fact that NaBH$_4$ can only reduce the Schiff base in the light (109), so that the investigation of the retinal binding site under non-photocycling conditions cannot be directly carried out. Their
study suggests that light acts to cause a conformational change in the protein that allows accessibility of NaBH₄ to either Schiff base (on LYS 41 or LYS 216) at 25°C. Additional evidence that retinal is initially bound to LYS 41 is shown by the work of Bridgen and Walker (40) who chemically reduced the Schiff base, and by the work of Sigrist and Zahler (116) who chemically modified LYS 216 with little change of the 568 nm chromophore. Additional evidence that retinal is located at LYS 216 in the light is evidenced by the work of Bayley et al. (119), who chemically reduced retinal in intense light. Since the Schiff base on LYS 41 is located only about 5 Å inward from the surface of bR in my model (see Figure 65), and the nitrogen on LYS 216 could be located about 11 Å below the surface, a conformational change of the protein is needed for these two residues to interact.

The question must be asked, by what reaction could LYS 41 transfer retinal to LYS 216, assuming that these two residues are closely juxtapositioned after the protein conformational change? The answer is that there are two possible reactions. The first is a direct amine substitution reaction, that was initially observed by Reddelien in 1920 (137). This type of "trans-imination reaction" is part of the mechanism of enzymes that use pyridoxal phosphate as a coenzyme. This reaction proceeds as follows:

\[
\begin{align*}
\text{All-trans Retinal} & + \text{LYS 41} \rightarrow \text{1,1-diamino-alkane (unstable intermediate)} \rightarrow \text{LYS 216} \\
\text{LYS 41} & \quad \text{LYS 216}
\end{align*}
\]
It has been found, that as the basicity of the displacing amine increases, the rate of displacement increases in nearly a linear manner (138). Thus if the relative basicity of LYS 41 and LYS 216 change during the photocycle, the equilibrium of this reaction will shift back and forth.

The other possibility which is suggested by a comparison to the photochemical reactions of rhodopsin (139), is hydrolysis of the Schiff base on LYS 216. The reaction is acid catalyzed; Jencks has shown that a proton is needed to remove -OH from the carbinolamine intermediate at neutral and mildly alkaline pH's (140). Formation of the Schiff base is just the hydrolysis reaction in reverse, except that a proton is donated to the OH group on the carbinolamine forming water, which is a better leaving group than OH. All the steps of the reaction are reversible. I have chosen tyrosine as a possible proton donor and acceptor since the double chemical modifications using iodination in this thesis implicate tyrosines in the vicinity of the Schiff base. The hydrolysis reaction proceeds as follows:

\[
\begin{align*}
\text{TYR-O} & + \text{H}_2\text{O} + \text{Retinal} \leftrightarrow \text{LYS 41} \\
\text{TYR-OH} & + \text{Carbinolamine (unstable intermediate)} \\
\text{TYR-OH} & + \text{H}_2\text{N} \leftrightarrow \text{H}_2\text{N} \\
\end{align*}
\]
If migration of retinal involves this mechanism, then the questions must be asked, is this reaction fast enough, and is this reaction energetically feasible? The data that is available on the rates of hydrolysis of Schiff bases in solution, while probably accurate, may not be indicative of the rate of hydrolysis of the Schiff base in the protein due to rate enhancement of many orders of magnitude by catalytic groups on the protein. The t₁/₂'s of hydrolysis of Schiff bases in solution at neutral pH vary according to the basicity of the amine and whether the OH⁻ ion or H₂O attacks, between 0.1 and 100 minutes (141). Thus without rate enhancement by bR, hydrolysis of the Schiff base does not appear to be a reasonable mechanism. Regarding the energetic feasibility of hydrolysis, the energy of activation also varies depending on whether OH⁻ or H₂O attacks the Schiff base in solution. In the case of an OH⁻ attack, Eₐ = 13-17 kcal/mole, and in the case of an attack by H₂O, Eₐ = 8 kcal/mole (141). Since this reaction must proceed in both directions in order to complete the photocycle, the maximum energy requirement would be 17 kcal/mole x 2 = 34 kcal/mole. In addition, the retinal must isomerize twice (30), which requires in retinals 20 kcal/mole x 2 = 40 kcal/mole (142). This adds to 74 kcal/mole. Since a photon absorbed by bR is only about 50 kcal/mole, the bR protein would have to significantly lower the energy barrier of activation of this process in order for hydrolysis to be possible. Since bR's role is similar to enzymatic catalysis, both the rate and Eₐ may be changed considerably by the protein, thus causing hydrolysis of the Schiff base. Comparable statistics for the amine substitution reaction in solution are not available. However, considering that loss of water from the unstable intermediate, which is the slow step in hydrolysis of the Schiff base, does not need to occur in the amine substitution reaction,
I favor this interpretation of the migration reaction. A small movement of helix B towards helix G would place these two residues in a proper position to react by an amine substitution reaction at the Shiff base.

An interesting question is, to what extent does intra- versus intermolecular cross-linking contribute to inhibition of the photocycle? The temperature experiment (see Results I.B.2., I.D.2.) shows that there is no correlation between a great extent of higher oligomers of bR formed by DMS at 60°C and inhibition of photocycling kinetics, suggesting that intramolecular cross-linking may be the primary cause of inhibition. However, Results I.D.1. shows the production of pentamers by DMA, and higher oligomers by DMS, which allows the possibility that some degree of intermolecular cross-linking in addition to intramolecular cross-linking may contribute to inhibition of the photocycle. If intermolecular cross-linking plays a role in inhibition of photocycling kinetics this would indicate that communication via conformational changes of three bR molecules in a trimer or in a higher polymer, is important for photocycling. Indeed, Hess et al. (136) have shown that the rate of the decay of $M_{412}$ depends on whether the nearest neighbor to bR is in the M state or the bR state; the rate of decay of $M_{412}$ was faster due to cooperativity of photocycling bR molecules within a trimer. An additional experiment that could be carried out to compare the contribution of intra- and intermolecular cross-linking to photocycling inhibition would be to chemically modify with DMA, then isolate electrophoretically the monomer band seen by SDS gel electrophoresis, and then use protein chemistry techniques (described below) to study the level of intramolecular cross-linking that had occurred. Given Model A
(see Discussion I.B.1.), the following intramolecular cross-links could be formed: LYS 40-lys 30, LYS 40-lys 216, LYS 172-lys 216, and perhaps LYS 40-lys 159 (see diagram below).

Possible Intramolecular Cross-Links by DMA
Model A(126)

Since proteolytic or cyanogen bromide cleavage does not readily occur in the presence of SDS detergent (143), SDS must be removed by extensive dialysis before cleavage is attempted. Initial cleavage could be carried out with chymotrypsin, which cleaves between helices B and C (144). The product could be analyzed electrophoretically. This cleavage would reveal if intramolecular cross-linking had occurred between LYS 40-lys 216 or LYS 40-lys 159. In order to investigate more closely all the cross-links more extensive cleavage would have to be carried out with CNBr as in (24). The fragments would be separated by Sephadex LH-60 chromatography and further derivatized with fluorescamine and separated by SDS polyacrylamide gel electrophoresis. Amino acid analysis could be used to identify the peptides. This procedure would reveal changes in the molecular weights of the CNBr peptides indicating that intramolecular cross-linking had occurred. Thus inhibition of the photocycle could be correlated with the extent of intramolecular cross-linking as well as intermolecular cross-linking.
II. A. 2. Availability of Lysines to EA in Bleached bR

The experiments of imidoester modification of bleached bR in this thesis also relate to the conformational changes that occur during photocycling. Results I.A.3. shows that the same number of lysines are modified by EA in bleached bR as in unbleached bR. The fact that no new lysines are made available to EA after treatment with hydroxylamine in the light, suggests that retinaloxime remains noncovalently bound at its original binding site, blocking accessibility of the Schiff base lysine to imidoesters. Hydroxylamine has been shown to react with Schiff bases as readily as it reacts with carbonyl compounds (145) to form the oxime, which in this case is retinaloxime. It is possible that hydroxylamine reacts with the Schiff base on LYS 41 after a light-induced conformational change has occurred that allows accessibility of hydroxylamine to LYS 41. The conformational change could occur during the K+L transition, which is supported by the work of Beece et al. (146) who have shown an effect of solvent viscosity on the K→L transition (κ = 0.25), but none on the L→M transition. Thus if the newly formed retinaloxime remains in the binding site of LYS 41, it will protect LYS 41 from reaction with EA. In addition, Beece et al. (146) have found much larger solvent viscosity effects on the steps M→O, O→bR, indicating a requirement for a conformational change of the protein in these steps as well. This model requires the order of molecular events to be as follows:

1) light absorption by bR,
2) conformational change of bR, K→L,
3) deprotonation, and simultaneous migration and isomerization of retinal from LYS 41 to LYS 216, L→M,
4) isomerization and migration of retinal from LYS 216 to LYS 41, and simultaneous reprotonation and conformational change of bR, \( M\rightarrow O \rightarrow bR \), or \( M \rightarrow bR \).

This model will be further extended and discussed throughout the Discussion.

II. A. 3. Inhibition of Regeneration by EA Modified Bleached bR

The regeneration experiments in this thesis of bleached bR can also be interpreted in terms of a photocycle that involves movement of retinal between LYS 41 and LYS 216. The question may be asked, what happens to retinal oxime during regeneration of the bleached chromophore with retinal? As shown in Results I.E.3., regeneration of the EA modified bleached sample is only about 3/4 complete in one hour, and less complete than regeneration in control bR, suggesting steric hindrance during the regeneration process. In addition, resonance Raman spectroscopy (see Results I.G.2.), shows a distortion of the chromophore in both regenerated samples, probably due to the presence of unremoved retinal oxime. Some clues to the answer to this question are obtained from a comparison to the work of Schreckenbach et al. (147). These investigators carried out a binding study of different retinal isomers to the oxime-free apomembrane. They determined that regeneration of the apomembrane with retinal occurs in 3 steps:

Retinal and Bacterio-opsin

<table>
<thead>
<tr>
<th>Step 1)</th>
<th>400 nm Chromophore</th>
<th>Step 2)</th>
<th>430/460 nm Chromophore</th>
<th>Step 3)</th>
<th>Purple Complex</th>
</tr>
</thead>
<tbody>
<tr>
<td>(seen in 5 secs)</td>
<td>(seen in 30 secs)</td>
<td>(seen in 2 mins at 0°C)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

at 0°C |

They described formation of the 400 nm chromophore as ring-chain planarization of the added retinal, but could not offer a molecular explanation
of the next step. My explanations for the molecular events of regeneration are as follows:

Retinal and Bacterio-opsin

Step 1) Retinal noncovalently bound to LYS 216
Step 2) Retinal covalently bound to LYS 41
Step 3) Retinal covalently bound to LYS 41 and protonated

I arrived at this conclusion from the work of Katre et al. (69) who interpreted their results as indicating the "M" state is retinal bound to LYS 216, from the work of Braiman and Mathies (30) who have shown that M is the 13-cis isomer of retinal, and also from the work of Oesterhelt and Schuhmann (148) who have shown that regeneration with 13-cis retinal occurs at a faster rate than regeneration with all-trans retinal. These investigations suggest that the added 13-cis isomer binds preferentially to LYS 216 (Step 1, above). The hydrophobic nature of the amino acids on helix G between LYS 216 and the cytoplasmic membrane surface might attract retinal to bind noncovalently more than the protonated LYS 40 attracts retinal in the aqueous phase next to LYS 41 (see Figure 65). In Step 2, retinal could move to the binding site of LYS 41. Isomerization should not occur in the absence of light in this step, since the reconstituted 13-cis chromophore was shown to have an absorbance at 548 nm, not 568 nm (81). Formation of the Schiff base linkage, catalyzed by the binding site of LYS 41, would then occur. In Step 3, protonation of the Schiff base would yield the purple complex. If retinaloxime were still present in the binding site of LYS 41, added 13-cis retinal would have to displace retinaloxime on LYS 41. The displaced retinaloxime could be retained at the hydrophobic LYS 216 binding site after regeneration. Indeed, it has been shown (10) that upon regeneration, the 366 nm absorbance band is lost as this peak
shifts to 362 nm. These results suggest that the binding site of LYS 41 is able to induce optical activity of retinal, while the binding site of LYS 216 is not. In the extensively EA modified sample, regeneration would have to bypass Step 1 in 57% of the bR molecules and proceed directly to Step 2. This might slow the regeneration process, as is observed in this sample.

II. A. 4. Effect on the Photocycle by Monofunctional Imidoester Modification of LYS 216

Given the proposed mechanism of light-induced movement of retinal between LYS 41 and LYS 216, the monofunctional imidoester modification of bR should be reexamined. The question must be asked, how can photocycling involve a shift of retinal from LYS 41 to LYS 216 if extensive monofunctional imidoester modification, which modifies 57% of LYS 216 (see Results I.A.1.), does not significantly affect the rate of $M_{12}$ decay? Since the amidine product of imidoester modification of LYS 216 will not be able to form a Schiff base with retinal, this apparent discrepancy needs an explanation. One possible answer to this question involves the following proposed mechanism for light-induced photocycling:

KEY

- Photoreaction
- Thermal Decay
- Isomerization and Migration, Conformational Change and Reprotonation
- Very Fast Migration and Isomerization
Ordinarily, light will cause retinal to be deprotonated with a formation time as is observed for the rise of M, 70 usecs (33). What may be formed first, however, is $M'$. The absorption spectrum of $M'$ has been produced by illuminating M with light of 420 nm (68). This intermediate is thought to be the deprotonated form of all-trans retinal (63) on LYS 41 (my interpretation). In the next step, isomerization of retinal and migration to LYS 216 will occur very quickly, perhaps in the nanosecond range. Since this is a fast step, it is not observed by absorption spectroscopy on the time scale of M formation at room temperature. M will thermally decay back to bR as is observed spectroscopically with a biphasic decay. However, if LYS 216 is modified by EA, $M'$ will not proceed to form M, but simply decay back to bR with the same rate as M decay. The evidence in this thesis that helped to formulate this mechanism is the resonance Raman spectrum of 20 mM EA modified bR (see Results I.G.1.). In the 20 mM EA Raman spectrum there is a marked increase in the 1174 cm$^{-1}$ band, which is thought to represent the $M'$ state (63). Thus the spectrum has more $M'$ due to EA blocking some of the LYS 216 residues. If this analysis is correct, then both the rise and decay rates observed at 412 nm would not be greatly affected by EA modification of LYS 216, as is observed. The photointermediate "0" has been left out of this scheme to emphasize the events of the first part of the photocycle. It is possible that one phase of the biphasic decay of M represents the decay of 0; but since the absorption spectrum of 0 is still controversial this intermediate was excluded.
II. A. 5. Light-to-Dark Adaptation

The light-to-dark adaptation kinetics of BD modified bR should also be discussed in terms of the conformational changes of the photocycle. As shown by other investigations (39,81,149) light-to-dark adaptation involves an isomerization of one-half of the retinal chromophores from the all-trans form to the 13-cis form. Dark-adapted bR has been shown to undergo a different photocycle from light-adapted bR, that does not pump protons (81). Since the kinetics of dark-adaptation are completely unchanged by BD modification while the photocycle is greatly slowed, it suggests that the conformational changes required of the protein are not related to those involved in photocycling. Thus the proposed migration of retinal from LYS 41 to LYS 216 may not occur in dark-adaptation. More likely is that there is a slow isomerization of all-trans to 13-cis retinal while retinal remains on LYS 41. A conformational change of the retinal alone would be less affected by a change in the conformation of the protein. This proposed mechanism for dark-adaptation could be tested by modifying LYS 216 with the hydrophobic reagent, azidophenylisothiocyanate (116), and then measuring the rate of light-to-dark adaptation. If LYS 216 is not involved in light-to-dark adaptation, then isomerization must occur while retinal is still on LYS 41.

II. A. 6. Role of Arginines in Ionic Interactions During Photocycling

As was shown in Results II.B.1., the inhibition of $M_{412}$ decay by increasing BD modification could be fit to a quadratic function, $100 + a_2x^2$. This quantitative analysis of the inhibition
indicates, as was stated in Results II.B.1., that modification of a single specific pair of arginines acts to inhibit photocycling by a cooperative mechanism. Given this analysis, the question must be asked, are the two arginines on the same or different bR molecules? If the arginines were on different bR molecules, they could still act cooperatively through protein conformational changes. However, it appears less likely that two arginines widely separated on two bR molecules would act cooperatively, than two arginines on the same bR molecule. Indeed, one would expect a cooperative mechanism of inhibition by arginines to result from two closely juxtapositioned arginines. In Figure 65, it is shown that ARG 225 and ARG 227 are separated by only one amino acid residue. As was described in Discussion I.A.5., these arginines are thought to form ionic linkages to ASP 38 and ASP 36, respectively, thus positioning them directly above helices B and G that contain retinal. Thus ARG 225 and ARG 227 are located such that their ionic character could influence movements of retinal.

The idea that ionic interactions between amino acid residues are important for photocycling activity is suggested by a similar importance of ionic forces in the mechanism of oxygen binding in hemoglobin. Determination of the structure of the hemoglobin molecule in various configurations at 2.8 Å resolution (150,151) has revealed that salt bridges at the surface and between the 4 polypeptides that compose the tetramer are essential for the cooperative interactions that allow the release of oxygen. It has been shown that removal of ARG 141 α, which is on the carboxy terminus of the α chains in hemoglobin, causes the Hill constant of oxygen binding to fall from 2.8 to 1.9 (152). ARG 141 α is thought to be involved in two salt bridges; one between the α-carboxyl of ARG 141 α and both the α-amino group of VAL 1 α and
and the ε-amino group of LYS 127 α on the opposite α chain, and the second between the guanidinium group of ARG 141 α and the carboxyl group of ASP 126 α on the opposite α chain. Further removal of HIS 146 β, which forms an important salt bridge to the carboxyl group of ASP 94 β on the same β chain, further reduces the Hill constant to 1.0 (152). Thus cooperative binding effects of oxygen in hemoglobin are totally dependent on these salt bridges.

It is, of course, entirely possible that salt bridges at the surface and interior of the bR molecule have nothing to do with the mechanism of photocycling and proton translocation. However, another example of this type of charge interaction is seen in the glycolytic enzymes. It has been shown that thirteen out of fourteen glycolytic enzymes contain arginines at their active site (153). Their role in this case is to bind the negatively charged phosphate groups of the glycolytic substrates. In addition, ARG 145 in carboxypeptidase has been shown to be essential in binding the terminal carboxyl group of the substrate (154). The only molecules that bR binds from the aqueous phase are protons and water, so the negatively charged groups must be ones that are already on the protein.

In the case of hemoglobin, the changes in quaternary structure on ligand binding involve large rotations combined with small translations of the subunits relative to each other (155). The salt-links on the C-terminal residues are completely destabilized in the oxy form since these residues are able to rotate freely and do not form new salt links. However, the total number of atoms in contact and the total number of hydrogen bonds remain fairly stable in converting from the deoxy to oxy forms (155), indicating that most of the contacts and hydrogen bonds are replaced by new ones.
In the case of bR, the molecular motion involved during photocycling should be smaller than that in hemoglobin upon oxygenation, since bR is constrained within the lipid matrix of the crystalline lattice of PM, and rotation of the surface groups may be restricted. Thus, instead of breaking salt links and rotating the groups so that no new links form as in the C-terminal links of hemoglobin, the surface groups in bR may be forced to substitute new links for initial salt links that may be broken during the conformational changes of bR. The initial salt links are thought to be ARG 227-ASP 36 and ARG 225-ASP 38 (see Figure 66). During photocycling, formation of new salt links may act to stabilize the photointermediate states and order the sequence of conformational changes. One possibility that is obvious from the 2° structure shown in Figure 65 is the formation of the new salt links, ARG 225-ASP 102, and ARG 227-ASP 104, since these two aspartates are separated by one amino acid residue as are ASP 36 and ASP 38. These changes are summarized in the diagram below:

\[
\begin{align*}
&\text{ASP 102} \\
&\quad (\text{new link})/ \\
&1) \quad \text{ARG 225} \quad \text{ASP 36} \\
&\quad (\text{new link})/ \\
&2) \quad \text{ARG 227} \quad \text{ASP 36}
\end{align*}
\]

This mechanism requires a closer approach of helix G to helix C during photocycling. The first negatively charged amino acid residue encountered by ARG 225 will be ASP 102. Once this ionic link is formed, ARG 227 may "flip" over to ionically link to ASP 104. If, in addition, retinal is shunted from LYS 41 to LYS 216 during photocycling, then helices B and G must also be close. Thus a close cluster of helices B, C and G is required. Model A (126) is the only model of the five models presented in Discussion I.B.1. that fits these criteria within one bR molecule.
One may ask, why must both arginines become modified before significant slowdown of the photocycle is observed? The answer may be related to the proximity of these residues to the helices that contain retinal during photocycling. If the initial salt links both act to stabilize the bR chromophore then rupture of only one of these links may not be sufficient to destabilize the function of the chromophore, i.e., migration of retinal between LYS 41 and LYS 216. It is possible that reformation of either one, or both of the initial ionic links is a lower energy configuration that is the driving force for M decay. In high salt, negative chloride ions may partially compete with the carboxyl groups which would act to slow photocycling. Indeed, it is shown in Appendix 10 that M decay is about 2 times slower in 4 M NaCl compared to 10 mM phosphate buffer.

The pH dependence of photocycling kinetics before and after BD modification can also be discussed in terms of two important salt links that involve arginine (see Results II.B.2.). In control bR the first phase of the decay is essentially unaffected by pH over the entire pH range studied. This is reasonable since the $t_{1/2}$ of this phase is on the order of 2 msecs and since it is thought that proton diffusion to the Schiff base has a $t_{1/2}$ of about 20 msecs (156). The second, slower phase is in the time range where it will be affected by proton diffusion to the Schiff base, and thus is expected to respond to the aqueous pH, as is observed. Slowdown of the second phase in control bR starts to occur at pH 11. It is possible that deprotonation of arginines in salt links starts to occur at this pH, which would contribute to this pH dependence. As the salt links are deprotonated, a delocalized conformational change may occur as caused by BD modification (see Discussion II.A.8.b.), which will result in the slowdown of
photocycling kinetics. The main role of the ionic links formed by arginines with carboxyl groups may be to maintain the protein structure, since deterioration of the chromophore structure also occurs at high pH's (see Appendix 4). The pH dependence of photocycling will be further discussed in Discussion II.B.2.a.

Another interpretation of the quadratic behavior of the inhibition should be discussed. Critics of my interpretation that two arginines inhibit cooperatively have suggested that modification of arginines alters the overall surface charge such that the inhibition is enhanced. First, the change in the overall surface charge in the dark that is produced by increasing BD modification was not quantitated. However, the discussion presented above can be thought about in terms of surface charge, since BD modification in the presence of borate buffer adds a negative charge onto each arginine and since all the modified arginines are thought to be at the surface. Thus it is possible that if negative charge in the dark produced by increasing concentrations of BD were graphed vs. $M_{412}^-$ decay, the inhibition would show a quadratic dependence. This criticism, then, may be essentially one of semantics; i.e., instead of saying arginines, one might say negative charge per arginine. Second, as for the light-induced negative surface charge, it was shown in Results II.B.2., that the light-induced negative surface charge/$[M_{412}]$ remains nearly constant after BD modification. These results indicate that when $M_{412}$ is present in higher concentrations due to the slowdown of $M_{412}^-$ decay, the light-induced negative charge also increases the same amount. The $M_{412}$ state is characterized by a negative surface charge due to proton release from the PM (see Discussion II.B.2.c.) that increases after BD modification simply due to the increased concentration of $M_{412}$.
If light-induced negative surface charge were graphed vs. $M_{412}$ decay, there would be almost no change.

Another possible interpretation of the quadratic behavior of the inhibition should be discussed. In a recent study (157), Ohno et al. found that at low light intensities, the concentration of the slow phase of $M$ decay depends in a quadratic manner on the light intensity. At higher light intensities, they found that the concentration of the slow phase increases if 2 or 3 neighboring bR molecules are excited. In a similar manner in the BD modification of bR, increasing concentrations of BD could cause 2, or 3 bR molecules to decay in an associated state due to ionic interactions, thus slowing the decay. However, if this were true, then the inhibition should fit the function $3x^3 - 2x^3$ or $x^3$, since all 3 of the bR molecules in a trimer are thought to be identical. Since these functions did not fit the data, this model of cooperativity is not a satisfactory explanation for the cooperative behavior of two arginines. Additional experiments to monitor $M_{412}$ decay as a function of light intensity in the BD modified samples would help to elucidate this explanation.

II. A. 7. Role of Carboxyl Groups and Lysines in Ionic Interactions During Photocycling

Although the role of carboxyl groups ionically bound via initial and transitory salt links to arginines was already discussed, two double modification experiments, BD/EDC and EA/EDC further explored the role of carboxyl groups by specific chemical modification of carboxyl residues in combination with modification of arginine or lysine residues. The significance of these experiments is to lend support to the postulated ARG-GLU ionic linkages discussed above.
The BD/EDC experiment provides evidence for the existence of at least three arginine-carboxyl salt links, since breaking these links by previous BD modification allows EDC to cross-link three carboxyls to three additional nearby lysines (see Results III.C.1.). As discussed, the three carboxyl-arginine salt links also in the vicinity of a lysine are thought to be ARG 225-ASP 38 near LYS 40, ARG 227-ASP 36 near LYS 30, and ARG 134-GLU 194 near LYS 129. It is reasonable to assume, although it is not proven by the BD/EDC experiment, that the two arginines not mentioned so far, ARG 7 and ARG 166, also form salt linkages that prevent modification of GLU 9 and GLU 166 by EDC in the absence of prior BD modification. Since both of these linkages contain an intervening proline residue, the possibility of forming these salt linkages was tested using Framework Molecular Models. As shown in Figure 72, these ionic linkages are quite feasible. Since ARG 7-GLU 9 and ARG 164-GLU 166 are not also near a lysine, however, evidence for these links is not obtained by the fluorescamine test used in this thesis. An additional experiment to support the postulated five ionic links between arginines and carboxyls in Figure 65 is to carry out amino acid analysis of the modified carboxyls as was described in Materials and Methods IV.B., following previous BD modification.

As discussed above, the salt links important for photocycling are thought to be as follows:

$$\begin{align*}
\text{ASP 102} \\
\text{(new link)/} \\
1) \quad \text{ARG 225} \longrightarrow \text{ASP 38} \\
\text{ASP 104} \\
\text{(new link)/} \\
2) \quad \text{ARG 227} \longrightarrow \text{ASP 36}
\end{align*}$$
Figure 72
Framework Molecular Model
of an ARG-PRO-GLU Salt Link
BD modification alone will destabilize the initial links, ARG 225-ASP 38 and ARG 227-ASP 36, causing the return of the photocycle to be markedly slowed (see Results II.B.1.). Modification of ASP 102 will destabilize the transitory salt links as well as the initial salt links. As shown in Results III.B.2., the M decay kinetics were only slightly slower after BD/EDC modification than after BD modification alone. This implicates the initial salt links as far more important in controlling the rate of photocycling than the postulated transitory salt links. This follows from the fact that the rate-determining steps of photocycling are between M and bR; thus it is reasonable that destabilization of the bR state, not the M_{412} state, will slow this pathway.

The EA/EDC double modification can also be interpreted in terms of these arginine-carboxyl salt links. As shown in Results III.B.1., EDC alone causes one cross-link to form between one lysine and a carboxyl (thought to be LYS 159-GLU 161 from proximity constraints). Inhibition of photocycling by EDC is about 1/3 that of extensive BD modification. If LYS 159 and GLU 161 are chemically cross-linked together, LYS 159 will be unable to separate from GLU 161 and may thus partially inhibit the conformational changes occurring in helices B, C and G. As shown in Results III.B.1. (Table 33), premodification with increasing concentrations of EA reverses the slowdown of the photocycle caused by EDC. The nearly complete reversal of the photocycle slowdown is accompanied by inhibition of the one and only cross-link formed between a carboxyl group and lysine, which is evidenced by competition for the single lysine modified by EDC. The reason for incomplete reversal of photocycling slowdown by EA may be due to incomplete modification of LYS 159 by EA (see Results I.A.1.). Thus both the BD/EDC
and EA/EDC double modifications support the postulated initial and
transitory salt linkages involving ARG 225 and ARG 227, and show that
the initial linkages control photocycling kinetics to a greater
extent than the postulated transitory linkages.

II. A. 8. Conformational Changes Caused by the Modifications
a. Imidoester Modification

Given this proposed mechanism for photocycling and accompanying conformational changes, it is essential to know if the modification itself has produced a conformational change. The spectroscopic techniques in this thesis that attempt to answer this question are absorption, tryptophan, resonance Raman, retinal fluorescence and circular dichroism. As shown in Results I.E.I., the 568 nm chromophore is essentially unchanged by monofunctional imidoester modification. This is expected even if these imidoesters can partially modify LYS 216, since the positive charge on LYS 216 is replaced by the positive charge of the imidoester, and therefore no shift of $\lambda_{\text{MAX}}$ is expected due to change of charge near the Schiff base (58,59,60,158) resulting from the modification. The bifunctional imidoesters also do not appreciably shift or decrease the 568 nm chromophore at low concentrations. Therefore, the differences in photocycling activity following bifunctional or monofunctional imidoester modification cannot be due to a dramatic effect of the bifunctional modifications on the chromophore structure. At higher concentrations, DMS but not DMA, does cause a marked decrease in the 568 nm chromophore and hence marked deterioration of protein structure. The inhibition of photocycling kinetics above this concentration of DMS is not considered to be significant for the mechanism of photocycling, since a nearly intact protein structure is
assumed to be a requirement.

pH titration of the 568 nm chromophore of bR with 1 lysine modified by DMS does reveal the interesting result that the "acid" form of bR is formed to a greater extent in this sample than in control bR. Since this form of bR occurs at low pH only, its formation is thought to be controlled by carboxyl groups on the protein. The $\lambda_{\text{MAX}}$ of the acid form is near 635 nm (159) and is thus thought to represent stabilization of the "0" photocycle intermediate that absorbs in the same region (54). The lysine that is thought to be most accessible to the imidoesters is LYS 40 (see Discussion I.A.1.). In the discussion of BD modification it is postulated that during photocycling two new salt links, ARG 225-ASP 102 and ARG 227-ASP 104 are formed. If these new links act to stabilize the M photocycle intermediate, then at low pH they may also control formation of the O photocycle intermediate that follows M in the photocycle. After DMS modification of LYS 40, the new link ARG 225-ASP 102 could be destabilized due to the steric hindrance of the hydrolyzed DMS reagent on LYS 40 (see diagram below):

At neutral and low pH, the imidoester hydrolysis product is primarily the ester form shown, but the hydrolyzed acid form is probably also present to some extent. Either form could cause the destabilization of the ASP 102-ARG 225 salt linkage, which would raise the pK of ASP 102,
thus enhancing its protonation.

As shown in Results I.F., only a small decrease in the quantum yield of tryptophan fluorescence and red shift of the fluorescence maxima were seen in bR modified by DMA, EA or DMS at low concentrations. The origin of the UV fluorescence is still controversial. The fact that only one broad band was seen in the fluorescence emission spectrum with no dependence of $\lambda_{\text{MAX}}$ on the wavelength of excitation, usually indicates that all of the tyrosines transferred their energy to the tryptophans (160). In a recent investigation (161), it was shown that all of the fluorescence in the UV region is due to only one or two tryptophans. The rest of the energy absorbed by tyrosine and tryptophan is transferred completely to retinal. These workers (161) postulate that tyrosines far from the chromophore transfer energy first to tryptophans closer to the chromophore, and then to retinal. In another study, however (162), the mechanism of fluorescence quenching of the UV fluorescence was interpreted as more complicated than just a Förster-type energy transfer process. Quenching may also involve dielectric, orientation-dependent and charge perturbation aspects. Suffice it to say that the UV fluorescence is very quenched even without chemical modification, compared to the fluorescence of 11 tyrosines and 8 tryptophans in solution, by mechanisms that are not completely understood. Since there were no marked differences between the extent of fluorescence quenching and the red shift caused by bifunctional and monofunctional imidoesters, differential inhibition of the photocycle cannot result from a different structural perturbation of the protein.

Resonance Raman spectroscopy (Results I.G.1.) shows a slightly more marked distortion of the C=C ethylenic stretches in the chromophore environment in a DMA modified sample than in an EA modified sample,
each with \( \sim 0.65 \) fraction of total lysines modified. Thus it is possible that the cause of photocycling inhibition by the bifunctional reagents is due to an initial distortion near the chromophore that is, however, not evidenced by absorption spectroscopy. The migration of retinal during the photocycle may be sterically hindered after bifunctional amidination.

II. A. 8. b. BD Modification

As shown in Results II.E.1., the 568 nm chromophore is only slightly decreased at all concentrations of BD, and no shift in \( \lambda_{\text{MAX}} \) is seen. Thus large changes in photocycling activity do not appear to be due to a dramatic change in the ground state bR_{568} chromophore structure.

Tryptophan fluorescence (Results II.E.2.) reveals a red shift in excitation and emission spectra with the red shift in the excitation \( \lambda_{\text{MAX}} \) more marked than in the case of imidoester modification. As mentioned in Results II.E.2., a red shift in tryptophan fluorescence may be due to a change of the 1) polarizability, 2) polarity and/or 3) mobility of the local environment. To understand polarizability changes, it is necessary to understand the physics of light excitation. The Franck-Condon principle states that upon excitation of a molecule the electron is raised to a new electronic level in much less time \( (10^{-15} \text{ sec}) \) than it takes the whole molecule to rearrange itself \( (10^{-10} \text{ to } 10^{-9} \text{ sec}) \) (163). During the \( 10^{-9} \text{ sec} \) that a singlet state remains excited, all kinds of processes can occur, including protonation or deprotonation reactions, solvent-cage relaxation, local conformational changes and any processes coupled to translational or rotational motion (164). Since the dipole moment in the Franck-Condon state is
different from the axis of the dipole moment in the ground state, the solvent molecules of the fluorescing tryptophans could be either the aqueous phase at the surface of the bR molecule, or the amino acid side chains surrounding tryptophans in the protein.

In the case of polarity, a fast change in the conformation of the protein is not needed, just a change in the dipole moment of the excited state of tryptophan. Such a charge effect on buried tryptophans could result indirectly from arginine modification near the aqueous phase, by causing a permanent change in the conformation of the protein, thus positioning other charged groups in the vicinity of tryptophans, or directly, by positioning the negative charge of the BD-borate adduct close to a tryptophan. As shown in Figure 65, it is possible that ARG 7 and ARG 134 could influence the polarity of the tryptophans near them directly. The one or two tryptophans in the environment of retinal (165) are probably not affected by a change in dipole moment, since the polarity of the retinal environment did not change enough to cause a shift in the $\lambda_{\text{MAX}}$ of the chromophore.

As regards increased mobility of the protein, local conformational changes of the protein might occur more easily in the BD modified bR samples, especially if the role of arginine is to stabilize the protein by salt bridge interactions with carboxyl groups. If these bridges are weakened, the protein may be more free to move.

Another explanation for a red shift is that selective quenching of 1 or 2 tryptophans emphasizes the contribution of others (57,58). It is possible that arginine modification in the aqueous phase permanently alters the bR conformation so that internal tryptophans in the hydrophobic environment are indirectly quenched. If some denaturation of the
protein has occurred, as suggested by the changes in the circular dichroism spectrum, then the aqueous phase could penetrate to some of the internal tryptophans, causing their fluorescence to be quenched. Or perhaps, an altered conformation of the protein causes more energy to be transferred from tryptophans to retinal.

Resonance Raman spectroscopy does not reveal marked changes in the chromophore structure after BD modification; the changes in intensity seen are primarily due to an increased amount of M. Some of the peak height changes, however, that are inconsistent with increased M, may be due to a slightly distorted chromophore structure, especially in the deprotonated Schiff base form.

Additional information about the retinal chromophore environment is seen from the retinal fluorescence. It will be recalled (see Results II.E.4.) that investigations in this area have shown a fluorescence with a $\lambda_{\text{MAX}}$ at 714 nm produced by low light intensities and a fluorescence with a $\lambda_{\text{MAX}}$ at 735 nm produced by high light intensities at room temperature (83). The difference in $\lambda_{\text{MAX}}$ between these two fluorescent environments indicates that the fluorescence produced by low intensity light is in a more hydrogen-bonded environment than is the fluorescence produced by high intensity light (92). After BD modification, the $\lambda_{\text{MAX}}$ of only the 735 nm fluorescence blue shifts 14 nm, indicating a more H-bonded environment, but not as H-bonded as the 714 nm fluorescence. In terms of the postulated photocycle described above (Discussion II.A.4.), the two different fluorescent species may reflect two environments of retinal. The first environment, seen by fluorescence produced by low intensity light, could be that of LYS 41; this fluorescence is produced simultaneously with the formation of photoproduct K (85–90). At high
steady state light intensities, the fluorescence may be produced by an excited state of a photointermediate, in addition to bR\textsubscript{568}. A possible source for the high-intensity fluorescence could be either the photointermediate 0, which has a λ\textsubscript{MAX} close to that of bR\textsubscript{568} (33), or the photointermediate P (83). It is possible that this fluorescence reflects the environment of LYS 216 before retinal moves back to LYS 41. This interpretation can be summarized with a modification of the previous diagram (Discussion II.A.4.):

As the fluorescence is produced by high intensity light, 0 (or P), could photoconvert to M or bR. If BD modification only blue shifts the photointermediate fluorescence, this could indicate that only the environment of LYS 216 is more H-bonded after BD modification. This could arise, for example, due to movement of ASP 212 closer to LYS 216. Thus the H-bonding character of LYS 216 may be important for the conformational changes during photocycling.
Additional information about the protein structure was obtained by the use of circular dichroism. As shown in Results II.E.5., the most marked change in the CD spectrum of BD modified bR is at 260 nm. As explained in (10), large changes in the near UV CD spectrum could represent 1) a change in the secondary and/or tertiary structure of the bR, resulting in changes in the environment of the \( \pi-\pi^* \) transitions of the aromatic amino acids, 2) a loss of possible dipole coupling between the \( \pi-\pi^* \) transitions of the retinal and the aromatic amino acids or 3) a change in contributions from \( \pi-\pi^* \) transitions of the chromophore in the near ultraviolet region. The first explanation is thought to be more important by comparing to rhodopsin and to light-dark adaptation in bR (100). In addition, the intensity of the near UV CD bands is comparable to proteins without prosthetic groups. Becher and Cassim (10) have called a large change in this region due to a delocalized conformational change, extending over the helices containing the aromatic residues. According to the CD spectrum the chromophore is included in the delocalized change since the 317 nm band indicates a greater freedom of the chromophore. In addition, the chromophore-chromophore distance or angle between transition dipole moments of bR monomers in a trimer is changed as evidenced by a shift in the negative exciton-exciton band. The tryptophan environments are also altered (166-168). Such a delocalized conformational change that causes a large change in the CD spectrum is different from the local conformational change caused by light adaptation of bR (10). A delocalized conformational change provides a means to transfer mechanical energy both intra- and intermolecularly in biological structures. This important result, that BD modification of surface
arginines causes a marked slowing of the photocycle (33-fold) plus a delocalized conformational change of the protein as seen by CD, emphasizes the importance of conformational changes in photocycling activity.

II. A. 8. c. EA/EDC Double Modifications

Since EDC does not shift the $\lambda_{\text{MAX}}$ of the 568 nm chromophore, this suggests that the carboxyl groups modified are near the surface and do not interact directly with the chromophore. Although modification by EDC alone and EA alone does not cause significant chromophore decrease, double modification with either order of reagents causes a 15% decrease in the 568 nm chromophore absorbance. These results indicate that the one carboxyl group that is cross-linked to a lysine is not involved in maintaining the initial ground state absorbance. Thus other carboxyls and lysines (ASP 102, ASP 104, GLU 74, LYS 30, LYS 40 and LYS 129) have a greater effect on the chromophore ground state. However, deterioration of the chromophore structure per se is not the cause of inhibition of photocycling activity, since the inhibition by EDC alone, which does not significantly affect the chromophore structure, is more marked than in any of the EA/EDC double modifications.

Tryptophan fluorescence quenching and red shifts in the excitation and emission $\lambda_{\text{MAX}}$'s (Results III.B.3.b.) indicate a significant protein structural change in the EDC/EA doubly modified samples, that could be the cause of the decrease in the 568 nm chromophore in these samples. The EDC sample also has red shifted fluorescence maxima, but no fluorescence quenching. This suggests that inhibition of photocycling kinetics is more closely correlated with red shifts of the fluorescence.
maxima than with fluorescence quenching. As discussed above, the red shifts have numerous explanations (Discussion II.A.8.b.), all of which indicate a change in the protein structure. The red shifts were more marked than following imidoester modification, and comparable to those following BD modification.

II. A. 8. d. BD/EDC Double Modification

After the BD/EDC double modification, the chromophore absorbance decreases about 10% compared to either chemical modification alone (see Results III.C.3.a.). This suggests that additional steric hindrance of the arginine-carboxyl ionic interactions is able to destabilize the chromophore. The role of EDC following previous BD modification is not to break salt linkages, since this was already accomplished by BD modification. Rather, the role of EDC is to add a bulky group to the additional carboxyls modified after previous modification and to cross-link three additional lysines to carboxyls. The carboxyl residues involved are thought to be ASP 36, ASP 38, GLU 166, GLU 9 and GLU 194. It is reasonable to implicate those carboxyls near retinal on LYS 41 (ASP 36, ASP 38 and GLU 9) as important in maintaining the chromophore structure.

Tryptophan fluorescence of the BD/EDC doubly modified sample reveals heterogeneity of fluorescing tryptophans after both arginines and carboxyl groups are modified. It is possible that modification of GLU 9 directly affects the polarity of TRP 80, or perhaps modification of GLU 194 affects the environment of TRP 137 and/or TRP 189. Alternatively, more tryptophans may fluoresce after this double modification, due to a change in protein structure that causes inhibition of fluorescence quenching. The significance of this result is that heterogeneity
of tryptophan environments is seen only with the double modification, BD/EDC but not with BD or EDC modification alone, which lends support to the idea that additional carboxyls are modified following BD modification causing a further change in protein structure. This protein structural change and the 568 nm chromophore decrease seen above are not correlated with a dramatic change in photocycling inhibition since photocycling kinetics after BD/EDC modification are not significantly slower than after BD modification alone.

II. B. Model for Mechanism of Proton Pumping in bR

1. Implications of Imidoester Modification

a. pH Changes

In an attempt to correlate the basic measurement in this thesis of bR's photocycling activity with proton movements during the photocycle, pH changes using a pH sensitive dye in a solution of PM were studied. Results I.C. shows that when the photocycle is slowed by bifunctional or monofunctional imidoester modification, proton uptake is slowed by a similar factor. The significance of these results is to confirm the work of others (36,51,52,53) that photocycling activity is a good indicator of proton uptake by bR.

Two questions should be asked of this technique: 1) Do proton movements measured with this pH sensitive dye accurately monitor the true proton release and uptake of bR, and 2) How do proton release and uptake relate to the actual protons pumped by bR? In answer to question 1), the work of Lozier et al. (36) should be discussed. These investigators also used 7-hydroxycoumarin to show that proton release occurred with a $t_{1/2}$ of 800 μsecs at 20°C. They estimated the response time of the dye to be diffusion-limited and complete at < 100 μsecs,
but the true response time of the dye is not known (Richard Lozier, personal communication). It is possible that the dye response time is affected by surface related phenomena, such as non-specific binding to the PM. Indeed, a recent study by Gutman et al. (169) showed that the response kinetics of the water-soluble pH indicators bromocresol green or neutral red were affected by absorption to micelles. By varying the electric charge on the micelle, the response time can be accelerated or decelerated, depending on the total charge on the micelle. In addition, the work of Gutman et al. (169) showed that the hydration shell on the micelle plays a role in proton transport to the dye, as has been suggested by D. Kell (170).

Another method to monitor proton release and uptake that does not use pH indicator dyes has been used by Ort and Parson (171). These investigators measured flash-induced volume changes in suspensions of bR-containing membrane fragments with a capacitor microphone transducer. By observing pH and buffer dependencies, they interpreted their results to indicate a fast initial release of a proton, followed by a slower movement of a proton on bR's surface. They found that the kinetics of these two steps changes as a result of the pH of the medium. The pH dependence of proton release could be due to protonation of groups on the surface of the PM that could release a proton if they were already protonated. It was seen in Appendix 5 that the most efficient pH for photocycling was pH 7.0; it is possible that the fastest rates of photocycling occur when proton release occurs readily and also when certain surface groups are partially deprotonated. At pH < 7.5, these investigators saw the fast proton release before the rise of M indicating a large discrepancy with the work of Lozier et al. (36) and this thesis work. Thus proton release and uptake monitored by pH sensitive
dye absorbance changes should only be considered as a rough estimate of the true proton release and uptake as monitored by volume changes.

In answer to question 2), the work of Lozier et al. (36) should again be cited. These investigators found that in suspensions of isolated PM, the kinetics of acidification and subsequent alkalinization produced by photocycling were similar to the rates of pH changes seen in suspensions of cell envelope vesicle preparations. Thus these researchers estimated proton release and uptake to be an indication of protons pumped across the PM, and correlated these changes roughly with the rise and decay of M*412*.

Another group of investigators has attempted to take a closer examination of protons pumped during the photocycle. Keszthelyi and Ormos (172) used electronic signals to monitor proton movements through oriented PM sheets. They interpreted their results to indicate that two protons move from the cytoplasmic to the external side of the PM with time constants similar to the K→L, L→M, M→O and O→bR transitions. In the K→L transition, the protons move backwards, while in the other transitions the protons move forward. Assuming that the only charged species that move are protons, they calculated the distances that two protons should move for each step. For example, they calculate that in the M→O step, two protons move 31 Å forward, and in the O→bR step, two protons move 15 Å forward, while the distances moved in the other steps are much smaller. Accepting their calculations, one should place the Schiff base and other proton donor as follows:

![Diagram showing proton movement through the PM](image-url)
However, the data in this thesis is in disagreement with this model since the Schiff base on LYS 41 is thought to be much closer to the cytoplasmic side as shown below:

This discrepancy in models leads to a reconsideration of the interpretation of Keszthelyi and Ormos (172). In their investigation, they measured volts as a function of time, which they show as being directly related to charge times distance. If they assume that the charge that moves is carried by one or two protons, then they can calculate the distances moved. However, if some of the charge displacement is due to movement of the configuration of the protein then their calculated distances will be incorrect. If we compare to the movement of protons in ice where ion migration has been well studied (173,174), it is known that for every forward movement of a proton another configurational movement in the backwards direction is needed. The movement of a configurational defect also carries a charge (negative in the backwards direction), that will appear to be a positive charge moving in the forwards direction. In ice the charge carried by the configurational defect is smaller (1/3 e) than the charge carried by the proton (2/3 e) (175). The factor of two in the distances calculated by Keszthelyi and Ormos (172) leads one to believe that the smaller distance (15 Å in the O→bR step) is actually movement of the configurational defect. Thus, unless the source of the charge displacement is known, these distances cannot
be accurately calculated. The significance of their work is to identify charge displacements that are correlated with each step of the photocycle.

Thus, if the model presented in this thesis and the data of Ort and Parson (171) are correct, one may ask how can one (or two) protons travel 40 Å between the Schiff base and the external side in about the same time as the L→M' step? Again, the model of ice is a well-studied system where proton mobilities are known. Given an extensively hydrogen-bonded structure as in ice, or perhaps in the hydrated interior of the bR molecule, it has been calculated that a proton can traverse a 50 Å thickness with a t₁/₂ = 2.5 μseconds (175). This transit time is rate-limited by the accompanying backwards movement of a configurational defect of the protein (175). Thus it is seen that movement of a proton could be fast enough in bR if there exists an extensively hydrogen-bonded network within the protein; one of the protons that appears at the external surface could then be the Schiff base proton. Therefore, the model in this thesis is consistent with the data of Ort and Parson (171) given the possibility of fast proton migration via a hydrogen-bonded network from the Schiff base to the external side of PM.

To summarize the significance of the results in this section, it is shown that both proton uptake and M₄₁₂ decay are slowed by a similar factor, indicating that monitoring the photocycle by M₄₁₂ decay is a good indicator of protons taken up by bR. Comparison to other workers in this section shows that protons are taken up during light-activated proton pumping. Proton pumping in bR involves step-wise movement of protons, and perhaps configurational defects, with kinetics that parallel the kinetics of absorption changes of the bR photocycle.
II. B. 1. b. Deuterium Isotope Effects During Photocycling

The deuterium oxide isotope effect is unchanged by the imidoester modifications that slow the $M_{412}$ decay (see Results I.B.4.). The observed range of isotope effects in this thesis (2-5) are smaller than the "normal" range of 6-10 (50) and are similar to the isotope effects on the bR photocycle observed by others (47,48,176). These results lead to an understanding of the mechanism of deprotonation and reprotonation of the Schiff base.

The effect of D$_2$O in slowing the photocycle is independent of the effect of slowing the photocycle by imidoester modification, i.e., $k_2/k_1$ = $k_4/k_3$ as shown in the following table:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Rates of $M_{412}$ Decay (Second Phase in msec$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>$k_1 = .187 \pm .025$</td>
</tr>
<tr>
<td></td>
<td>$k_2 = .090 \pm .025$</td>
</tr>
<tr>
<td>EA Modified bR</td>
<td>$k_3 = .126 \pm .021$</td>
</tr>
<tr>
<td></td>
<td>$k_4 = .045 \pm .017$</td>
</tr>
</tbody>
</table>

This is consistent with the following scheme of thermally activated proton transfer as the primary step in $M$ decay:

Free Energy Barrier for Proton Transfer to the Schiff Base

![Diagram showing free energy barriers and reaction coordinate](image-url)
Using transition state theory (32),

\[ k_1 = A \exp \left(-\frac{\Delta G_1^\dagger}{RT} \right) \]

\[ k_2 = A \exp \left(-\frac{\Delta G_2^\dagger}{RT} \right) \exp \left(-\frac{\Delta G_0^\dagger}{RT} \right) \]

\[ k_3 = A \exp \left(-\frac{\Delta G_2^\dagger}{RT} \right) \]

\[ k_4 = A \exp \left(-\frac{\Delta G_2^\dagger}{RT} \right) \exp \left(-\frac{\Delta G_0^\dagger}{RT} \right) \]

From these formulae the Boltzmann factors due to deuteration are independent of the Boltzmann factors due to modification, and, in particular, \( k_2/k_1 = k_4/k_3 \).

In transition state theory, \( \Delta G^\dagger = \Delta H^\dagger + T\Delta S^\dagger \), which states that the change in the free energy of activation is related to both the activation enthalpy and activation entropy. Imidoester modification could cause a higher free energy barrier for proton transfer due to different entropy or enthalpy requirements in the modified samples.

In a separate investigation (177) we have studied the entropy of activation and energy (enthalpy) of activation of EA and DMA modified bR samples compared to control bR. The EA sample was the only chemically modified sample that showed a more favorable (positive) entropy of activation for the decay of M. The more favorable entropy of activation could be due to imidoester modification of LYS 30. As will be discussed below (Discussion II.B.3.b.), TYR 43 is thought to be the direct proton donor to the Schiff base. By placing the hydrolyzed EA reagent on LYS30, the protein may force TYR 43 into a better position as a proton donor to the Schiff base. Since there was a higher energy (enthalpy) of activation in this sample, however, the overall free
energy of activation was less favorable and the photocycle was slower than control. Thus even though TYR 43 may have been closer to the Schiff base, more energy was required perhaps due to a change in the pK of TYR 43. In the case of the DMA sample, the entropy of activation was more negative than in the control, indicating a less favorable arrangement for proton donation to the Schiff base.

There are several interpretations of a smaller than normal isotope effect. This may indicate a highly asymmetric transition state, especially for the decay. In an asymmetric transition state, the hydrogen atom can vibrate, whereas in a symmetric transition state, there is no stretching vibration of the hydrogen atom. To the extent that the difference in zero-point energy of the starting material is maintained in the transition state by this vibration, the observed isotope effect will be reduced from the theoretical maximum (178). Other possible causes of a small isotope effect are the development of a significant new bending vibration in the transition state (179), and/or masking of primary isotope effects by secondary isotope effects (180). In addition, the proton may not be undergoing translation at the saddle point of energy which represents the transition state for proton transfer (181). This last possibility cannot be true for the rise or decay of M, since the proton transfer step is the reaction. Of the other explanations it is not possible to single out the contribution of any one of these in particular from the data.

The question may be asked, how many lysines participate directly in the proton transfer steps? The roles of LYS 41 and LYS 216 have already been discussed. In addition, it is likely that LYS 172 and the other three lysines have a direct role in the transfer of protons due to their ability to be protonated and deprotonated, but this role is not
tested by the imidoester experiments. The pK of the product of amidination is similar to that of lysine and so it will be able to transfer protons nearly as well as lysine. Another chemical modification that would better test the role of lysine as proton donor and acceptor is pyridoxal phosphate modification of lysines. Although this experiment was attempted (see Appendix 9), the modification was not able to be stabilized by NaBH₄, and reversed. Extensive pyridoxal phosphate modification would change the charge of all the lysines and their pK's, and hence would markedly affect photocycling kinetics if lysines were directly on the H-bonded chain. It would be interesting to carry out this modification successfully and then monitor the decay of M₄₁₂.

II. B. 2. Implications of BD Modification

a. Light-Induced pH Changes

As in the imidoester experiments, the use of a pH-sensitive dye, 7-hydroxycoumarin, monitors proton release and uptake in the 400 mM BD modified bR sample. Although the BD modification is almost completely reversed upon removal of borate buffer, a small inhibition of photocycling remains (see Results II.C.1.). Monitoring pH changes of BD modified bR, with a slower rise of M₄₁₂ as well as decay of M₄₁₂, shows that proton release parallels the kinetics of the rise of M, and proton uptake parallels the kinetics of the decay of M. Thus BD modification supports the conclusions of the imidoester experiments, that photocycling kinetics are a good indicator of proton release and uptake.

In order to correlate the photocycling changes caused by BD modification with light-induced proton pumping across the PM,
pH changes in solutions of bR-lipid vesicles with BD modified bR incorporated were monitored. Initial rates of light-induced alkalization are shown to be about 7-fold slower in the BD modified bR samples, although the great extent of photocycling inhibition (33-fold) is not reflected in these pH changes. As explained in Results II.C.2. several factors may cause this discrepancy, such as orientation of PM in the lipid vesicles, response time of the pH meter, and an interfering protein-lipid interaction. As stated in Results II.C.2. these results are considered to be qualitatively, but not quantitatively, significant. The importance of these results is that the proton pump is also slowed down in the predicted direction following BD modification.

The question may be asked, do arginines participate directly in the proton transfer steps? The direct role of an arginine residue in proton pumping was first suggested by Lewis et al. (117) in 1978. These investigators postulated that photon absorption by the retinal chromophore causes an arginine on the external side of the membrane to donate its proton to a carboxyl group, and then this arginine accepts a proton from a lysine group that simultaneously accepts the proton of the Schiff base lysine (117). Thus they believe that arginine is directly involved in the deprotonation of the Schiff base but not in the reprotonation of the Schiff base during the decay of M. As described in Materials and Methods III.A., BD modification is thought to add a negatively charged BD borate adduct onto arginine. At neutral pH, the borate adduct retains its negative charge and arginine retains its positive charge (182). Placement of a negative charge near the guanidinium group is expected to raise the pK of arginine, which may have been the cause of the increased pK of M, decay after BD modification.
This result could be interpreted as evidence for the direct participation of arginines in proton donation to the Schiff base, since the pK changes were in the same direction.

Ehrenberg and Lewis (183) have shown the pK of the Schiff base during M\textsubscript{412} decay to be > 12 which correlates well with proton transfer from arginine. Since the 568 nm chromophore was unaltered after BD modification, the modified arginines are not in the direct environment of the Schiff base, but are probably near the surface of bR. Hence one or two amino acid residues may be required to transfer arginine's proton from the cytoplasm to the Schiff base on the proton pathway (175) in bR.

Other evidence that favors the interpretation that arginine residues participate directly in proton donation to the Schiff base is that it has been shown that the number of protons transported per photocycle is two (136, 172). If arginine donates directly to the Schiff base via the proton wire (175), then the justification for two important arginines could be since there are two protons per photocycle. Proton translocation through the PM could occur by two hydrogen-bonded chains, only one of which includes the Schiff base (184). A recent study (185) has implicated helices B, C and E as participating in the formation of a proton wire. It is possible that the proton from ARG 227 enters the proton wire near helix E while the proton from ARG 225 enters near the Schiff base.

Another interpretation of the parallel changes of pK of arginine and the Schiff base reprotonation that must be considered is that arginine modification causes a change in the secondary and/or tertiary structure of the protein such that the pK of the Schiff base reprotonation is raised. This is certainly a possibility, since it is shown...
in Appendix 4 that the 568 nm chromophore begins to deteriorate about pH 11, thus preventing the titration of photocycling activity at pH's above 12.4. If this were the case, arginine would not necessarily be implicated as a direct donor of protons to the Schiff base.

Concerning the deprotonation of the Schiff base, the present study cannot rule out the possibility that arginine participates directly as Lewis et al. (117) proposed. A dramatic slowdown in the K+L step of the photocycle might result in the small slowdown of the rise of M that was observed. Since, in addition, proton release and uptake kinetics parallel the photocycle inhibition (see Results II.C.1.), it is possible that arginines participate directly in both deprotonation and reprotonation of the Schiff base. The participation of ARG 82 and ARG 175 is not directly studied by this BD modification, since these two arginines are located near the center of the PM and are not modified by BD. A future experiment to try to modify ARG 82 and ARG 175 by BD might reveal the direct participation of these arginines in proton transfer steps. BD modification of ARG 82 and ARG 175 might be accomplished by first loosening the protein structure with a detergent such as CTAB, octyl-β-D-glucoside, or Triton X-100 (52,75, 79,186) and then modifying with BD for three hours. Removal of the detergent by dialysis could restore the initial protein structure for photoactivity measurements. Amino acid analysis as described in Materials and Methods III.C.2. would determine if all the arginines were modified by BD under these conditions. If an increase in the slowdown of photocycling resulted from the modification of ARG 82 and ARG 175, and no further increase was noted in the delocalized conformational change monitored by CD spectroscopy, this would suggest that ARG 82
and ARG 175 participate directly in proton transfer steps.

II. B. 2. b. Deuterium Isotope Effects During Photocycling

The fact that BD modification slows the rise as well as the decay of M (see Results II.B.1.) signifies that the formation of the transition state for proton transfer is slowed in both cases. As will be discussed (Discussion II.B.3.b.), the proton acceptor for the Schiff base proton is thought to be TYR 26 and the proton donor is thought to be TYR 43. BD modification at the surface of bR may act to inhibit the rotation of the tyrosine residues from interacting with the Schiff base. Indeed, we have shown (177) that the entropy of activation is more negative for the rise (-0.8 e.u./mole) and for the decay (-38 e.u./mole) in 500 mM BD modified bR, than in control bR (rise = 3 e.u./mole, decay = -2.0 e.u./mole). The fact that the deuterium isotope effect for both the rise and decay of M is unchanged following BD modification signifies that only the rate of formation of the transition has been slowed, but the difference in stretching frequencies between deuterium and hydrogen remain the same.

II. B. 2. c. Surface Charge Changes During Photocycling

Results II.B.3. shows that when the steady state concentration of M increases due to BD modification, the increase in the negative charge at the surface of bR increases in the same proportion. This negative charge has been interpreted to be caused by steady-state proton release and uptake from the bR surface during photocycling (74). Thus the M state is associated with a fixed number
of protons released at a given pH and salt concentration, and when the concentration of M increases, the kinetics of protons released and taken up changes.

The questions may be asked, 1) How does arginine modification lead to light-induced increased negative surface charge, and 2) Does it involve modification of arginines directly or is it an indirect effect simply due to increased M? In an attempt to answer these questions, the amino acid residues responsible both for the light-induced surface charge changes and for M decay must be identified. As shown in Results II.B.2., at room temperature, there is no pH dependence of the rate of decay of the fast phase over the pH range 7-12, and the slow phase is unaffected by pH until pH 11. These results can be interpreted to indicate that arginines, perhaps together with tyrosines and lysines with altered higher pK's, are responsible for the pH dependence. The light-induced surface charge changes, however, have been observed by Carmeli et al. (74) to show a marked pH dependence at room temperature, with one pK about 4 and one pK about 9. Their study implicates lysines and tyrosines with altered lower pK's as the amino acid groups that regulate proton release. Although they do not discuss arginines, their work does not disprove a contribution from surface arginines to the surface charge changes, since their pH titration was only carried out up to pH 10. In another study, Renthal has shown that light-activated proton release has a pK of 9.9 (187). The discrepancy between the pH dependence of M decay shown here, and the pH dependence of surface charge changes (74) and proton release (187), leads to the conclusion that different amino acid residues dominate in their contribution to the pH dependence of these two events. A clue to which amino acid
residues control the surface charge changes and proton release stems from the work of Ort and Parson (171). These workers showed the room temperature pK of the slow step of a flash-induced volume expansion to be 8.6 (171), which is close to the pK of 9 observed by Carmeli et al. (74). Ort and Parson (171) interpreted this step to be the movement of a proton from one group to another on the membrane surface, which occurred just after the rise of M and before the decay of M. This implicates amino acid groups which are associated with the rise of M and proton release from PM to be responsible for the pK of 9-10 of surface charge changes. These groups are most likely tyrosines and lysines. M decay, on the other hand, may be regulated primarily by arginines, but also tyrosines and lysines with altered higher pK's. Although protons are released at the external side, it is not possible to implicate amino acid residues on the external side as responsible for controlling proton release, since control of the photocycle may be primarily from groups on the cytoplasmic surface. Thus these data do not specify the location of the amino acid groups involved in these two events.

In another study, Ehrenberg and Lewis (183) monitored light-activated deprotonation of the Schiff base by kinetic resonance Raman spectroscopy as a function of pH. They found the pK of Schiff base deprotonation to be about 10, which is fairly close to the pK of surface charge changes (74) and proton release (187). If the Schiff base donates its proton to the amino acid residues located on the external side, it is reasonable that these are the residues that have a combined pK of about 10. When the light is off, the pK of the Schiff base has been shown to be greater than 12. It is also reasonable that
with such a high pK, the Schiff base could accept a proton from groups at the external surface with a pK also near 12. Thus one explanation for the changes in pK at the Schiff base caused by light (180) may be to allow the Schiff base proton to interact with different sides of the PM.

To summarize the significance of the results of this section, it is shown that the amino acid residues that dominate in the control of M decay are different from those that participate in the control of surface charge. It is thought that lysines and tyrosines with lowered pK's control surface charge changes (release of protons) in the pH region 9-10, while arginines, and lysines and tyrosines with altered higher pK's control the reprotonation of the Schiff base. Thus the light-induced negative charge that is associated with the M state does not appear to be due to the direct placement of a negatively charged adduct on arginine. Rather, the negative charge is characteristic of the M state and increases due to the increased concentration of M_{412}.

II. B. 3. Implications of EA/I^- and BD/I^- Double Modifications

a. Iodination of Tyrosines Blue Shifts the 568 nm Chromophore

As shown in Results III.A.3.a., as the time of iodination following EA or BD modification increases up to 24 hours, a marked blue shift occurs which may result from placing negative charges near the chain of retinal (103) or Schiff base (104-106), or perhaps from an increased dipole moment in the micro-environment of the chromophore (107,108). This marked sensitivity of
the chromophore \( \lambda_{\text{MAX}} \) to iodination of tyrosines suggests that the tyrosines modified by iodination include those that are close to the chromophore. The tyrosines closest to retinal on LYS 41 are TYR 43 and TYR 26. Model building with Framework Molecular Models reveals that TYR 26 is located just below LYS 30 on helix A which is at nearly the same height as LYS 41 on helix B. TYR 43 on helix B is also in a position to interact with LYS 41. A possible mechanism for the interaction of TYR 26 and TYR 43 with the Schiff base during photocycling will be described in the next section.

It is shown in Figures 61 and 62 (see Results III.A.3.a.) that the new absorbance peak in the heavily iodinated samples is near 360 nm. By comparing to the work of Bayley et al. (119), the chromophore that absorbs at 360 nm is probably retinal on LYS 216. These authors used protein chemistry to identify the binding site of retinal as LYS 216 in bR treated with CTAB or reduced by NaBH₄ in the light. Since either CTAB or NaBH₄ in the light can alter the 568 nm chromophore to absorb in the region near 360 nm (109), it suggests that after extensive iodination some of the retinal molecules are permanently positioned on LYS 216. The broad peak at 500 nm in the heavily modified samples may indicate that some of the retinal molecules remain on LYS 41, but since the environment of LYS 41 is now more negatively charged, the \( \lambda_{\text{MAX}} \) of LYS 41 has blue shifted.

The continued decrease in tryptophan fluorescence as the time of iodination increases up to 180 minutes, can be interpreted in terms of a continued increase in polarity near the fluorescing tryptophans. This is reasonable since the one or two fluorescing tryptophans could be TRP 10, TRP 80, TRP 137 and/or TRP 189 which are all located on
the external half of the bR molecule with most of the tyrosines. If TRP 80 fluoresces, its fluorescence is expected to change markedly due to iodination of tyrosines, since it is right next to TYR 79 (see Figure 65). An alternative explanation for a decrease in the fluorescence yield could be that there is an increase in energy transfer to the chromophore after iodination, perhaps due to a closer approach of tyrosines to retinal.

II. B. 3. b. Role of Tyrosine in Proton Transfers

Results III.A.2. shows that iodination further changes both the rise and decay of M$_{412}$ after previous modification with either EA or BD. Iodination alone causes the rise of M to become faster, and the decay of M to become slower (27). Both EA and BD pre-modifications increase the rate and the magnitude of the changes in the rise and decay of M caused by subsequent iodination. These results will be interpreted in terms of a stepwise movement of protons through bR.

As postulated by Scherrer et al. (27) tyrosine may be the primary acceptor for the proton on the Schiff base on LYS 41. According to the postulated mechanisms in this thesis, deprotonation of the Schiff base occurs in the L→M step, just after a conformational change of the protein in the K→L step. The conformational change of the protein is thought to be necessary in order to position the protein for the transfer of retinal from LYS 41 to LYS 216, so it is possible that LYS 41 moves slightly downwards (see Figure 65) to meet LYS 216, or that LYS 216 moves slightly upwards to meet LYS 41, or that both movements occur. The total distance needed to be covered for these lysines
to react by the substitution reaction described above is about 6 Å.

Since in the L→M' step the protons are transferred forwards (towards the external side) (172), the proton acceptor must be further towards the external side than LYS 41 when the Schiff base deprotonates. Model building with Framework Molecular Models shows that TYR 26 is in a good position to accept the Schiff base proton. This mechanism for Schiff base deprotonation can be summarized in terms of a diagram:

1) \[ \text{Schiff base on LYS 41} \]
\[ \text{HO} \downarrow \text{TYR 26} \]
\[ \text{Proton Acceptor} \]

2) \[ \text{Schiff base on LYS 41} \]
\[ \text{HO} \downarrow \text{TYR 26} \]
\[ \text{Proton Acceptor} \]

In the case of the deprotonation of the Schiff base, both double modifications, EA/I" and BD/I" cause a fast deprotonation \( (t_{1/2} = 25 \ \mu\text{sec}) \) after only 30 minutes of iodination. In the case of iodination alone, such a fast deprotonation is observed after 120 minutes of iodination (27). Thus the tyrosine that is responsible for the deprotonation is made more accessible to the iodinating reagents following either BD or EA modification. TYR 26 is located about 9 Å within the hydrophobic phase of the membrane so it may become easily
accessible to iodination reagents only following EA or BD premodification. After iodination, the pK of TYR 26 will be lower and so it will more readily give up its own proton and accept the proton of the Schiff base.

For the reprotonation of the Schiff base, the tyrosine that is in a position to transfer a proton back to the Schiff base in the Framework Molecular Model is TYR 43. The chain of events could be the simultaneous hopping of a proton from the cytoplasm to ARG 225, to TYR 43, and then to the Schiff base on LYS 41. This scheme is summarized in the diagram below:

1) Cytoplasm

\[
\begin{align*}
\text{Cytoplasm} & \quad \xrightarrow{\text{M+BR Step}} \\
H_2C = N & \quad \text{ARG 225} \\
H_2C = N & \quad \text{TYR 43} \\
& \quad \text{Schiff base on LYS 41} \\
& \quad \text{(or on LYS 216 if the 0 photointermediate is present)}
\end{align*}
\]

2) Cytoplasm

\[
\begin{align*}
\text{Cytoplasm} & \\
H_2C = N & \quad \text{ARG 225} \\
H_2C = N & \quad \text{TYR 43} \\
& \quad \text{Schiff base on LYS 41}
\end{align*}
\]

This scheme requires TYR 43 to have a pK near 12 as do ARG 225 and the Schiff base on LYS 41. Although they have not been included in this diagram, one or two water molecules may be necessary to bridge the distances covered by this proton chain.

Iodination alone will act to slow the decay of M, since if the pK of TYR 43 is lowered by iodination, it will be more frequently
deprotonated and unable to donate a proton to the Schiff base. The different shapes of the EA/1" and BD/1" inhibition curves seen in Figure 60, may indicate a different mechanism of inhibition caused by prior lysine or arginine modification. This suggests that lysines do not participate directly in translocating protons. The pK of arginine may be raised one pK unit, thus slowing this chain of events if arginine donates its proton to TYR 43. The combined effect of raising the pK of arginine and lowering the pK of tyrosine results in the greatest inhibition of the photocycle of all the chemical modifications in this thesis. Alternatively, the conformational changes of the protein caused by BD modification, together with the lowered pK of tyrosine, could be the cause for the great slowdown of photocycling after these two modifications.

An alternative explanation of the role of tyrosine has been proposed recently by Kalisky et al. (188), based primarily on evidence obtained by Bogomolni et al. (111) and Hess and Kuschmitz (189). Kalisky et al. (188) believe that one tyrosine deprotonates just before the Schiff base deprotonation, and that this tyrosine and the Schiff base both reprotonate during M decay. Their model requires two proton acceptors, one for tyrosine and one for the Schiff base, and two separate proton wires. The iodination results in this thesis could be explained by this model, although I do not believe it to be the correct model for several reasons: 1) a deprotonated tyrosinate ion will leave a negative charge in the interior of the protein, which will tend to destabilize the protein, not lead to the metastable M state; 2) the observed UV fluorescence changes (111) may signify conformational changes.
in the protein, or increased tryptophanyl-retinal resonance energy exchange as well as deprotonation of tyrosine; 3) although the 275 nm absorption peaks observed by Hess and Kuschmitz (189) could be due to the protonated and deprotonated forms of tyrosine, respectively, they could also be due to changes in the tryptophan environments. Indeed, Hess and Kuschmitz (189) showed that 412 nm excitation inhibited formation of the 275 nm absorption, but had no effect on the 295 nm absorption, suggesting that these two peaks are not caused by the state of ionization of the same tyrosine. For these reasons, the model postulated in this thesis, that implicates TYR 26 as the direct acceptor of the Schiff base proton, and TYR 43 as participating in proton donation to the Schiff base, is thought to be the correct model. The second proton translocated may bypass the Schiff base, and enter the proton wire below the Schiff base. As mentioned above, ARG 227 may be a direct donor of the second proton translocated.
This thesis applied and developed the chemical modification technique and its analysis to study the membrane bound protein, bacteriorhodopsin. These techniques included optimization of the reaction conditions regarding protein concentration, reagent concentration, pH, buffer concentration and type of buffer, and number of treatments needed to complete the modification. In addition, changes in the reaction conditions in order to obtain more information about the protein were made, such as carrying out the reaction in D$_2$O, at different temperatures, and with the protein in an altered state (bleached, or in cell envelope vesicles). After the chemical modification, analysis of the product by chemical and spectroscopic techniques yielded information about the alteration in chemical properties of the protein. These techniques included determination of the presence of any remaining reagent by absorption spectroscopy, determination of the conditions needed for reagent removal and reaction reversal by centrifugation and column chromatography, determination of the number of amino acids modified by the fluorescamine technique and amino acid analysis, and determination of the amount of polymerization caused by the imidoester modification by gel electrophoresis.

The spectroscopic techniques that were used in order to determine if a conformational change had been caused by the chemical modification were absorption spectroscopy, tryptophan fluorescence, and, with collaborators, resonance Raman spectroscopy, retinal fluorescence and circular dichroism.

Once the chemical and structural changes caused by the modification had been assessed, the activity was measured, primarily by analysis of the decay of the $M_{412}$ photocycle intermediate, produced by flash photolysis. To analyze the traces of $M_{412}$ decay, I developed a
new pencil-and-paper technique that fits two exponential decays to the $M_{412}$ decay curve by means of successive approximations (Materials and Methods V.B.1.b.). Other measures of protein activity used were proton pumping in lipid vesicles monitored by pH electrode, pH changes in PM solutions using a pH-sensitive dye, and light-induced surface charge changes using an EPR spin label. In addition, another protein activity was investigated by absorption spectroscopic methods: light-to-dark adaptation of bR.

The specific conclusions to be drawn from the chemical modifications of bR relate first to the structure of bR and second to the mechanism of photocycling and proton pumping in bR.

**Structure of bR**

**Secondary Structure:**

1) Analysis of the number of lysines modified by the monofunctional imidoesters using the Scatchard and Hill plots revealed that one lysine reacts to a far greater extent than the other five lysines. By comparing to other investigators, this result implicates LYS 40 as totally exposed to the aqueous phase, while the other lysines are all buried to some degree within the hydrophobic domain (see Discussion I.A.1.).

2) Analysis of the number of arginines using the Scatchard and Hill plots revealed that all the arginines modified (7 out of 9) have the same reactivity with 2,3-butanedione. This result, together with the interpretation that the two additional arginines in my strain of bR are located in the aqueous phase, leads to the conclusion that all 7 of the arginines modified by BD are located in the aqueous phase and the remaining 2 arginines are buried and inaccessible to BD.
(see Discussion I.A.3.).

3) Analysis of the number of lysines modified by EDC revealed that only one lysine can be cross-linked to a carboxyl by EDC. By proximity constraints and the imidoester/EDC double modification, LYS 159 is most likely cross-linked to GLU 161 by EDC. Analysis of the number of additional lysines cross-linked by EDC following previous modification with BD revealed that 3 carboxyl groups form ionic salt links to arginines that are also in the vicinity of a lysine. The existence of additional carboxyl-arginine salt links that are not also in the vicinity of a lysine is implicated by this result. The most likely carboxyl-arginine salt links also in the vicinity of a lysine are ARG 225-ASP 38, ARG 227-ASP 36 and ARG 134-GLU 194. These results constrain the placement of LYS 30, LYS 129 and LYS 159 to be buried by only one or two residues within the hydrophobic domain so that they can cross-link to carboxyl groups (see Discussion I.A.5.). All of the chemical modification results were used to construct a new model of the secondary structure of bR (see Figure 65).

Tertiary Structure:

1) Analysis by gel electrophoresis of the polymers (oligomers) produced by DMA and MA showed that Model A of Agard and Stroud (126) is the best model to fit the patterns of oligomerization. Model A fits the helices A-G of bR into the pattern seen by X-ray diffraction and electron diffraction as shown in Figure 65. Thus these results help to decide the current controversy between Model A and Model E (see Discussion I.B.1. and I.B.2.).
Quaternary Structure:

1) Analysis by gel electrophoresis of the polymers produced by the 2.3 Å cross-link of MA showed that in bleached bR the bR molecules in a trimer are closer than in native bR. This indicates that one structural role of retinal is to increase the intermolecular distances between bR molecules in a trimer. This result focuses attention on the bR trimer as the primary structural unit (see Discussion I.B.2.).

Mechanism of Photocycling

Conformational Changes of the bR Protein:

1) Inhibition of the photocycle by bifunctional, and not monofunctional, imidoesters indicates that a conformational change of the protein is needed for photocycling. All of the possible intramolecular cross-links could inhibit the conformational changes needed to transfer retinal 6 Å from LYS 41 to LYS 216. In addition, some level of intermolecular cross-linking may be needed to inhibit photocycling (see Discussion II.A.1.).

2) Analysis of the $M_{412}$ decay after BD modification by curve fitting techniques indicated that two specific arginines of the 7 surface arginines modified by BD are responsible for the 33-fold slowdown of $M_{412}$ decay. The closely juxtapositioned ARG 225 and ARG 227 that could be located just above helices B and G that contain retinal are the best candidates for the two important arginines. Part of the mechanism of inhibition of photocycling by these two arginines may be to destabilize ionic linkages between arginine and aspartic acid groups that causes a delocalized conformational change of the protein (see Discussion II.A.6.).
3) Several experimental results suggest that there are two environments of retinal: retinal fluorescence after BD modification, regeneration experiments of EA treated bleached bR, resonance Raman experiments of EA treated bR, and $M_{412}$ decay kinetic analysis of extensively EA modified bR. These results can be accounted for by retinal migration from LYS 41 to LYS 216 and back during photocycling (see Discussion II.A.1.,II.A.2., II.A.3., II.A.4., and II.A.8.).

4) Double modification experiments using BD and EDC, and EA and EDC suggest that destabilization of the initial arginine-glutamate salt links has a greater effect on photocycling kinetics than destabilization of any of the postulated new ionic links (see Discussion II.A.7.).

Proton Pumping:

1) pH dye experiments of chemically modified bR with slowed photocycling confirmed that photocycling activity is a good indicator of light-induced proton release and uptake by bR in PM. By comparison to other workers, proton movements are produced by light-induced proton pumping (see Discussion II.B.1.a. and II.B.2.a.).

2) With the use of $D_2O$ it was found that the difference in stretching frequencies between deuterium and hydrogen is maintained even when the transition state barrier is increased by the chemical modification. This indicates that chemical modification causes a slowdown of the formation of the transition state for proton transfer which is independent of the slowdown caused by $D_2O$ (see Discussion II.B.1.b and II.B.2.b.).

3) The pH dependence of $M_{412}$ decay after BD modification suggests that one role of arginines is as direct donor of protons to the
Schiff base. Since proton movements were also slowed after BD modification when monitored by a pH indicator dye, this supports the direct involvement of arginine in both deprotonation and reprotonation of the Schiff base (see Discussion II.B.2.a.).

4) The experiments exploring light-induced surface charge changes showed that the amino acid residues that dominate in the control of $M_{412}$ decay are different from those that participate in the control of surface charge (74). In particular, arginines do not control surface charge changes in the pH region 9-10, but lysines and tyrosines will have the major influence over proton release and surface charge changes. Arginines appear to dominate in the control of $M_{412}$ decay (see Discussion II.B.2.c.).

5) Double modifications with EA/I$^-$ and BD/I$^-$ indicate that tyrosines in the vicinity of the Schiff base can be readily modified after previous EA or BD modification due to a loosening of the bR structure. A postulated mechanism suggested by model building indicates TYR 26 is the best candidate for the acceptor of the Schiff base proton. By comparison to other workers and model building, reprotonation of the Schiff base is thought to involve a pathway of a proton from the cytoplasm to ARG 225, then to TYR 43 and then to the Schiff base on LYS 41. One or two water molecules may be necessary to bridge the distances involved in this proton chain (see Discussion II.B.3.b.).
APPENDICES

Appendix 1. Calculations for Determining the Converting Wavelengths for Light Intensity Measurements

The purpose of this appendix is to describe the calculations used to determine which wavelength should be used to convert from μEinsteins/m²·sec measured with the LI-COR probe to mW/cm². Since the sensitivity of the LI-COR Photometer is constant over the range 400-700 nm, the converting wavelength would just be the average, 550 nm, if the lamp also had a constant output. However, the spectrum of all quartz halogen lamps increases rapidly between 400 and 700 nm as shown below:

Assuming a linear relationship between intensity (I) and λ described by \( I(\lambda) = a\lambda + b \), the average wavelength, \( \lambda \), is given by

\[
\frac{\int_{400}^{700} \lambda(a\lambda + b) \, d\lambda}{\int_{400}^{700} (a\lambda + b) \, d\lambda} = \frac{\frac{a\lambda^3}{3} + \frac{b\lambda^2}{2}}{\frac{a\lambda^2}{2} + b\lambda} \bigg|_{400}^{700}
\]

The value of \( b \) may be determined as follows:

\(-(a_{400} + b = 0.08)\)

\(+ (a_{700} + b = 0.85)\)

\( a_{500} = 0.77, a = 2.57 \times 10^{-3}, \text{ so } b = -0.95.\)
Solving the integral,

\[
\frac{(2.57 \times 10^{-3})(700)^3}{3} + \frac{(-.95)(700)^2}{2} - \frac{(2.57 \times 10^{-3})(400)^3}{3} + \frac{(-.95)(400)^2}{2}
\]

\[
\frac{(2.57 \times 10^{-3})(700)^2}{2} + (-.95)(700) - \frac{(2.57 \times 10^{-3})(400)^2}{2} + (-.95)(400)
\]

= 592 nm.

A sample conversion calculation using this result of 592 nm is given below:

\[
\nu = \frac{c}{\lambda} = \frac{3 \times 10^8 \text{ M/sec}}{592 \times 10^{-9} \text{ M}} = 5.068 \times 10^{14}/\text{sec}
\]

\[E = h\nu, \text{ for one photon}\]

\[E = (6.624 \times 10^{-34} \text{ joule sec})(5.068 \times 10^{14}/\text{sec}) = \]

\[3.3568 \times 10^{-19} \text{ joules/photon} \times 6.023 \times 10^{23} \text{ photons/einstein} = \]

\[2.0218 \times 10^5 \text{ joules (wattsec)/einstein} = \]

\[202.18 \text{ mwatt sec/einstein}\]

LI-COR measured intensity = 20,000 \(\text{weinsteins}/\text{M}^2\text{sec} = \]

\[20,000 \left(202.18 \text{ mwatt}/\text{M}^2 \times \text{M}^2/(100 \text{ cm})^2 = \right.\]

\[404.4 \text{ mW/cm}^2\]
Appendix 2. Descriptions of Chemical Modification Reactions

A. Imidoester Reactions

The purpose of this section of this appendix is to summarize what is known about imidoester reactions and to justify the choice of pH 10.0 for most of the experiments. Imidoesters, or imidates, react in alkaline solution with amines to form imidoamides, or amidines, raising the pK of lysine without changing its positive charge. Two papers published in 1962 discussed this reaction. The results of Hunter and Ludwig (190) showed that amidine formation from amino acids proceeds with sharp pH-rate maxima which are different for different amino acids, so that it is possible to control product formation in reactions with proteins or mixtures of amino acids by suitable control of pH. Hand and Jencks (191) studied the reaction mechanism of ethyl benzimidate and ethyl m-nitrobenzimidate with several amines as a function of pH, showing that at low pH the decomposition of the tetrahedral intermediate (shown in Figure 3) is rate-limiting, while at high pH the formation of the tetrahedral intermediate is rate limiting. On the alkaline side of the pH maxima, the rate law is described by:

\[ v = k_1 \hat{[RNH_2][IEH^\ominus]} \]

\[ [RNH_2] = \text{deprotonated amine} \]

\[ [IEH^\ominus] = \text{protonated imidoester} \]

and on the acidic side by

\[ v = k_2 \hat{[IE][RNH_2]} + k_3 \hat{[IEH^\ominus][RNH_2]} + k_B [IEH^\ominus][RNH_2][B] \]

\[ [B] = \text{concentration of buffer} \]

Hunter and Ludwig (190) analyzed the reaction products by ultraviolet spectroscopy and by pH titration and found the \( t_{1/2} \) of the reaction of MA (10 mM) with \( \epsilon-NH_2 \) caproic acid (50 mM) at 25°C at pH 8.0 in water to be 6.8 minutes. The \( t_{1/2} \) of hydrolysis of MA
(12.5 mM) under these conditions was 23 minutes.

In a more recent study by S. Kent (9) using $^1$H-NMR, the $t_{1/2}$ of the reaction of MA (0.4 M) with ε-NH$_2$ caproic acid (0.4 M) at 22$^\circ$C, pH 8.2 in 2 M NaPi buffer was found to be << 1 minute. The $t_{1/2}$ of hydrolysis of MA under these conditions was 1.3 minutes.

Kent also compared the effect of pH on the reaction of a similar imidoester, EA, with ε-NH$_2$ caproic acid by $^1$H-NMR. At pH 8.2 in 2 M NaPi buffer at 22$^\circ$C, EA (0.4 M) reacted with ε-NH$_2$ caproic acid (0.4 M) with a $t_{1/2}$ of 1-1/4 minutes, and simultaneous acetimidate hydrolysis occurred with a $t_{1/2}$ of 3 minutes. 30% of the initial amine produced the desired product, N-alkyl acetamidine. At pH 10 in 0.5 M borate buffer, EA (0.4 M) reacted with ε-NH$_2$ caproic acid (0.4 M) with a $t_{1/2}$ of about 1 minute, but the acetimidate hydrolysis $t_{1/2}$ was 30 minutes, i.e., 30 times slower than the reaction. In this case, 70% of the initial amine produced N-alkyl acetamidine. It therefore appeared from the literature that the most efficient reaction between EA and ε-NH$_2$ caproic acid was at pH 10, where the reaction was fastest compared to hydrolysis of imidoester, and a higher percentage of the initial amine formed the desired product.

Kent's investigation confirmed the rate-limiting steps as a function of pH to be as Hand and Jencks (191) had described them. He also envisioned how a monofunctional imidoester might act as a cross-linking reagent (9). At pH 8.2, 85% of EA reacted to form the N-alkyl acetimidate intermediate (see Figures 3 and 4), while at pH 10.0, only 5% formed the N-alkyl acetimidate, and this was completely gone within 6 minutes of mixing. The mechanism shown in Figure 4 allows a second protein group to interact with the N-alkyl acetimidate intermediate as if it were an imidoester. In addition, several monofunctional...
imidoesters may react in a chain-like fashion with the tetrahedral intermediate, producing an elongated N-alkyl amide. Thus more cross-linking by a monofunctional imidoester would be expected at pH's near 8.0, where this intermediate is present. This is another reason why pH 10 was chosen as the optimal pH for the imidoester modification of bR, to avoid cross-linking with a monofunctional reagent. Fortunately, bR is stable between pH 5 and 10, as evidenced by little change of the 568 nm chromophore absorption in this region (see Appendix 4).

Amidines are stable in neutral or acidic solution, but slowly hydrolyze at high pH. The reaction can be reversed in concentrated ammonia-acetic acid (15:1) at pH 11.3 or at pH 9 using 0.6 to 1.2 M hydrazine (192). Amidines are not stable to the conditions of protein acid hydrolysis.

Chemical Formulae of Imidoesters Employed

**Monofunctional Imidoesters**

- Ethyl Acetimidate (EA), $\text{H}_2\text{CCH}_2\text{COCH}_3$
- Methyl Butyrimidate (MB), $\text{H}_2\text{C(CH}_2\text{)}_2\text{COCH}_3$
- Methyl Acetimidate (MA), $\text{H}_3\text{COCH}_3$

**Bifunctional Imidoesters**

- Dimethyl Suberimidate (DMS), $\text{H}_3\text{COC(CH}_2\text{)}_6\text{COCH}_3$
- Dimethyl Adipimidate (DMA), $\text{H}_3\text{COC(CH}_2\text{)}_4\text{COCH}_3$
Appendix 2

B. 2,3-Butanedione Reaction

The 2,3-butanedione (BD) reaction has been well studied by Riordan (15,193,194). The reaction of BD with free arginine is 96% complete in 15 minutes. The concentration of borate buffer that leads to maximal change is 50 mM; more or less borate buffer is less effective (15). At higher concentrations of borate, borate interacts with BD thereby reducing BD's effective concentration. This is suggested by the change in pH from 8.6 to 3.8 in mixing 1.5 ml of BD with 8.5 ml of 50 mM borate. In the case of carboxypeptidase A, 3 hours is more than sufficient to maximally change peptidase and esterase activities. The pH of reaction that leads to maximum change of both of these activities is about pH 8.0.

As shown in Figure 12, borate buffer is needed to stabilize the imidazoline adduct; other buffers such as Tris, Hepes, Veronal or bicarbonate are less effective. The product of the BD reaction in the presence of borate buffer has both a positive charge on the guanidinium group and a negative charge on the borate adduct at neutral pH's (182). After one hour of reaction with BD in the presence of borate, removal of BD but retention of borate maintains the modification. Removal of both BD and borate causes reaction reversal.

Riordan (15) assayed for the number of arginines modified by BD by amino acid analysis. In the absence of borate, BD modification of arginines produced a new peak in the amino acid analysis emerging just before lysine. In the presence of borate buffer this peak was not visible (see Results II.A.1.), but the disappearance of the arginine peak was a good indication of BD modification since it is stable to acid hydrolysis.
BD is able to be detected in the reaction mixture since it has two absorptions: at 408 nm ($\varepsilon = 1.15$) and at 284 nm ($\varepsilon = 9.75$).

Appendix 2

C. Phenylglyoxal Reaction

The phenylglyoxal (PGO) reaction has been studied by K. Takahashi (17,18) who found that two moles of PGO reacted with one mole of arginine (see Figure 13). The pH and buffer dependence of this reaction have been studied by Cheung and Fonda (195). It was found that the rate of reaction increased 37-fold with increasing pH between 7.5 and 11.5, so it was suggested that only the non-protonated form of arginine reacts with PGO. The reaction was fastest in bicarbonate buffer and much slower in borate, Veronal and N-ethylmorpholine buffers. The PGO-arginine adduct absorbs at 340 nm (195). The derivative slowly decomposes at neutral or alkaline pH's, and incubation under such conditions in the absence of excess PGO can regenerate most of the initial guanidino groups (17).
Appendix 3. Calculations of Errors Associated with the Relative Fluorescence and Lowry Protein Results

An error analysis was carried out to determine the average error associated with the standard curve that is drawn from the fluorescence of bR controls. Assuming no error in the fluorescence of a single modified bR sample, the relative fluorescence would also have this percent error since relative fluorescence = modified bR fluorescence + control bR fluorescence + standard error. This analysis does not take into consideration the standard deviation of the fluorescence of duplicate and triplicate samples of modified bR samples; these standard deviations will be presented in Results.

The standard error of the control fluorescence was calculated using a formula (196) incorporating the standard deviation of the fluorescence of duplicate control bR samples, the standard error of the slope, $\hat{\beta}$, in the fluorescence test, and the standard error of the protein value as follows:

$$\frac{SE_{(CF)}}{CF} = \sqrt{\left(\frac{SD_{(F)}}{F}\right)^2 + \left(\frac{SE_{(\hat{\beta})}}{\hat{\beta}}\right)^2 + \left(\frac{SE_{(P)}}{P}\right)^2}$$

where

- $CF$ = fluorescence of a single point on the standard curve drawn from control bR samples
- $SE$ = standard error
- $SD$ = standard deviation
- $F$ = fluorescence of a single control bR sample
- $\hat{\beta}$ = best estimate of the slope of the standard curve in the fluorescence test
- $P$ = protein value determined in Lowry test
The errors were calculated as follows:

**Fluorescence Standard Deviation**

\[
SE_{(F)} = \sqrt{(SD_{(F)})^2 + (\hat{\beta} SE_{(P)})^2}
\]

where \(SD_{(F)}\) = the standard deviation of fluorescence of duplicate bR control samples in the fluorescamine test.

Rearranging,

\[
SD_{(F)} = \sqrt{(SE_{(F)})^2 - (\hat{\beta} SE_{(P)})^2}
\]

This equation subtracts out the protein error from the standard error of fluorescence to give the standard deviation of fluorescence of duplicate bR control samples. \(SE_{(F)}\), \(\hat{\beta}\), and \(SE_{(P)}\) will be calculated below.

**Standard Error**

\[
\hat{\beta} = \frac{\sum X_i Y_i}{\sum X_i^2}
\]

where \(X_i\) and \(Y_i\) are the protein and fluorescence values, respectively, of a single bR control sample in the fluorescamine test.

\[
SE_{(F)} = \sqrt{\frac{\sum(Y_i - \hat{Y}_i)^2}{n - 1}}
\]

\[
SE_{(\hat{\beta})} = \sqrt{\frac{SE_{(F)}^2}{\sum X_i^2}}
\]

\(\alpha\), in the linear equation, \(\alpha + \hat{\beta} X_i = \hat{Y}_i\), was assumed to be zero in both the fluorescence test and the Lowry protein assay, since the background was subtracted. \(\hat{Y}_i\) is the "fitted" \(Y\) value to the best estimate of the slope.
Protein Standard Error

\[
SE(Y) = \sqrt{\frac{\sum(Y_i - \hat{\beta}X_i)^2}{n-1}}
\]

(197)

where

\( Y \) = absorbance in the Lowry protein assay

\( \hat{\beta} = \) best estimate of the slope of the standard curve in the protein assay = \( \frac{\sum X_i Y_i}{\sum X_i^2} \)

\( \hat{\beta} X_i = \hat{\beta} Y_i \)

is therefore the "fitted" \( Y \) value to the best estimate of the slope.

The \( SE(Y) \) can be converted to protein units by dividing by \( \hat{\beta} \):

\[
\frac{SE(Y)}{\hat{\beta}} = SE(X) = SE(P)
\]

By placing these calculated errors into the first formula given in this appendix, a range of errors from the highest to the lowest concentration of control bR in the fluorescence test was obtained. By averaging the highest and lowest errors, the average standard error of the standard curve in the fluorescence test was obtained. When calculated as percent standard error of the control fluorescence, this average percentage was also the percent standard error of the relative fluorescence (see above). At the highest concentration of bR protein on the fluorescence standard curve, a bR control sample had a standard error of 3.8%. A bR control sample at the lowest protein concentration had a standard error of 21.1%. The average error for this test was thus 12.5%. Since this error analysis was not carried out on each fluorescence test performed, a conservative estimate of the standard error for all the tests, considering larger errors in the standard curves of the bR samples, might be ±13%. 
Thus it should be noted that the fluorescence results presented may be inaccurate by ± 13%.

An additional source of error in the fluorescamine calculation must be considered. It has been shown that the quantum yield of the fluorophor produced in the fluorescamine reaction can vary by a factor of 10 if the amine is free in solution or is located in a protein (13). Also, the quantum yield is sensitive to the solvent and percentage of acetone. As was described in Materials and Methods II.C.1. the bR protein was first solubilized as completely as possible using an SDS/bR mole ratio of about 3500/1, and letting the solution remain at room temperature for 30 minutes. Under these conditions, the protein is expected to completely dissociate from the lipid and expose all of the amino groups to fluorescamine, since SDS is a well-known protein and lipid solubilizer (198). By solubilizing this way it is expected that a homogeneous environment will be provided for all the lysines, since the excess negative charges of SDS will "swamp" any charge effects between neighboring amino acids. As long as the modified bR is always compared to a standard curve constructed from solubilized bR control, the actual quantum yields of the fluorescence do not need to be determined.

By a similar procedure to the above calculations, the Lowry protein results were determined to be 6.7% inaccurate at the lowest protein concentration, and 2% inaccurate at the highest protein concentration in the Lowry test, yielding an average error of ± 5%.
Appendix 4. Stability of bR's 568 nm Chromophore

Figure 73 shows a typical absorption spectrum of light-adapted bR. The pH dependence of the 568 nm chromophore was studied as described in Materials and Methods V.A.1. Figure 74 shows the pH dependence of bR in distilled water. This graph is the average of two sets of bR samples. It can be seen that there was little change in bR's 568 nm chromophore peak height between pH 5 and 10. In the presence of a high buffer concentration, 0.2 M phosphate buffer, there was also little change in the 568 nm peak height up until pH 10.0 (see Figure 75).
Figure 73

Typical Absorption Spectrum of Light-Adapted bR

Absorbance
Units

0.3
0.2
0.1

Wavelength in Nanometers
Figure 74

pH Dependence of bR's 568 nm Chromophore

Absorbance at 568 nm
Figure 75

pH Dependence of bR's 568 nm Chromophore

in 0.2 M Phosphate Buffer

Absorbance at 568 nm

pH
Appendix 5. 412 nm Flash Photolysis and 412 nm Photostationary Steady State Spectroscopy Assay Conditions

A. pH Dependence

The pH dependence of the 412 nm flash photolysis kinetics and photostationary steady state spectroscopy in the neutral pH area, where most of the flash photolysis assays were carried out, is shown in Figure 76. As can be seen, the pH of the fastest 412 nm decay was pH 7.0. As described in Materials and Methods V.B.I., these results were obtained by computer analysis of 20 flashes at each pH. The computer did not usually distinguish two phases in control bR, due to noise obliteration of data at longer times. However, this method of closely examining the neutral pH area, especially at 5°C, where the decay is slowed 5-fold compared to room temperature, revealed that *H. halobium* is well-adapted to physiological pH, since the most efficient photocycle occurred at this pH.

The M$_{412}$ photostationary steady state is expected to reflect the decay of M, as was shown in Results I.B.1. As shown in Figure 76, the smallest photostationary steady state absorbance occurred at pH 7.0, where the M$_{412}$ decay was also fastest. Thus this measurement was another indication of the pH dependence of the M$_{412}$ decay.

The fact that the rise of M$_{412}$ changed in one direction only suggests that the most efficient pH for the rise may not occur in the neutral pH area. Since the rise of M is not the rate-determining step of the photocycle, this does not appear to have physiological significance.
Figure 76

pH and Temperature Dependence of 412 nm Rise and Decay Kinetics

412 nm Decay \(t^{1/2}\) in microseconds, \(\Delta\) O

412 nm Rise \(t^{1/2}\) in microseconds, \(\Delta\), ●

412 nm Photostationary Steady State Absorbance

Absorbance Units,

\[ .003 \]
\[ .002 \]
\[ .001 \]

\(\triangle, \Box = 5^\circ C\)

\(\bigcirc, \bullet = 20^\circ C\)

\(\square = 22^\circ C\)
Appendix 5

B. Protein Concentration Dependence

The protein concentration dependence of the 412 nm photostationary steady state is shown in Figure 77. At low concentrations of protein there is almost a linear increase in the 412 nm absorbance with increasing protein concentration. The slight curvature in this figure was compensated for by comparing the light-induced 412 nm absorbance of chemically modified bR samples to control bR samples at the same protein concentration. The 412 nm absorbances shown in Figure 77 were in the range predicted by the 568 nm chromophore absorbances, calculated as follows: the 568 nm absorbance can be converted to molarity using $\varepsilon = 63,000 \text{ M}^{-1}\text{cm}^{-1}$ (2). Assuming 1 % as the fraction of bR photocycling, the molarity of $M_{412}$ is 1 % $[bR_{568}]$. This can be converted to absorbance at 412 nm using $\varepsilon = 32,800 \text{ M}^{-1}\text{cm}^{-1}$ (34) which is a molar extinction coefficient obtained from the low temperature spectra of M minus the contribution from $bR_{568}$. The actual fraction cycling as monitored by $M_{412}$ photostationary steady state absorbance varied between 0.5 and 4 % for bR control.

Appendix 5

C. Wavelength Dependence

The effect of changing the wavelength of the measuring light when reading the 412 nm photostationary steady state absorption was examined. The procedure for measuring the photostationary steady state was as described in Materials and Methods V.B.2., but the measuring wavelength was changed between 396 and 420 nm to determine the maximum absorbance of the bR sample. The sample was bR in 10 mM phosphate buffer, pH 7.0, 50 μg/ml (see Figure 78).
Figure 77

Effect of Protein Concentration on 412 nm Photostationary Steady Steady Absorbance

412 nm Absorbance

bR Protein Concentration in μg/ml
Figure 78

412 nm Photostationary Steady State Absorption Spectrum

Absorbance Units

Wavelength in Nanometers
Appendix 6. Imidoester Reaction at pH 8.0

Imidoester modification of bR was first carried out at pH 8.0 using 1 mM and 7.5 mM DMS. Before imidoester modification, no decrease or shift of the 568 nm chromophore was observed after light-adaptation for 30 minutes with the bR sample receiving 25 mW/cm² of visible light at 20-25°C. Following imidoester modification at pH 8.0, the position of the light-adapted 568 nm chromophore maximum did not shift and the height did not decrease compared to the Lowry protein, indicating no deterioration of the chromophore (see Table 41). Following imidoester modification and reagent removal by centrifugation, examination of the UV absorbance spectra was undertaken to detect the presence of imidoesters which absorb at 250-260 nm (ε = 500 M⁻¹cm⁻¹). The UV absorbance of the imidoester-treated, washed bR samples always appeared similar to control bR.

At pH 8.0, both 1 mM and 7.5 mM DMS modified only about 1 lysine (see Table 41). Thus, the small extent of reaction was not increased by the addition of fresh reagent, since hydrolysis of imidoester is nearly as fast as reaction at this pH (see Appendix 2.A.). The inhibition of the 412 nm decay kinetics was similar in both samples since both had one lysine modified (see Table 41). Due to the inability to increase the modification at this pH with increasing reagent, the reaction was subsequently carried out at pH 10.0.
Table 41

Imidoester Modification of bR at pH 8.0

<table>
<thead>
<tr>
<th>bR Sample</th>
<th>568 nm Chromophore As % Control</th>
<th>Number of Lysines Modified</th>
<th>412 nm Decay Kinetics</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lowry Protein</td>
<td></td>
<td>t&lt;sub&gt;1/2&lt;/sub&gt; - 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>msecs % Control</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>t&lt;sub&gt;1/2&lt;/sub&gt; - 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>msecs % Control</td>
</tr>
<tr>
<td>Control</td>
<td>100</td>
<td>0</td>
<td>4.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>17.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>1 mM DMS</td>
<td>100</td>
<td>1.0 ± 0.4</td>
<td>7.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>156</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>44.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>262</td>
</tr>
<tr>
<td>7.5 mM DMS</td>
<td>100</td>
<td>0.99 ± 0.6</td>
<td>7.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>156</td>
</tr>
<tr>
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<td>54</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>318</td>
</tr>
</tbody>
</table>
Appendix 7. Protein Concentration Dependence of Imidoester Treatment at pH 10

The protein concentration dependence during the imidoester modification was studied in order to determine if aggregation of PM occurred at any protein concentration. Aggregation of PM could change the number of lysines modified and also the pattern of oligomerization seen by gel electrophoresis.

Fluorescamine analysis revealed that the number of lysines modified by 10 mM DMS remained constant at 2.8 lysines within the error of the test (see Appendix 3) over the bR protein concentration range of 0.02 to 0.32 mg/ml. This is expected if aggregation does not occur, since this is a pseudo first order reaction with imidoester in great excess. The pattern of oligomerization also did not change over this protein range as shown in Figure 79. At 0.005 mg/ml, however, higher polymers were formed that did not enter the gel, as was quantitated with the cytochrome C internal standard. Thus it appeared that aggregation only occurred at a very low bR protein concentration. This may have been the result of the domination of hydrophobic forces at this DMS/bR molar ratio. This could have occurred if the repulsive forces between the individual patches of PM were overcome, for example, by neutralization of the negative charges on the acidic lipids and acidic amino acids by excess imidoester. Two or more PM patches could then aggregate and cause higher polymers of bR to form. None of the experiments in this thesis were carried out at 0.005 mg/ml, but rather at 0.2 mg/ml. Thus aggregation was not a factor during the amidination reactions carried out herein.
Figure 79

Effect of Protein Concentration on Oligomerization of bR by 10 mM DMS

Percent Total Protein Applied to Gel

-x1 = Monomer
-x2 = Dimer
-x3 = Trimer
-x4 = Tetramer
-x5 = Pentamer

\[
\frac{\text{DMS}}{\text{bR}} \quad \text{Molar Ratio:}
\]

<table>
<thead>
<tr>
<th>Control</th>
<th>0.4 mg/ml</th>
<th>0.32 mg/ml</th>
<th>0.13 mg/ml</th>
<th>0.02 mg/ml</th>
<th>0.005 mg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>650</td>
<td>812</td>
<td>1955</td>
<td>13,000</td>
<td>52,000</td>
<td></td>
</tr>
<tr>
<td>Higher Polymers</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

bR Protein Concentration During Amidination
Appendix 8. Effects of Successive Imidoester Treatments at pH 10.0

Imidoester modification with DMS, DMA and MA was carried out 3 or 4 times on the same bR sample in order to determine the number of treatments needed to achieve the most complete modification. The criteria for completion was to observe no change in chromophore deterioration, number of lysines modified, oligomerization and 412 nm decay kinetics. The results of successive treatments are shown in Tables 42, 43 and 44, and Figure 80. Table 45 presents a summary of all the results, indicating the number of treatments needed for completion as judged by each criteria. As shown in Table 45, modification with DMS showed the most variability in the number of treatments needed for completion. Depending on the criteria used for judgment and the imidoester concentration, this number varied from 1 to 3, with an average of about 2. The number of treatments needed for completion with DMA modification was more consistently 2, and with MA, 1. Thus the cross-linking reagents took, on the average, 2 attempts with fresh reagent to modify the full complement of lysines available to that reagent. This could be explained by a decrease in the accessibility of bifunctional reagent to lysines as the modification progresses; i.e., the first lysines modified with a long chain, bulky group may hinder the modification of the rest of the lysines. In the meantime, the reagent hydrolyzes and fresh reagent is needed to reach the more inaccessible lysines. Rates of hydrolysis compared to reaction rates for the bifunctional imidoesters were not studied by Browne and Kent (199).
### Table 42
Effect of Successive Imidoester Treatment on 568 nm Chromophore and Number of Lysines Modified

<table>
<thead>
<tr>
<th>bR Sample</th>
<th>568 nm Chromophore as % Control</th>
<th>Lowry Protein Treatment Number</th>
<th>1st</th>
<th>2nd</th>
<th>3rd</th>
<th>4th</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.5 mM DMS</td>
<td></td>
<td></td>
<td>87</td>
<td>90</td>
<td>99</td>
<td>99</td>
</tr>
<tr>
<td>5.0 mM DMS</td>
<td></td>
<td></td>
<td>92</td>
<td>100</td>
<td>76</td>
<td>-</td>
</tr>
<tr>
<td>7.0 mM DMS</td>
<td></td>
<td></td>
<td>90</td>
<td>96</td>
<td>93</td>
<td>100</td>
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<tr>
<td>10.0 mM DMS</td>
<td></td>
<td></td>
<td>79</td>
<td>89</td>
<td>70</td>
<td>-</td>
</tr>
<tr>
<td>5.0 mM DMA</td>
<td></td>
<td></td>
<td>99,100</td>
<td>89,93</td>
<td>90,92</td>
<td>-</td>
</tr>
<tr>
<td>10.0 mM DMA</td>
<td></td>
<td></td>
<td>93,100</td>
<td>100,79</td>
<td>96,97</td>
<td>-</td>
</tr>
<tr>
<td>10.0 mM MA</td>
<td></td>
<td></td>
<td>100,94</td>
<td>100,96</td>
<td>100,96</td>
<td>-</td>
</tr>
<tr>
<td>20.0 mM MA</td>
<td></td>
<td></td>
<td>94</td>
<td>88,100</td>
<td>95,100</td>
<td>-</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>bR Sample</th>
<th>Number of Lysines Modified</th>
<th>Treatment Number</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1st</td>
<td>2nd</td>
</tr>
<tr>
<td>3.5 mM DMS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.0 mM DMS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.0 mM DMS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.0 mM DMS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.0 mM DMA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.0 mM DMA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.0 mM MA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20.0 mM MA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 43

Effect of Successive Treatment with Imidoesters on Oligomerization

<table>
<thead>
<tr>
<th>bR Sample</th>
<th>% Total Protein in Gel</th>
<th>Treatment Number</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1st</td>
</tr>
<tr>
<td>3.5 mM DMS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monomer</td>
<td></td>
<td>78</td>
</tr>
<tr>
<td>Dimer</td>
<td></td>
<td>20</td>
</tr>
<tr>
<td>Trimer</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>5.0 mM DMS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monomer</td>
<td></td>
<td>53</td>
</tr>
<tr>
<td>Dimer</td>
<td></td>
<td>24</td>
</tr>
<tr>
<td>Trimer</td>
<td></td>
<td>15</td>
</tr>
<tr>
<td>Tetramer</td>
<td></td>
<td>8</td>
</tr>
<tr>
<td>Pentamer</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>7.0 mM DMS</td>
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<td></td>
</tr>
<tr>
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<td>80</td>
</tr>
<tr>
<td>Dimer</td>
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<td>17</td>
</tr>
<tr>
<td>Trimer</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Tetramer</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>Pentamer</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>10.0 mM DMS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monomer</td>
<td></td>
<td>38</td>
</tr>
<tr>
<td>Dimer</td>
<td></td>
<td>21</td>
</tr>
<tr>
<td>Trimer</td>
<td></td>
<td>17</td>
</tr>
<tr>
<td>Tetramer</td>
<td></td>
<td>24</td>
</tr>
<tr>
<td>Pentamer</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>5.0 mM DMA</td>
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</tr>
<tr>
<td>Monomer</td>
<td></td>
<td>40</td>
</tr>
<tr>
<td>Dimer</td>
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<td>23</td>
</tr>
<tr>
<td>Trimer</td>
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<td>20</td>
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<tr>
<td>Tetramer</td>
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<td>9</td>
</tr>
<tr>
<td>Pentamer</td>
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<td>8</td>
</tr>
<tr>
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</tr>
<tr>
<td>Monomer</td>
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<td>42</td>
</tr>
<tr>
<td>Dimer</td>
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<td>Pentamer</td>
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<td>6</td>
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<tr>
<td>10.0 mM MA</td>
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<td></td>
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<tr>
<td>Monomer</td>
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<td>80</td>
</tr>
<tr>
<td>Dimer</td>
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<td>20</td>
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<td>Trimer</td>
<td></td>
<td>7</td>
</tr>
<tr>
<td>20.0 mM MA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monomer</td>
<td></td>
<td>90</td>
</tr>
<tr>
<td>Dimer</td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>Trimer</td>
<td></td>
<td>-</td>
</tr>
</tbody>
</table>
Table 44

Effects of Successive Imidoester Treatment on 412 nm Decay Kinetics

<table>
<thead>
<tr>
<th>bR Sample</th>
<th>412 nm Decay $t_{1/2}$ as % Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Treatment Number</td>
</tr>
<tr>
<td></td>
<td>1st</td>
</tr>
<tr>
<td>3.5 mM DMS</td>
<td></td>
</tr>
<tr>
<td>$t_{1/2}^{-1}$</td>
<td>120</td>
</tr>
<tr>
<td>$t_{1/2}^{-2}$</td>
<td>137</td>
</tr>
<tr>
<td>5.0 mM DMS</td>
<td></td>
</tr>
<tr>
<td>$t_{1/2}^{-1}$</td>
<td>146±26</td>
</tr>
<tr>
<td>$t_{1/2}^{-2}$</td>
<td>168±51</td>
</tr>
<tr>
<td>7.0 mM DMS</td>
<td></td>
</tr>
<tr>
<td>$t_{1/2}^{-1}$</td>
<td>140</td>
</tr>
<tr>
<td>$t_{1/2}^{-2}$</td>
<td>168</td>
</tr>
<tr>
<td>10.0 mM DMS</td>
<td></td>
</tr>
<tr>
<td>$t_{1/2}^{-1}$</td>
<td>196±19</td>
</tr>
<tr>
<td>$t_{1/2}^{-2}$</td>
<td>288±57</td>
</tr>
<tr>
<td>5.0 mM DMA</td>
<td></td>
</tr>
<tr>
<td>$t_{1/2}^{-1}$</td>
<td>310±30</td>
</tr>
<tr>
<td>$t_{1/2}^{-2}$</td>
<td>275±53</td>
</tr>
<tr>
<td>10.0 mM DMA</td>
<td></td>
</tr>
<tr>
<td>$t_{1/2}^{-1}$</td>
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<td>$t_{1/2}^{-2}$</td>
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<tr>
<td>$t_{1/2}^{-1}$</td>
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<tr>
<td>$t_{1/2}^{-2}$</td>
<td>176±63</td>
</tr>
<tr>
<td>20.0 mM MA</td>
<td></td>
</tr>
<tr>
<td>$t_{1/2}^{-1}$</td>
<td>149±23</td>
</tr>
<tr>
<td>$t_{1/2}^{-2}$</td>
<td>187±4</td>
</tr>
</tbody>
</table>
Figure 80

Imidoester Successive Treatment Gel Patterns

Imidoester Concentration in mM

10,20/5,10
10,20/5,10
10,20/5,10

MA DMA
MA DMA
MA DMA
Table 45

Summary of Effects of Successive Imidoester Treatments at pH 10

<table>
<thead>
<tr>
<th>Criteria for Completion</th>
<th>bR Sample</th>
<th>Number of Treatments Needed for Completion</th>
</tr>
</thead>
<tbody>
<tr>
<td>568 nm Chromophore Deterioration</td>
<td>3.5 mM DMS</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>5.0 &quot;</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>7.0 &quot;</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>10.0 &quot;</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>5.0 mM DMA</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>10.0 &quot;</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>10.0 mM MA</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>20.0 &quot;</td>
<td>1</td>
</tr>
<tr>
<td>Number of Lysines Modified</td>
<td>3.5 mM DMS</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>5.0 &quot;</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>7.0 &quot;</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>10.0 &quot;</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>5.0 mM DMA</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>10.0 &quot;</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>10.0 mM MA</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>20.0 &quot;</td>
<td>1</td>
</tr>
<tr>
<td>Oligomerization of bR</td>
<td>3.5 mM DMS</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>5.0 &quot;</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>7.0 &quot;</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>10.0 &quot;</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>5.0 mM DMA</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>10.0 &quot;</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>10.0 mM MA</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>20.0 &quot;</td>
<td>1</td>
</tr>
<tr>
<td>412 nm Decay Kinetics</td>
<td>3.5 mM DMS</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>5.0 &quot;</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>7.0 &quot;</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>10.0 &quot;</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>5.0 mM DMA</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>10.0 &quot;</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>10.0 mM MA</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>20.0 &quot;</td>
<td>1</td>
</tr>
</tbody>
</table>
The results of this successive amidination experiment also showed the full potential of modification with each of these reagents. DMS could not modify more than $\frac{4}{6}$ fraction of total lysines available even after 3 attempts, suggesting that not all 6 lysines are available to DMS. The most complete reaction by DMA and MA was only $\frac{5}{6}$ or 0.83 fraction of total lysines available; i.e., even successively amidinating bR did not modify 100% of the lysines available for reaction.
Appendix 9. Pyridoxal Phosphate Modification of bR at pH 7.2

The results from the pyridoxal phosphate modification of bR at pH 7.2 are included here in Appendix 9, since the modification cannot be considered successful. Due to inability to reduce the pyridoxal phosphate adduct with NaBH₄, the actual number of lysines modified with pyridoxal phosphate in situ was not determined accurately, and the 412 nm kinetics may represent bR samples that have mostly reversed to control bR.

This conclusion was drawn primarily from absorption spectroscopy. Figure 81 shows the visible absorption spectrum of bR with pyridoxal phosphate in situ. The absorption maximum at low wavelengths peaks at 418 nm, which represents the Schiff base intermediate pyridoxal phosphate adduct (see Materials and Methods II.B., Figure 5). In addition, this peak is a broad peak at lower wavelengths near 388 nm, where pyridoxal phosphate absorbs, indicating the presence of excess reagent. No peak near 330 nm could be detected, although it may have been obscured by the 418 nm peak; the 330 nm peak represents the aldamine pyridoxal phosphate adduct (see Materials and Methods II.B., Figure 6). Whether pyridoxal phosphate forms the 418 nm adduct or the 330 nm adduct depends on the geometry of the lysine binding site (11), and cannot be predicted from the sequence. After removal of reagent by one centrifugation, absorption spectroscopy revealed the complete disappearance of the 418 nm peak and 388 nm absorbance, and the presence of a small peak at 330 nm. This suggested that a small amount of 330 nm aldamine adduct had always been present but not visible before removal of the Schiff base adduct. Loss of the Schiff base adduct was not unexpected, since this reaction is readily reversed upon removal of reagent (11). The 568 nm chromophore peak height decreased to 83 %
Figure 81

Visible Absorption Spectrum of bR with Pyridoxal Phosphate In Situ

Wavelength in Nanometers

2.0 Absorbance Units

1.0
of control bR after this procedure, indicating some deterioration of the chromophore structure.

In an attempt to stabilize the modification, bR samples that had been treated with pyridoxal phosphate were first subjected to 100 mM NaBH₄ to reduce the Schiff base adduct. After this treatment, the peak at 418 nm is expected to shift to 340 nm, where the reduced Schiff base pyridoxamine absorbs (195) (see Materials and Methods II.B., Figure 5). Unfortunately, this did not occur. After addition of 100 mM NaBH₄, the pyridoxal phosphate bR samples were left to reduce at room temperature for 10 minutes and were then centrifuged as described (see Materials and Methods II.B.). Absorption spectroscopy of the resuspended pellets revealed the complete disappearance of the 418 nm adduct as before, with no new peak at 340 nm. The small peak at 330 nm was unaffected. Thus it appeared as though the Schiff base pyridoxal phosphate adduct was completely removed even under these conditions of reduction. Possible explanations are: aged NaBH₄ that no longer could chemically reduce the Schiff base adduct, an insufficient quantity of NaBH₄, or not enough time allowed for reduction, although 5-10 minutes is usually sufficient (200).

When the "reduced" samples were analyzed by the fluorescamine method to detect the number of lysines modified, the results showed an average of 0.9 ± 0.5 lysines modified for four pyridoxal phosphate modified bR samples. This was thus another indication that the reaction had largely reversed, since up to 6 lysines had been available to the imidoester reagents. It is likely that the one lysine that remained modified was represented in the absorption spectrum by the 330 nm aldamine peak.
The 412 nm kinetics are shown in Table 46. The bR sample that was assayed by flash spectroscopy was not reduced with NaBH₄, since this also will reduce the retinal Schiff base linkage in the light (109). Rather it was centrifuged once, resuspended, and the flash photolysis activity measured within 15 minutes of resuspension. As shown, the 412 nm decay kinetics and photostationary steady state absorbance were slightly more inhibited than the control bR. The rise kinetics were also slightly slower than control.

This chemical modification could have been one of the most informative regarding the direct role of lysine in proton transport, since by changing lysine's charge from positive to negative, its pK is also greatly changed. Imidoester modification, on the other hand, only slightly raises the pK of lysine and may thus not significantly affect its proton transporting capabilities. Changing the charge of 6 lysines would have tested the role of lysine to carry protons to a much greater extent than changing the charge of only one lysine. For future experiments, quantitative reduction of the 418 nm peak should be carried out in the dark (so the retinal Schiff base is also not reduced), before the chemical and kinetic analysis is continued. Perhaps more NaBH₄, more time, or a fresher reagent is needed to accomplish this.
Table 46

Effect of Pyridoxal Phosphate on 412 nm Kinetics

<table>
<thead>
<tr>
<th>bR Sample</th>
<th>412 nm Decay Kinetics</th>
<th>412 nm Rise Kinetics</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$t_{1/2}^{-1}$</td>
<td>$t_{1/2}^{-2}$</td>
</tr>
<tr>
<td></td>
<td>As % Control</td>
<td>As % Control</td>
</tr>
<tr>
<td>Control</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Pyridoxal Phosphate</td>
<td>102 ± 8</td>
<td>132 ± 8</td>
</tr>
<tr>
<td>Modified</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

412 nm Mole Ratio as % Control

<table>
<thead>
<tr>
<th></th>
<th>412 nm Mole Ratio as % Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
</tr>
<tr>
<td>Pyridoxal Phosphate</td>
<td>132 ± 9</td>
</tr>
<tr>
<td>Modified</td>
<td></td>
</tr>
</tbody>
</table>
Appendix 10. 2,3-Butanedione, Reagent Removal and Reaction Reversal

Reagent Removal

The purpose of removing the reagent after a chemical modification is to find out if the changes seen in structure with the reagent in situ were not just the effect of excess reagent, or were real changes in the protein structure caused by a covalent modification. Since BD absorbs at 284 nm (see Appendix 2.B.), the 284 nm/568 nm peak height ratio was used to assess the amount of remaining BD. As shown in Table 47, with BD in situ this ratio increased above the control, even in the 10 mM BD sample, due to increased absorbance at 284 nm. All of the removal procedures listed in Table 47 were effective in removing BD. The sample that retained the most BD was the 500 mM BD sample, centrifuged once, and then loaded with very little dilution onto a Sephadex G-25 column. The 500 mM BD sample that had been centrifuged once, and resuspended in 5 ml of 50 mM borate buffer did not retain BD. Thus one or two washings and centrifugations appeared to be sufficient to remove BD, but usually three centrifugations were carried out to insure reagent removal.

Reaction Reversal

Reaction reversal has been well studied by Riordan (15) who found that removal of borate buffer will reverse a BD modification. As shown in Table 48, several procedures were used to reverse the BD modification. bR samples were initially centrifuged once and resuspended in 50 mM borate buffer. The kinetics shown in 1) in Table 48 should represent the maximum photocycling inhibition at these concentrations of
Table 47

BD Reagent Removal

<table>
<thead>
<tr>
<th>Removal Procedures</th>
<th>bR Sample</th>
<th>284 nm/568 nm Peak Height</th>
<th>284 nm/568 nm Peak Height Ratio as % Control</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BD In situ</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>10 mM BD</td>
<td>1.59</td>
<td>1.59</td>
</tr>
<tr>
<td></td>
<td>50 &quot;</td>
<td>1.75</td>
<td>1.75</td>
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<tr>
<td></td>
<td></td>
<td>2.39</td>
<td>2.39</td>
</tr>
<tr>
<td>Centrifuged</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>10 mM BD</td>
<td>1.58, 1.59</td>
<td>1.58, 1.59</td>
</tr>
<tr>
<td>Once After Reaction</td>
<td>50 &quot;</td>
<td>1.56, 1.59</td>
<td>1.56, 1.59</td>
</tr>
<tr>
<td></td>
<td>100 &quot;</td>
<td>1.46, 1.49</td>
<td>1.46, 1.49</td>
</tr>
<tr>
<td></td>
<td>200 &quot;</td>
<td>1.50, 1.50</td>
<td>1.50, 1.50</td>
</tr>
<tr>
<td></td>
<td>500 &quot;</td>
<td>1.63, 1.56</td>
<td>1.63, 1.56</td>
</tr>
<tr>
<td>Centrifuged</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Twice After Reaction</td>
<td>50 &quot;</td>
<td>1.59</td>
<td>1.59</td>
</tr>
<tr>
<td></td>
<td>100 &quot;</td>
<td>1.52</td>
<td>1.52</td>
</tr>
<tr>
<td></td>
<td>200 &quot;</td>
<td>1.55</td>
<td>1.55</td>
</tr>
<tr>
<td></td>
<td>500 &quot;</td>
<td>1.55</td>
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</tr>
<tr>
<td></td>
<td>1 M BD</td>
<td>1.56</td>
<td>1.56</td>
</tr>
<tr>
<td>Centrifuged</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Three Times After Reaction</td>
<td>50 &quot;</td>
<td>1.64</td>
<td>1.64</td>
</tr>
<tr>
<td></td>
<td>100 &quot;</td>
<td>1.63</td>
<td>1.63</td>
</tr>
<tr>
<td></td>
<td>200 &quot;</td>
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<td></td>
<td>400 &quot;</td>
<td>1.53</td>
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</tr>
<tr>
<td></td>
<td>500 &quot;</td>
<td>1.56</td>
<td>1.56</td>
</tr>
<tr>
<td>Centrifuged</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Once After Reaction, Then Eluted From Sephadex G-25</td>
<td>50 &quot;</td>
<td>1.68, 1.69</td>
<td>1.68, 1.69</td>
</tr>
<tr>
<td></td>
<td>100 &quot;</td>
<td>1.63</td>
<td>1.63</td>
</tr>
<tr>
<td></td>
<td>200 &quot;</td>
<td>1.60, 1.59</td>
<td>1.60, 1.59</td>
</tr>
<tr>
<td></td>
<td>500 &quot;</td>
<td>1.77, 1.80</td>
<td>1.77, 1.80</td>
</tr>
</tbody>
</table>

284 nm/568 nm Peak Height Ratio as % Control
Table 48

BD Reaction Reversal in Borate and Bicarbonate Buffers

<table>
<thead>
<tr>
<th>Reversal Procedures</th>
<th>bR Sample</th>
<th>412 nm Decay</th>
<th>t_{1/2} \text{-}1</th>
<th>t_{1/2} \text{-}2</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>msecs % Control</td>
<td>msecs % Control</td>
<td></td>
</tr>
</tbody>
</table>

1) bR Samples Centrifuged Once after 10 mM BD Reaction, Resuspended in 50 mM Borate Buffer
   - Control
   - 10 mM BD
   - 50 mM Borate
   - 100 mM
   - 200 mM
   - 500 mM

<table>
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<th>100</th>
<th>2.5</th>
<th>100</th>
<th>2.5</th>
<th>100</th>
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<td>3.8</td>
<td>153</td>
<td>7.8</td>
<td>180</td>
<td>18.0</td>
<td>735</td>
<td>22.0</td>
<td>800</td>
<td>30.0</td>
<td>1225</td>
<td>49.0</td>
<td>1980</td>
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<tr>
<td></td>
<td>12.0</td>
<td>485</td>
<td>15.0</td>
<td>400</td>
<td>18.0</td>
<td>735</td>
<td>22.0</td>
<td>800</td>
<td>30.0</td>
<td>1225</td>
<td>49.0</td>
<td>1980</td>
</tr>
<tr>
<td></td>
<td>66.0</td>
<td>236</td>
<td>153.0</td>
<td>552</td>
<td>210.0</td>
<td>757</td>
<td>538.0</td>
<td>1937</td>
<td>725.0</td>
<td>2613</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>100.0</td>
<td>100</td>
<td>100.0</td>
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<td>100</td>
<td>100.0</td>
<td>100</td>
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<td></td>
</tr>
</tbody>
</table>

2) Samples from 1), Centrifuged and Eluted from Sephadex G-25 Column in 100 mM Bicarbonate Buffer
   - Control
   - 10 mM BD
   - 50 mM Bicarbonate
   - 100 mM
   - 200 mM
   - 500 mM

<table>
<thead>
<tr>
<th></th>
<th>2.0</th>
<th>100</th>
<th>2.0</th>
<th>100</th>
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<th>100</th>
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<th>100</th>
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</thead>
<tbody>
<tr>
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<td>4.2</td>
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<td>1.5</td>
<td>75</td>
<td>2.0</td>
<td>100</td>
<td>14.0</td>
<td>700</td>
<td>30.0</td>
<td>1225</td>
<td>100.0</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>21.0</td>
<td>206</td>
<td>23.5</td>
<td>168</td>
<td>20.0</td>
<td>143</td>
<td>172.0</td>
<td>1229</td>
<td>700.0</td>
<td>5000</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

3) Samples from 1), Centrifuged and Eluted from Sephadex G-25 Column in 50 mM Borate Buffer
   - Control
   - 10 mM BD
   - 50 mM Borate
   - 200 mM
   - 500 mM

<table>
<thead>
<tr>
<th></th>
<th>2.0</th>
<th>100</th>
<th>2.0</th>
<th>100</th>
<th>2.0</th>
<th>100</th>
<th>2.0</th>
<th>100</th>
<th>2.0</th>
<th>100</th>
<th>2.0</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>14.0</td>
<td>100</td>
<td>14.0</td>
<td>100</td>
<td>14.0</td>
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</tr>
<tr>
<td></td>
<td>20.0</td>
<td>143</td>
<td>23.5</td>
<td>168</td>
<td>20.0</td>
<td>143</td>
<td>172.0</td>
<td>1229</td>
<td>700.0</td>
<td>5000</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

4) Samples from 2), Centrifuged and Washed 3 Times in 100 mM Bicarbonate Buffer
   - Control
   - 10 mM BD
   - 200 mM
   - 500 mM

<table>
<thead>
<tr>
<th></th>
<th>2.0</th>
<th>100</th>
<th>2.0</th>
<th>100</th>
<th>2.0</th>
<th>100</th>
<th>2.0</th>
<th>100</th>
<th>2.0</th>
<th>100</th>
<th>2.0</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.5</td>
<td>75</td>
<td>14.0</td>
<td>700</td>
<td>30.0</td>
<td>1225</td>
<td>100.0</td>
<td>100</td>
<td>100.0</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>23.5</td>
<td>168</td>
<td>172.0</td>
<td>1229</td>
<td>700.0</td>
<td>5000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>


BD. These samples were then divided in half, recentrifuged and loaded onto two Sephadex columns, one equilibrated with 50 mM borate buffer and one equilibrated with 100 mM bicarbonate buffer. As shown in Table 48, the samples from the bicarbonate column showed more reversal than the samples from the borate column. It is perhaps more illuminating to compare the $t_{1/2}$'s as % control, since the control $t_{1/2}$'s varied quite considerably between experiments. When the samples from the bicarbonate column had been washed by diluting in 25 ml of bicarbonate buffer and centrifuged 3 times, the reversal was more evident, especially at low concentrations of BD (see 4) in Table 48. It was surprising that the 500 mM BD sample remained quite inhibited in the decay even after all these procedures. These results suggest that bicarbonate buffer may have some stabilizing properties, although not as much as borate buffer.

As described in Materials and Methods III.A.1.c. several procedures were carried out to determine the effect of NaCl on the extent of the BD reaction and also on reaction reversal. This was a necessary control for the BD modification of cell envelope vesicles which must be carried out in 4 M NaCl. As shown in Table 49, control bR had a slightly slower decay in 4 M NaCl than a bR control in 10 mM phosphate buffer. All the samples in Table 49 were compared to the control in 4 M NaCl, even though 5) was resuspended in distilled water. The actual $t_{1/2}$'s are also provided in Table 49 for comparison.

The kinetics for 200 mM BD modified bR in 4 M NaCl were much less inhibited than 200 mM BD modified bR without NaCl, as shown in 7) in Table 49. Thus the BD reaction was slowed down in the presence of
**Table 49**

**Effect of NaCl on Reaction Reversal**

<table>
<thead>
<tr>
<th>Reversal Procedures</th>
<th>$t_{1/2}^{-1}$ msecs</th>
<th>$t_{1/2}^{-2}$ msecs</th>
<th>% Control</th>
<th>% Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) None; bR Control in 4 M NaCl</td>
<td>5.5</td>
<td>24.8</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>2) None; bR Sample with 200 mM BD in situ, 4 M NaCl and 50 mM Borate Buffer</td>
<td>9</td>
<td>148</td>
<td>164</td>
<td>597</td>
</tr>
<tr>
<td>3) Sample from 2) Centrifuged and Supernatant Left at 4° C for 2 Days; Recentrifuged and Resuspended in 4 M NaCl</td>
<td>8</td>
<td>52</td>
<td>145</td>
<td>210</td>
</tr>
<tr>
<td>4) Sample from 2) Centrifuged and Pellet Resuspended in 4 M NaCl; Left at 4° C for 1 Day</td>
<td>4</td>
<td>58</td>
<td>73</td>
<td>234</td>
</tr>
<tr>
<td>5) Sample from 4) Centrifuged and 1/2 Pellet Resuspended in H$_2$O; Left at 20° C for 1 Day</td>
<td>4.5</td>
<td>37.5</td>
<td>82</td>
<td>151</td>
</tr>
<tr>
<td>6) Sample from 4) Centrifuged and 1/2 Pellet Resuspended in 4 M NaCl; Left at 20° C for 1 Day; Recentrifuged and Resuspended in 4 M NaCl</td>
<td>6</td>
<td>44</td>
<td>109</td>
<td>177</td>
</tr>
<tr>
<td>7) 200 mM BD-Modified bR in 50 mM Borate Buffer Without 4 M NaCl</td>
<td>30</td>
<td>538</td>
<td>545</td>
<td>2170</td>
</tr>
</tbody>
</table>
NaCl, perhaps due to the intervening charge of NaCl disrupting the reaction or the stabilization by borate buffer. The remainder of Table 49 shows that progressive removal of borate buffer caused the reaction to reverse. Since there was not much difference between 5) and 6) in Table 49, NaCl did not appear to slow down or accelerate BD reaction reversal.
Appendix 11. Effect of Modifying bR with BD in Cell Envelope Vesicles on Photocycling Kinetics

The purpose of carrying out BD modification in cell envelope vesicles was to find out the orientation of the arginines which are important for activity, since cell envelope vesicles contain bR in primarily one orientation only (1). Thus this study might be able to tell if the single pair of important arginines (see Results II.B.1.) is located on the external side of bR in the PM.

BD modification of cell envelope vesicles needs to be carried out in 4 M NaCl in addition to 50 mM borate buffer, pH 8.2, since the cell envelope vesicles require high salt to maintain their structure (1). As a control for this experiment, BD modification was carried out on purified PM in 4 M NaCl and 50 mM borate buffer, pH 8.2. The flash photolysis kinetics were carried out in 10 mM phosphate buffer, pH 8.8. As shown in Table 50, inhibition of M_{412} decay by 500 mM BD in 4 M NaCl was not as extensive (about 1/3) as inhibition of M_{412} decay by BD in 50 mM borate buffer only. This may have been due to charge shielding of the positively charged guanidinium groups by the chloride ions of NaCl, thus interfering with the attraction of the polar BD molecule. In addition, the modification of bR in cell envelope vesicles caused a greater inhibition than in purified PM, indicating the accessibility to bR by BD was at least as good in PM in cell envelope vesicles. Thus this experiment did not reveal any information about the position of the arginines involved in the inhibition, but rather indicated that the BD molecule is sufficiently small to traverse the membrane of the cell envelope vesicles.
Table 50

Effect of Modifying bR in Cell Envelope Vesicles on M_{412} Decay Kinetics

<table>
<thead>
<tr>
<th>bR Sample</th>
<th>$t_{1/2}^{-1}$</th>
<th>$t_{1/2}^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>msecs % Control</td>
<td>msecs % Control</td>
</tr>
<tr>
<td>Control (Unmodified)</td>
<td>4.2 100</td>
<td>18.8 100</td>
</tr>
<tr>
<td>500 mM BD in Cell Envelope Vesicles (Modified in 4 M NaCl and 50 mM borate buffer)</td>
<td>45 1071</td>
<td>285 1516</td>
</tr>
<tr>
<td>500 mM BD (Modified in 4 M NaCl and 50 mM borate buffer)</td>
<td>12.4 295</td>
<td>208 1106</td>
</tr>
<tr>
<td>500 mM BD (Modified in 50 mM borate buffer only)</td>
<td>48 1143</td>
<td>725 3856</td>
</tr>
</tbody>
</table>
Appendix 12. Possible Modification of Lysine Residues by BD

Since the nitrogens of the guanidinium group react with BD, possible reaction of lysine amino groups in bR with BD was checked. The fluorescamine test revealed that no lysines were modified at any concentration of BD. This was expected since it is thought that borate buffer stabilizes the imidazoline adduct that can only be formed with 2 close-lying nitrogens (see Figure 12) as in the guanidinium group of arginine (15). It is possible that two lysines next to each other could each contribute one nitrogen, but this was not seen. Thus this reagent is thought to modify only arginines, so the effects on photocycling and other activities of bR are due to a single type of charge and molecular alteration.
Appendix 13. Phenylglyoxal Modification of Arginine Residues in bR

The results of the PGO modification of arginine residues in bR are included here in Appendix 13, since this was a trial experiment which determined that the PGO modification should not be explored in detail. The disadvantages of this modification were: 1) presence of UV absorption after modification that interferes with tryptophan fluorescence studies, 2) reversibility of the modification when PGO was removed, and 3) nonspecificity, since 2 lysines were modified as well as arginines.

The results of absorption spectroscopy are divided into two experiments in Table 51 as described in Materials and Methods III.B.2. As shown in Table 51, the 568 nm chromophore had decreased to 63% of the control in the PGO modified sample after two centrifugations and one dialysis for two days indicating considerable chromophore deterioration. UV spectroscopy revealed considerable absorbance at 260 nm and below, but the 340 nm peak which should represent the PGO-arginine adduct (201) was not present. A similar absorption was seen at 260 nm and below by Yang et al. (202) when they used PGO to modify cobra toxin. These investigators saw an increase in this UV absorption as they increased the number of arginines modified by increasing the pH. Thus it is likely that in some proteins the PGO-adduct absorbs below 260 nm. This was also suggested from Table 51, since the 260 nm and below UV absorbance was not easily removed by repeated centrifugations or column chromatography on Sephadex G-25. The problem with an adduct that absorbs in the UV region is that tryptophan also absorbs in the same region. As a result, tryptophan
Table 51

Effect of PGO on Absorption Spectroscopy

<table>
<thead>
<tr>
<th>Reagent Removal Procedures After PGO Reaction</th>
<th>bR Sample</th>
<th>568 nm Chromophore As % Control</th>
<th>260 nm Lowry Protein As % Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>None; Diluted Reagent In Situ</td>
<td>Control</td>
<td>100</td>
<td>100 ± 6</td>
</tr>
<tr>
<td></td>
<td>PGO-Modified</td>
<td>93 ± 2</td>
<td>266 ± 76</td>
</tr>
<tr>
<td>Centrifuged and Resuspended</td>
<td>Control</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>PGO-Modified</td>
<td>-</td>
<td>141 ± 3</td>
</tr>
<tr>
<td>Recentrifuged and Dialyzed for Two Days</td>
<td>Control</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>PGO-Modified</td>
<td>63 ± 3</td>
<td>133 ± 9</td>
</tr>
</tbody>
</table>

Experiment # 2

<table>
<thead>
<tr>
<th>Centrifuged and Resuspended</th>
<th>Control</th>
<th>-</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PGO-Modified</td>
<td>-</td>
<td>182 ± 45</td>
</tr>
</tbody>
</table>

Chromatographed on Sephadex G-25

<table>
<thead>
<tr>
<th>In Borate Buffer</th>
<th>Control</th>
<th>-</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>In Carbonate Buffer</td>
<td>PGO-Modified</td>
<td>-</td>
<td>139</td>
</tr>
<tr>
<td></td>
<td>PGO-Modified</td>
<td>-</td>
<td>145</td>
</tr>
</tbody>
</table>
fluorescence measurements, which are a sensitive probe of the protein environment, cannot be carried out.

In Table 52 is shown the effect of PGO on the 412 nm kinetics. With the diluted reagent in situ after the reaction, the 412 nm decay was as inhibited as BD modification of arginine, in both phases of the decay. With only one centrifugation, however, the PGO modification reversed to less than 1/2 of the initial inhibition. This was consistent with other investigations that have shown that the PGO modification will reverse if PGO is removed at neutral or slightly alkaline pH (17). It is undesirable to work with a modification that reverses if this can be avoided, since the activity must be measured with the reagent in situ, and the photocycling activity may be affected by excess reagent as well as by a covalent modification.

Table 52 also lists the effect of D<sub>2</sub>O on the PGO modification. The values of D<sub>2</sub>O/H<sub>2</sub>O in Table 52 are quite similar to the bR control values of 2.1 for the 412 nm decay, 4.8 for the 412 nm rise, and 3.2 for the 412/568 mole ratio. They are included in this table simply to emphasize that PGO modification did not show an unusual or new D<sub>2</sub>O effect.

Finally, the fluorescamine test revealed 2.2 ± 0.1 lysines modified by PGO. PGO is thought to react with lysines during extensive modification of proteins (203). Thus the third drawback to the PGO reaction is that it is non-specific for arginines. The modification of lysines as well as arginines obscures the contribution of each amino acid's modification to the inhibition of the 412 nm kinetics.
Table 52
Effect of PGO on 412 nm Kinetics

<table>
<thead>
<tr>
<th>Reagent Removal Procedures After PGO Reaction</th>
<th>bR Sample</th>
<th>412 nm Decay Kinetics</th>
<th>412 nm Rise Kinetics</th>
</tr>
</thead>
<tbody>
<tr>
<td>None; Diluted Control</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Reagent In Situ PGO-Modified</td>
<td>3565 ± 1034</td>
<td>3464 ± 771</td>
<td></td>
</tr>
<tr>
<td>Centrifuged and Resuspended PGO-Modified</td>
<td>1236 ± 121</td>
<td>843 ± 255</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>D₂O Effect</th>
<th>bR Sample</th>
<th>412 nm Decay Kinetics</th>
<th>412 nm Rise Kinetics</th>
</tr>
</thead>
<tbody>
<tr>
<td>D₂O/H₂O</td>
<td>53</td>
<td>308</td>
<td>133</td>
</tr>
<tr>
<td>PGO-Modified in H₂O</td>
<td>145</td>
<td>724</td>
<td></td>
</tr>
</tbody>
</table>

Mole Ratio as % Control

| D₂O/H₂O                                       | 34.0      | 54.4                  |
| PGO-Modified in H₂O                           |           |                       |
| PGO-Modified in D₂O                           |           |                       |
Appendix 14. Probability Calculations Used to Analyze the Inhibition of $M_{412}$ Decay by BD Modification of Arginines

The following probability relations are adapted from (204). Let the probability of modifying an arginine be $x$, which is the fraction of arginines modified determined by amino acid analysis. As a first simple analysis, consider that there are three arginines capable of being modified. Then the random probabilities of modification of arginine are:

- none modified = $(1-x)^3$
- one modified = $3x(1-x)^2$
- two modified = $3x^2(1-x)$
- all three modified = $x^3$

Next consider the following cases:

Case 1: Only one specific arginine is needed to inhibit, so the inhibition of $M_{412}$ decay ($I(x)$) is equal to $I(x) = x$.

Case 2: Any one out of three arginines can inhibit. Since this depends on random modification, the probabilities given above that will be able to inhibit should be added:

$$I(x) = 3x - 3x^2 + x^3$$

Case 3: Only a specific pair can inhibit, so $I(x) = x^2$.

Case 4: Any two out of three can inhibit. Since this depends on random modification, the probabilities given above that will be able to inhibit should be added:
\[ 3x^2(1-x) \quad \text{(two modified)} \]
\[ + \frac{x^3}{3} \quad \text{(three modified)} \]

\[ I(x) = 3x^2 - 2x^3 \]

Case 5: All three are needed to inhibit, so \( I(x) = x^3 \).

In a slightly more complicated analysis, consider that there are four arginines capable of being modified. Then the random probabilities of modification of arginine are:

- none modified \( = (1-x)^4 \)
- one modified \( = 4x(1-x)^3 \)
- two modified \( = 6x^2(1-x)^2 \)
- three modified \( = 4x^3(1-x) \)
- all four modified \( = x^4 \)

Case 6: Any two out of four arginines modified can inhibit.

Since this depends on random modification, the probabilities given above that can inhibit should be added:

\[ 6x^2(1-x)^2 \quad \text{(two modified)} \]
\[ + \quad 4x^3(1-x) \quad \text{(three modified)} \]
\[ + \frac{x^4}{4} \quad \text{(four modified)} \]

\[ I(x) = 6x^2 - 8x^3 + 3x^4 \]

In the analysis of the actual BD modification of bR as carried out in this thesis, consider that there are seven arginines capable of being modified. Then the random probabilities of modification of arginine are:

- none modified \( = (1-x)^7 \)
- one modified \( = 7x(1-x)^6 \)
- two modified \( = 21x^2(1-x)^5 \)
- three modified \( = 35x^3(1-x)^4 \)
- four modified \( = 35x^4(1-x)^3 \)
- five modified \( = 21x^5(1-x)^2 \)
- six modified \( = 7x^6(1-x) \)
- seven modified \( = x^7 \)
Case 7: Any two out of seven arginines modified can inhibit.

Since this depends on random modification, the probabilities given above that can inhibit should be added:

\[ I(x) = 21x^2 - 70x^3 + 105x^4 - 84x^5 + 35x^6 - 6x^7 \]

When this function was graphed vs. the real first phase $M_{412}$ decay data as were the functions in Figure 48, a straight line was not obtained, but rather the plot curved in a manner similar to the plot of $3x^2 - 2x^3$. Thus this choice was also not correct; rather $x^2$ was the correct function since it yielded a straight line in Figure 48.
REFERENCES

15. Riordan, J. (1973), Biochem. 12, 3915-3923.


63. Stockburger, M., Klusmann, W., Gattermann, H., Massig, G. and Peters, R. (1979), Biochem. 18, 4886-4900.
Kernforschungsanlage Jülich Nr. 1374, Zentralbibliothek der
Kernforschungsanlage, Jülich, F.R.G. 1-42.

82. Lewis, A., Spoonhower, J. and Perreault, G. (1976), Nature
260, 675-678.

Acta. 546, 106-120.

84. Alfano, R., Yu, W., Govindjee, R., Becher, B. and Ebrey, T.
(1976), Biophys. J. 16, 1399-1409.

Biophys. J. 16, 1399-1409.


200, 1279-1281.

22, 67-77.


90. Kaufmann, K., Sundstrom, V., Yamane, T. and Rentzepis, P.

57-60.

Yamamoto, N. and Waddell, W. (1976), J. Phys. Chem. 80,
2265-2273.

93. Talbot, B. (1970), in: Optical Methods In Biology, p. 588,


95. Johnston, E. and Zand, B. (1973), Biochem. 12, 4631.

New Biol. 233, 149.


Res. Commun. 67, 897-903.


110. Means, G. and Feeney, R., Ibid., p. 11.


180. Ibid., p. 268.

181. Ibid., p. 264.


196. (Formula derived in consultation with the Statistics Department Counseling Service, University of California, Berkeley.)


