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STRUCTURE-FUNCTION STUDIES OF BOVINE RHODOPSIN

by

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Submitted to the Department of Chemistry on March 16,
1994 in partial fulfillment of the requirements for the
degree of Doctor of Philosophy

ABSTRACT

Rhodopsin is an integral membrane glycoprotein that is the G protein-coupled receptor of the rod cell. 11-*cis* Retinal, a vitamin A analog, binds to the apoprotein, opsin, via a protonated Schiff base linkage with lysine 296. Bovine rhodopsin has three major covalent modifications: 1) A single disulfide bond between cys110 and cys187; 2) Two palmitoylated cysteine residues at 322 and 323; 3) Two asparagine(N)-linked glycosylation sites at asn2 and asn15.

Previously in our laboratory opsin has been expressed in COS-1 cells, regenerated with 11-*cis* retinal and immunoaffinity purified. The yield of opsin from these cells was about 10 $\mu\text{g}/1.25\text{-}1.5 \times 10^7$ cells. In an attempt to obtain greater quantities of protein, bovine opsin was expressed in the baculovirus/insect cell system. A method was developed to obtain pure rhodopsin. Furthermore insect cell rhodopsin had N-linked glycosylation similar to rod cell rhodopsin, was palmitoylated and triggered transducin like it as well. The total amount of opsin produced by insect cells was about ten-fold greater than COS-1 cells. However the amount of purified rhodopsin was 1/10 on a cell-per-cell basis. A large fraction of insect cell opsin did not regenerate and was misfolded. Despite this, expression of rhodopsin mutants in insect cells may be an attractive alternative expression system since they can be grown at high density in suspension.

Our laboratory has established the role of the single disulfide bond and of palmitoylation by site-directed mutagenesis and biochemical studies. Using a similar approach the role of glycosylation in rhodopsin folding, transport and function has been studied. Complementing this approach wild-type opsin has been expressed in the presence of tunicamycin (TM), a well-known inhibitor of N-linked

glycosylation. Glycosylation does not affect the folding or transport of opsin. However unglycosylated opsin is 1/10 less efficient in triggering transducin, the cognate G-protein of the rod cell.

Retinitis Pigmentosa (RP) is a hereditary retinal degeneration. It is the most common cause of blindness in people under the age of 50. Its prevalence is 1/3000 live births. RP can be inherited as an autosomal dominant, autosomal recessive or X-linked trait. The clinical hallmarks of this disease include nightblindness, mid-peripheral visual field loss, progressive diminution of electroretinogram (ERG) signals and eventual blindness, most often before the age of 60.

Within the past four years over 40 mutations in the opsin gene have been described to be associated with autosomal dominant retinitis pigmentosa (ADRP). Most of these mutants are single amino acid substitutions, a few are small deletions. Some amino acid positions have multiple substitutions. Nearly all of the currently known mutations have been constructed by site-specific cassette mutagenesis of the bovine rhodopsin gene. After confirming the presence of the mutation by DNA sequencing, the mutant genes have been expressed in COS-1 cells, immunoaffinity purified and studied biochemically. There is significant heterogeneity among the mutant proteins. They can be classified into three groups: Class I (e.g. L125R) had wild-type levels of expression and chromophore formation. These mutants also had wild-type glycosylation and were transported to the cell surface. Class II mutants (e.g. P171L) were expressed at 1/2 to 1/3 of wild-type, did not form any chromophore and had abnormal glycosylation. They were retained in the endoplasmic reticulum (ER). They represented misfolded forms of opsin. Class III (e.g. P23H) mutants were expressed at levels similar to the Class II mutants but they formed some chromophore but the amount was less than 1/2 to 1/3 of wild-type. These mutant proteins were also abnormally glycosylated and retained in the ER. They probably represent slow folding forms of opsin. The majority of the ADRP mutant proteins fell into the class II and class III categories. The photobleaching behavior of all the mutants that formed chromophore was also studied as well as their ability to trigger transducin. Nearly all the mutants bleached more quickly upon illumination i.e. they released the photoisomerized chromophore, all-*trans* retinal, more rapidly than wild-type rhodopsin. These mutants also triggered transducin less efficiently than wild-type. This finding provides a biochemical basis of nightblindness in these patients.

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Even though my father will never see me receive my doctorate he is still very much part of my life. The simple values he taught us three children, work hard, be truthful, be happy and always remember the importance of your family, are very much a part of me.

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Table of Contents

Abstract	3
Acknowledgements	5
Table of Contents	7
List of Figures	11
List of Tables	13
Abbreviations	14
1. Introduction	
1.1 The Eye and Retina	15
1.2 The Photoreceptor Cells: Rods and Cones	17
1.3 The Rod Inner and Outer Segments	19
1.4 Rhodopsin- The Photoreceptor Protein	
1.4.1 Rhodopsin Structure	20
1.4.2 The G Protein-Coupled Receptor Superfamily	23
1.4.3 Spectral Properties of Rhodopsin	23
1.4.4 Covalent Modifications of Rhodopsin	26
1.4.5 The Three Regions of Rhodopsin	28
1.4.6 Light Activation of Rhodopsin	30
1.5 The Visual Cascade	30
1.6 Transducin- The G Protein of the Rod Cell	33
1.7 Phosphodiesterase	35
1.8 The cGMP-Gated Channel	35
1.9 Inactivation of the Visual System	36
1.10 Goals of Thesis	36
2. Purification and Characterization of Bovine Opsin Expressed in Insect Cells	
2.1 Introduction	42
2.2 Materials and Methods	
2.2.1 Materials	43

2.2.2	Construction of pVL1393-rho and Purification of Recombinant Viral Particle	43
2.2.3	Detergent Extraction	47
2.2.4	Quantitation of Opsin from Whole Cell Extracts	47
2.2.5	Time Course of Expression	48
2.2.6	Glycosylation of Insect Cell Opsin	48
2.2.7	Palmitoylation of Insect Cell Opsin	49
2.2.8	Whole Cell Regeneration and Purification of Rhodopsin	50
2.2.9	Membrane Preparation and Purification of Rhodopsin	50
2.2.10	Purification of Rhodopsin and Transducin	51
2.2.11	Transducin Activation	51
2.3	Results	
2.3.1	Detergent Extraction	52
2.3.2	Quantitation of Opsin	52
2.3.3	MOI and Time Course of Expression	54
2.3.4	Glycosylation	57
2.3.5	Palmitoylation	57
2.3.6	Purification	62
2.3.7	Functional Activity	65
2.4	Discussion	65
3.	The Role of N-Linked Glycosylation in Rhodopsin Structure and Function	
3.1	Introduction	71
3.2	Materials and Methods	
3.2.1	Materials	75
3.2.2	Construction of Rhodopsin Mutants	75
3.2.3	Expression and Purification of Mutants	76
3.2.4	<i>In vivo</i> Labelling of Opsin in COS-1 Cells with ^3H -Mannose and ^3H -Palmitic Acid	77
3.2.5	Determination of Molar Extinction Coefficients	78
3.2.6	Binding and Activation of Transducin	78
3.2.7	Determination of Metarhodopsin II Half-life	79
3.2.8	Phosphorylation of Rhodopsin and Mutants	79
3.3	Results	
3.3.1	Replacement of Asn by Gln at Glycosylation Sites in Rhodopsin	80

3.3.2	Sugar Labelling of Glycosylation Mutants	82
3.3.3	Cellular Localization of Mutants	82
3.3.4	Hydroxylamine Sensitivity of Mutants	85
3.3.5	Characterization of COS Cell Rhodopsin Expressed in the Presence of Tunicamycin	85
3.3.6	Characterization of Other Glycosylation Site Mutants	88
3.3.7	Phosphorylation of Rhodopsin and Mutants	91
3.4	Discussion	92
4.	Structural and Functional Characterization of Rhodopsin Mutants Associated with Autosomal Dominant Retinitis Pigmentosa (ADRP)	
4.1	Introduction	98
4.2	Materials and Methods	
4.2.1	Materials	104
4.2.2	Construction of Rhodopsin Mutants	105
4.2.3	Expression and Purification of Mutants	106
4.2.4	Binding and Activation of Transducin	107
4.2.5	Sodium Carbonate Extraction of COS cell Membranes	107
4.2.6	Phase Separation of Proteins in TritonX-114 Solution	108
4.2.7	Immunofluorescence Microscopy and Fluorescence Activated Cell Sorting (FACS)	109
4.3	Results	109
4.3.1	Mutants at the N-Terminus	111
4.3.2	Mutants Found in the Intradiscal Loops	117
4.3.3	Mutants Found in the Transmembrane Region	119
4.3.4	Mutants at the C-Terminus	123
4.3.5	Expression of Wild-Type Opsin in the Presence of Brefeldin A (BFA)	124
4.3.6	Bleaching Properties of the ADRP Mutants that Regenerated	125
4.3.7	Transducin Activation	129
4.3.8	Purification of Mutants on K16-50 Sepharose4B Resin	131
4.3.9	Extraction of COS-1 Cell Membranes	133
4.3.10	Phase Separation of Opsin with TritonX-114	135
4.4	Discussion	137
5.	Appendices	

5.1 Spectra of Glycosylation Mutants	148
5.2 Bleaching Spectra of the Glycosylation Mutants	155
5.3 Hydroxylamine Sensitivity of Glycosylation Mutants	164
5.4 GTP γ S Exchange Assay of other Glycosylation Mutants	172
5.5 Expression of N2Q, N15Q and N2,15Q in the Presence of Tunicamycin	174
5.6 UV/visible Absorption Spectra of ADRP Mutants	177
5.7 Bleaching Spectra of ADRP Mutants	199
References	210

Figures

1. Introduction

- 1-1 Anatomy of the eye
- 1-2 Anatomy of the sensory retina
- 1-3 Structure of the rod cell
- 1-4 Secondary structure model of bovine opsin
- 1-5 UV/visible absorption of rhodopsin
- 1-6 UV/visible absorption spectra of a model Schiff base
- 1-7 Structure of 11-*cis* retinal
- 1-8 Photobleaching sequence of rhodopsin
- 1-9 The visual cycle

2. Purification and Characterization of Bovine Opsin Expressed in Insect Cells

- 2-1 Schematic of isolating recombinant virus
- 2-2 Dot-blot of recombinant virus
- 2-3 Detergent solubilization of insect opsin and purification with 1% DTAB
- 2-4 Standard curve for opsin quantitation
- 2-5 Time course of expression
- 2-6 UV/visible absorption spectra of regenerated insect cell opsin 24 and 48 hrs post-infection
- 2-7 Sensitivity of insect cell opsin to N-glycosidase F
- 2-8 Carbohydrate structure of insect cell opsin
- 2-9 Palmitoylation of insect cell opsin
- 2-10 UV/visible absorption spectra of insect cell and ROS rhodopsin
- 2-11 Gel characteristics of insect cell opsin purified to a $A_{280}/A_{500}=3.0$
- 2-12 Purification of insect cell rhodopsin by a membrane fractionation protocol
- 2-13 Light-dependent transducin activation of insect cell and ROS rhodopsin

3. The Role of N-Linked Glycosylation in Rhodopsin Structure and Function

- 3-1 Synthetic pathway of glycosylation structure of oligosaccharides isolated from bovine opsin

- 3-2 Secondary structure model of bovine opsin
- 3-3 UV/visible absorption spectra of nonglycosylated wild-type rhodopsin and glycosylation site mutants
- 3-4 Characterization of glycosylation-site mutants of opsin
- 3-5 Cellular localization of glycosylated and non-glycosylated opsins by immunofluorescence
- 3-6 Characterization of wild-type opsin expressed in the absence and presence of tunicamycin
- 3-7 Transducin activation by nonglycosylated wild-type rhodopsin and glycosylation-site mutants
- 3-8 UV/visible absorption spectra of glycosylated, non-nonglycosylated wild-type and N15Q rhodopsins upon illumination
- 3-9 Phosphorylation of glycosylated, nonglycosylated and glycosylation-site mutants

4. Biochemical Characterizations of Rhodopsin Mutants Associated with Autosomal Dominant Retinitis Pigmentosa (ADRP)

- 4-1 The funduscopy and electroretinographic features of RP
- 4-2 A secondary structure model of bovine opsin
- 4-3 UV/visible absorption spectra of Class III mutants
- 4-4 Characterization of the three types of ADRP mutants by Western blots
- 4-5 Cellular localization of ADRP mutant opsins by immunofluorescence
- 4-6 UV/visible absorption spectra of Class II mutants
- 4-7 UV/visible absorption spectra of wild type and Class I mutants
- 4-8 Expression of opsin in the presence of Brefeldin A
- 4-9 Bleaching characteristics of ADRP mutants
- 4-10 Transducin activation by ADRP mutants
- 4-11 UV/visible absorption spectra of wild-type opsin and K296E purified on K16-50 antibody column
- 4-12 Extraction of opsin with 0.1 M sodium carbonate
- 4-13 Phase partitioning of wild-type opsin and ADRP mutants with 1% TritonX-114

Tables

- 1-1 G-protein coupled receptors
- 4-1 Clinical features of Retinitis Pigmentosa
- 4-2 Mutations associated with ADRP
- 4-3 Classification of ADRP Mutant Phenotypes
- 4-4 Cell Surface Expression of ADRP Mutants by FACS

Abbreviations

AcMNPV	Autographa californica nuclear polyhedrosis virus
BFA	Brefeldin A
BSA	Bovine serum albumin
CD	Circular dichroism
CHO	Chinese hamster ovary
DM	Dodecyl maltoside
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethyleneglycol-bis(β -aminoethyl ether) N,N,N',N'-tetraacetic acid
ER	Endoplasmic reticulum
FACS	Fluorescence Activated Cell Sorting or Sorter
LM	Lauryl maltoside also known as dodecyl maltoside
m I	Metarhodopsin I
m II	Metarhodopsin II
MOI	Multiplicity of infection
NEM	N-ethyl maleimide
PBS	Phosphate buffered saline
PDE	Phosphodiesterase
PDI	Protein disulfide isomerase
PMSF	Phenylmethyl sulfonyl chloride
RIS	Rod inner segment
ROS	Rod outer segment
RP	Retinitis pigmentosa
Sf9	<i>Spodoptera frugiperda</i> (insect cells)
SDS	Sodium dodecyl sulfate
TCA	Trichloroacetic acid

Chapter 1

INTRODUCTION

1.1 The Eye and Retina

The human eye is the sensory modality that mediates light perception. As such it has specific receptors for detecting low light intensities as well as color. The eye is composed of many transparent tissues including the lens, aqueous humor and the vitreous humor whose primary purpose is to filter and focus the incoming light stimulus to the retina (Figure 1-1) (Schichi, 1983).

The retina is composed of both non-neural and neural components. Immediately anterior to it is the vitreous humor. Posterior to the retina is the retinal pigment epithelium (RPE) followed by the choroid and sclera. These tissues provide the metabolic and nutritional requirements of the neural retina. The RPE does not directly contact the neural retinal cells but plays a critical role in the phagocytosis and subsequent turnover of both photoreceptor cell types, the rods and the cones.

Structurally the retina is organized in various layers (Schichi, 1983). Beginning from the vitreous side the six layers include the inner limiting membrane, the nerve fiber layer, the inner plexiform

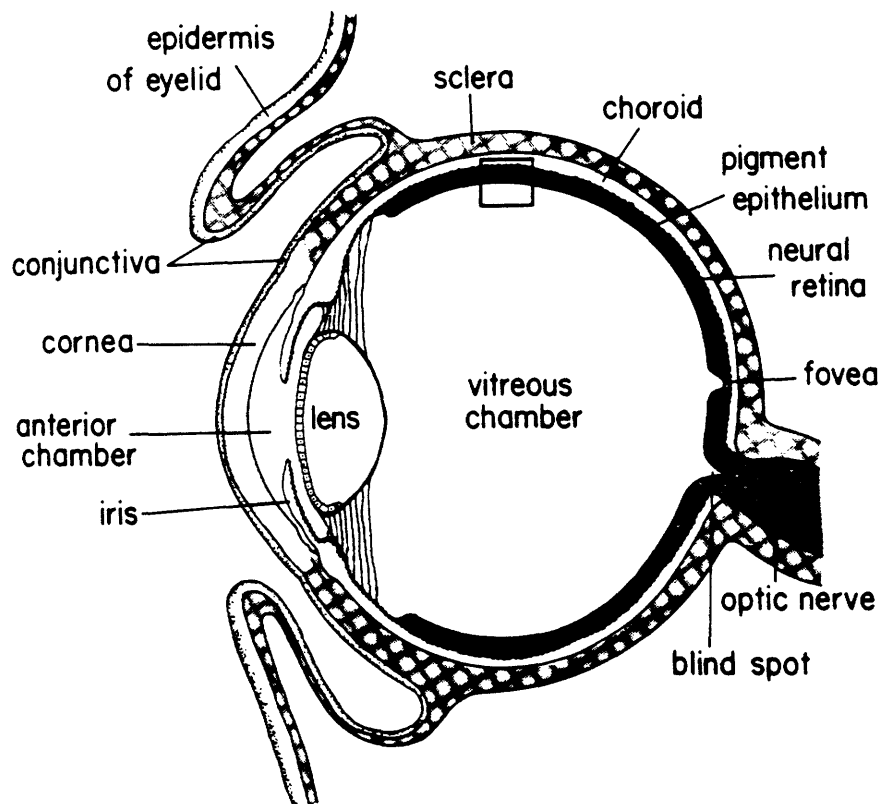


Figure 1-1 Anatomy of the eye. The neural retina is located next to the transparent vitreous humor.

layer, the outer plexiform layer- it contains the photoreceptor cells predominantly, the outer limiting membrane and Bruch's membrane (Figure 1-2). Light traverses nearly the entire neural retina before it strikes the photoreceptor cells. Interestingly the photoreceptor cells face the posterior aspect of the eye so that light must travel nearly the entire length of the rod or cone before it interacts with the appropriate receptor. In many ways the structural architecture of the eye is similar to that of a camera. The iris is a shutter that controls the amount of light which is received by the lens. The lens itself focuses the light stimulus onto the retina. It along with the aqueous humor, vitreous humor and the RPE serve to filter ultraviolet light as well as absorb reflected light within the eye, thereby preventing internal glare. The retina, of course, serves as the photographic film.

1.2 The Photoreceptor Cells: Rods and Cones

As mentioned previously the cells which mediate the primary events in visual transduction are the rods and cones. The cones are pyramidal shaped cells which allow for the perception of color. They are found in the central retina and are the major photoreceptor type found in the macula- the region of highest visual acuity. In humans, there are three distinct populations of cones- one subtype absorbs light primarily in the 445 nm region whereas the other two detect light in the 535 and 570 nm region respectively. These correspond to the absorption maxima of the three known human color pigments. Cones are 1-1.5 μm in thickness and are about 75 μm in length. The average eye contains about 6.5×10^6 cones. Morphologically they

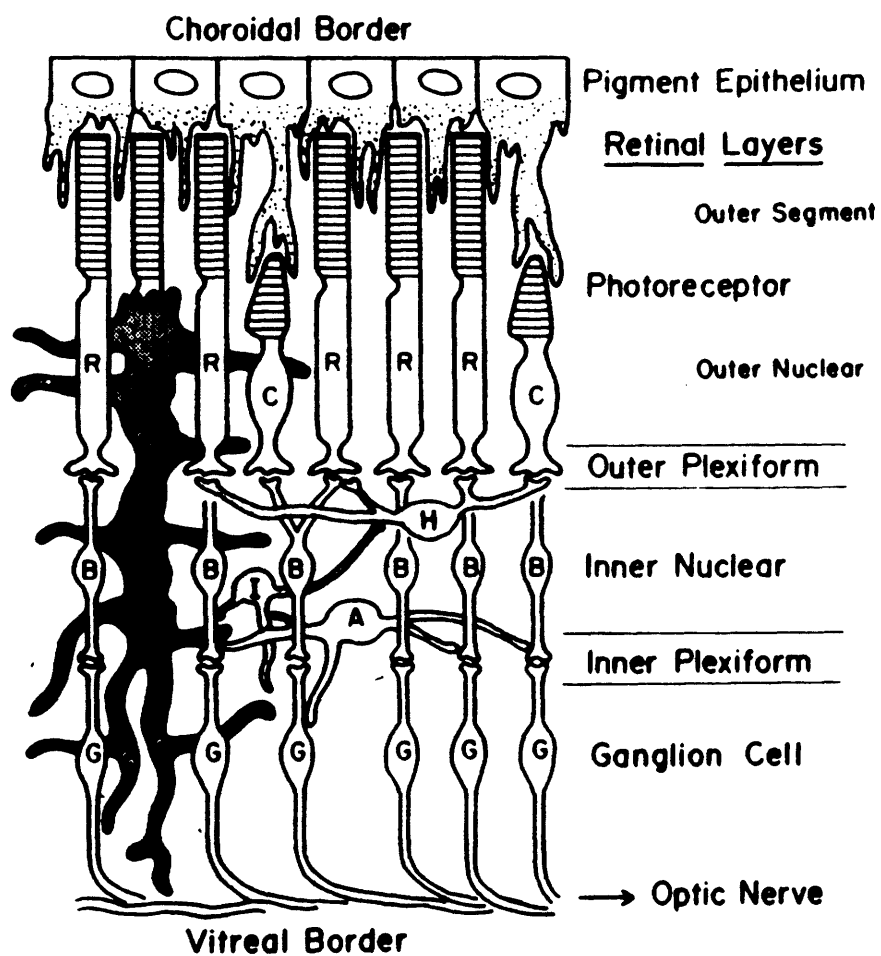


Figure 1-2 Anatomy of the sensory retina. The rods and cones are located furthest from the vitreous so that light must transverse the entire length of the retina before being absorbed by the visual proteins.

consist of an inner segment and an outer segment. The outer segment is where the color pigment receptors are found in a series of invaginated membrane structures called disks that are contiguous with the plasma membrane of the inner segment.

Rod cells are approximately 1-3 μm in thickness and 40-60 μm in length. They are found predominantly at the periphery of the retina in contrast to the cones. Their primary purpose is to mediate vision in dim light. There are approximately 120×10^6 rod cells in the eye. They absorb light maximally at 500 nm- the absorption maximum of rhodopsin, the visual receptor of these cells.

1.3 The Rod Inner and Outer Segments

The rod cell, like its cone counterpart, consists of an inner segment as well as an outer segment (Figure 1-3). They are referred to as the rod inner segment (RIS) and the rod outer segment (ROS). The inner segment is where rhodopsin is synthesized and inserted into the membrane of the endoplasmic reticulum (ER). While in the ER compartment opsin is covalently modified by being glycosylated (Young, 1976, O'Brien, 1978; Papermaster and Schneider, 1982), disulfide bonded (Gething and Sambrook, 1992) and palmitoylated (Pfanner *et al.*, 1989). 11-*cis* Retinal binds to the properly folded, mature opsin before it exits from the ER (St. Jules *et al.*, 1989). Subsequently rhodopsin is shuttled to the Golgi apparatus via membrane transport vesicles where the carbohydrate moieties are trimmed back and further modified by the addition of other sugars

(e.g. galactose) (Smith *et al.*, 1991). Finally rhodopsin is transported from the inner segment through a narrow structure called the cilium to the outer segment (Papermaster *et al.*, 1985; Deretic and Papermaster, 1991).

In the outer segment rhodopsin is found either within the plasma membrane or invaginated disks (Molday and Molday, 1987). Unlike the cone disks, the rod disks are not contiguous with the plasma membrane (Figure 1-3). Because of the topology of membrane fusion and budding the N-terminus of rhodopsin faces into the disk membrane's lumen whereas the C-terminus faces the cytoplasm of the outer segment where all the accessory proteins of visual transduction reside (Hargrave and Fong, 1977; Adams *et al.*, 1978; Clark and Molday, 1979; Dratz *et al.*, 1979).

1.4 Rhodopsin- The Photoreceptor Protein

1.4.1 Rhodopsin Structure

Bovine rhodopsin is an integral membrane protein of 348 amino acids (Ovchinnikov *et al.*, 1982; Hargrave *et al.*, 1983; Nathans and Hogness, 1983). A secondary structure model is shown in Figure 1-4. It has seven transmembrane segments, as determined by hydropathy analysis, resulting in the N and C terminus being on opposite sides of the lipid bilayer (Ovchinnikov *et al.*, 1982, Hargrave *et al.*, 1983). From circular dichroism (CD) measurements the helical content of rhodopsin is about 60%, as determined in the disk membrane or in

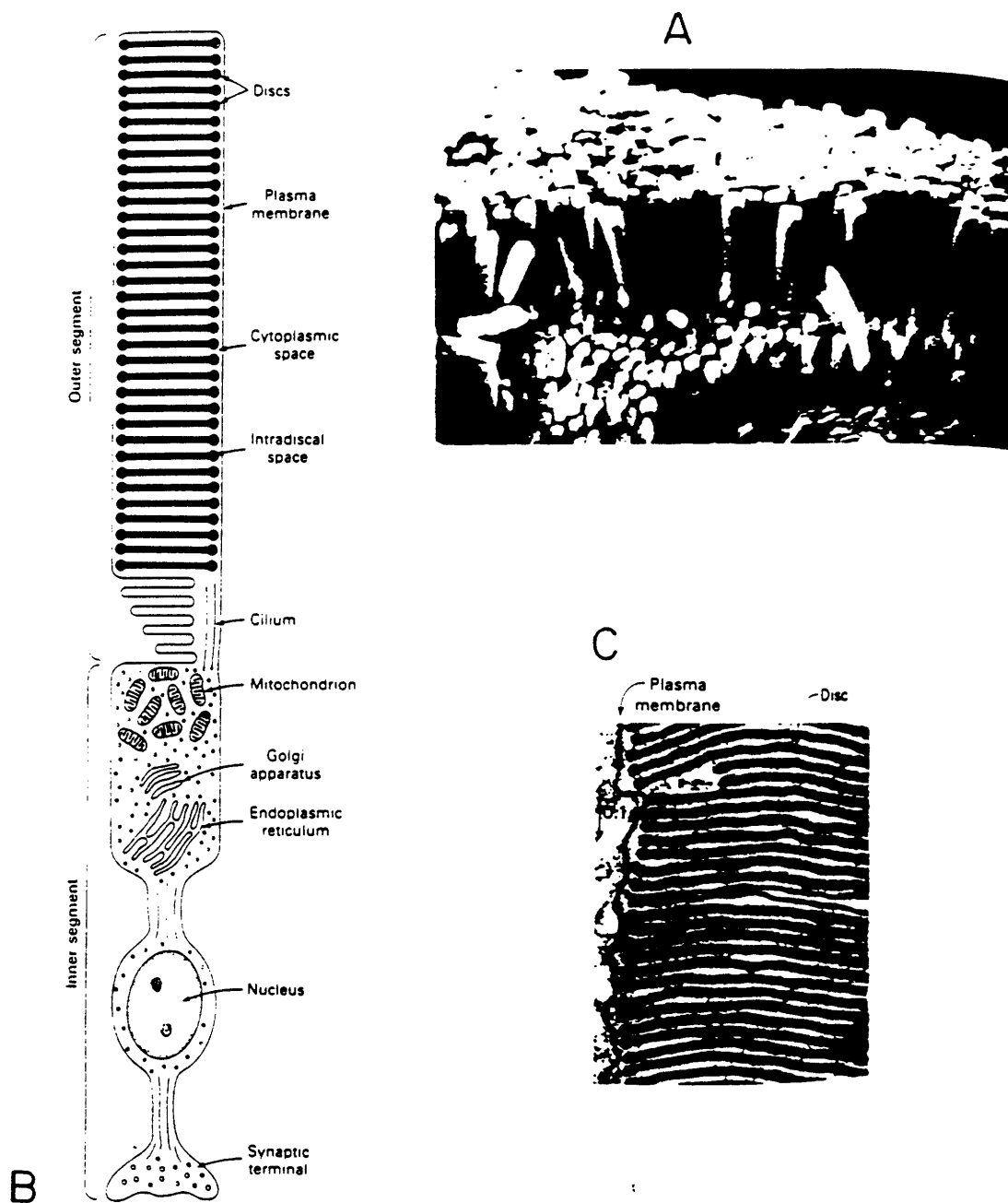


Figure 1-3 A) Scanning micrograph of rod cells; B) Schematic diagram of rod cells; C) Electron micrograph of rod outer segment with stacks of discs.

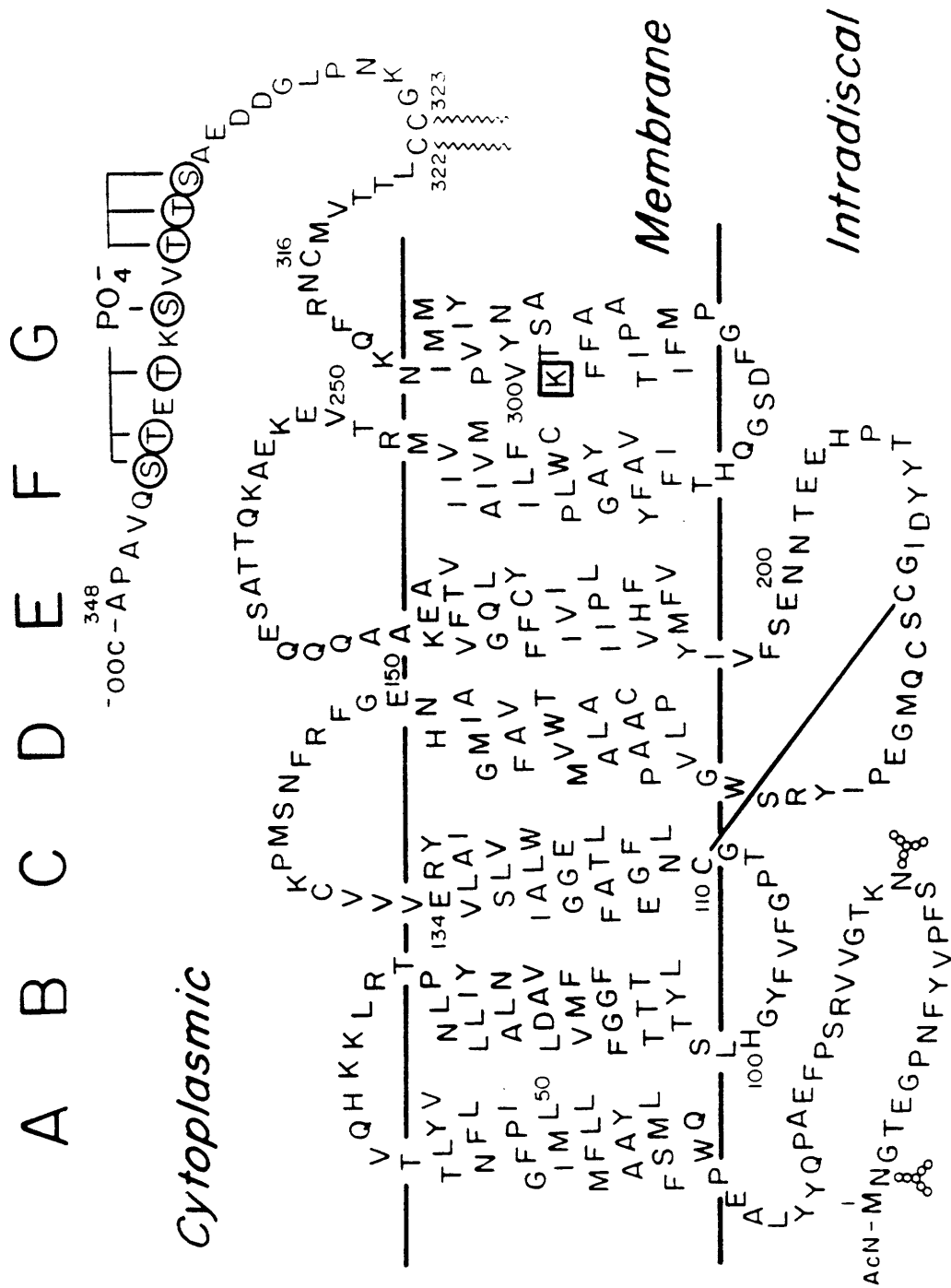


Figure 1-4 A proposed secondary structure model of bovine opsin based on hydrophathy analysis. The circles at asn2 and asn15 represent sugar moieties.

detergent micelles (Schichi *et al.*, 1969; Albert and Litman, 1978). There is a conformational change in rhodopsin after photoexcitation resulting in the loss of some helical content and the exposure of additional cysteine residues to the alkylating agent N-ethylmaleimide (NEM) (deGrip *et al.*, 1973). Recently, a low resolution projection structure of about 7 Å has been determined for rhodopsin (Schertler *et al.*, 1993). It confirms there are indeed seven transmembrane segments.

1.4.2 The G Protein-Coupled Receptor Superfamily

Rhodopsin belongs to a superfamily of G protein receptors which have seven transmembrane segments. The list of these receptors is ever-growing (Table 1-1) (Iismaa and Shine, 1992). The best studied members of this family include rhodopsin and the α and β -adrenergic receptors. Rhodopsin has been extensively studied since it can be purified in large quantities from the rod outer segment. It constitutes about 70% of the total protein of the outer segment and nearly 90% of the disk membrane (Hargrave and McDowell, 1992).

1.4.3 Spectral Properties of Rhodopsin

The chromophore, 11-*cis* retinal, is attached by a protonated Schiff base linkage to lysine 296 in helix G (Bownds, 1967). Once this occurs rhodopsin acquires a distinct UV/visible spectrum in which there are three distinct peaks at 280 nm, 340 nm and 500 nm (Figure 1-5). The 280 nm peak represents the collective contribution of all

Table 1. Cloned G protein-coupled receptors categorized according to endogenous ligand

Peptides/peptide hormones
Angiotensin II
Bombesin/gastrin-releasing peptide
Bombesin/neuromedin B
Bradykinin
C5a anaphylatoxin
Calcitonin
Endothelin (2)
Follicle-stimulating hormone
N-formyl peptide (2)
Interleukin-8 (2)
Luteinizing hormone/chorionic gonadotropin
Neurokinin A (substance K)
Neurokinin B (neuromedin K)
Neuropeptide Y/peptide YY (3)
Neurotensin
Parathyroid hormone/parathyroid hormone-related peptide
Secretin
Somatostatin (2)
Substance P
Thyroid-stimulating hormone (Thyrotropin)
Thyrotropin-releasing hormone
Vasoactive intestinal polypeptide
Neurotransmitters
Adenosine (2)
Adrenergic (12)
Dopamine (6)
Glutamate (3)
Histamine (2)
Muscarnic acetylcholine (5)
Octopamine
Serotonin (5)
Tyramine
Other regulatory factors
Cannabinoids
Cyclic AMP
Cytomegalovirus proteins (3)
Platelet-activating factor
Prostanoid thromboxane A ₂
Thrombin
Yeast mating factors (2)
Sensory stimuli
Light (4)
Odorants (> 100)
Orphan receptors (> 6)

Numbers in brackets refer to the number of molecular subtypes cloned to date.

Table 1-1 Members of the seven transmembrane segment G-protein coupled receptors.

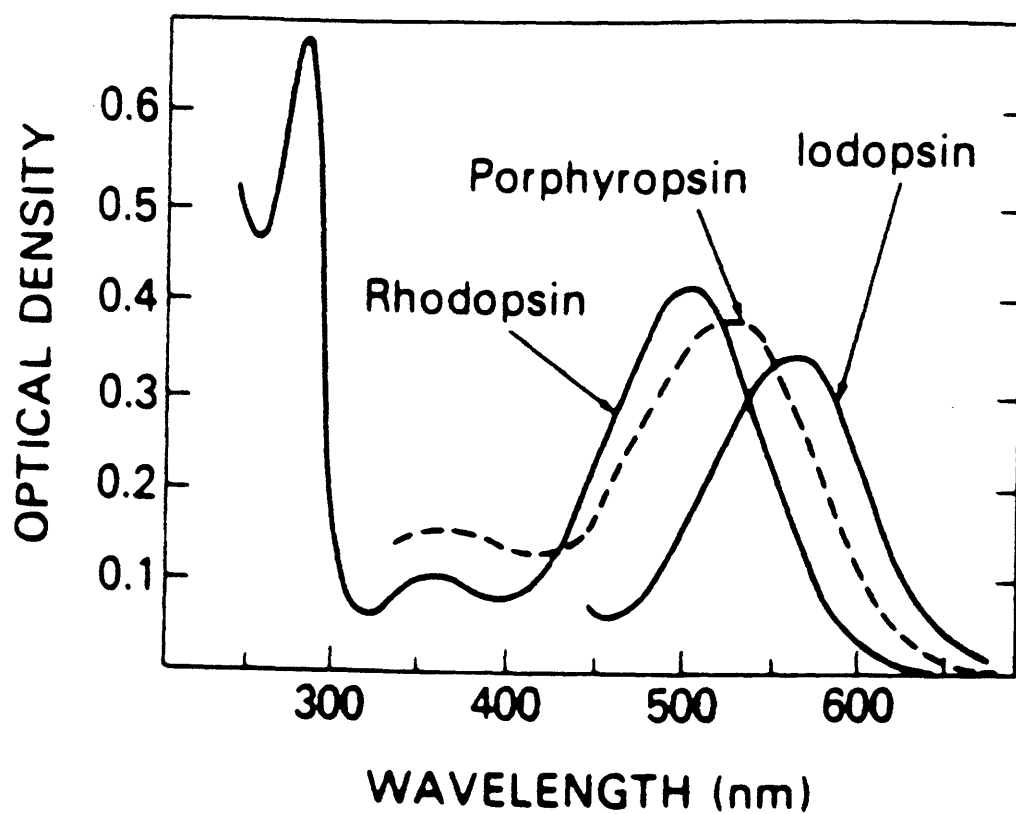


Figure 1-5 UV / visible absorption spectra of rhodopsin, porphyropsin, and iodopsin. All three pigments have α and β bands due to the retinylidene chromophore and γ band due to the opsin protein.

the tyrosine and tryptophan residues in the protein. The other two, of which the 500 nm peak is the largest and most distinctive for rhodopsin, represent the interaction between the amino acid residues that form the binding pocket for retinal. Model studies have demonstrated a protonated Schiff base in solution has an absorption maximum at 440 nm (Figure 1-6) (Blatz *et al.*, 1972). Denaturation of rhodopsin with either acid or sodium dodecyl sulfate (SDS) results in such a species (Kito *et al.*, 1968), suggesting the 60 nm red shift must be due to specific interactions between the residues of the retinal binding pocket and the chromophore.

1.4.4 Covalent Modifications

Rhodopsin has three known covalent modifications. First, a single disulfide bond exists between cys110 and cys187 . The presence and importance of this linkage for the folding and stability of opsin has been established by both biochemical and mutagenesis studies (Karnik *et al.*, 1988; Karnik and Khorana, 1990). Second, rhodopsin is palmitoylated at cys322 and cys323 (Ovchinnikov *et al.*, 1988, Papac *et al.*, 1992). From previous mutagenesis studies the replacement of these cysteine residues with serines has little consequence on the folding and function of rhodopsin, despite the lack of palmitoyl groups (Karnik *et al.*, 1993). Third, bovine rhodopsin has two asparagine (N) -linked glycosylation sites at asn2 and asn15 (Fukuda *et al.*, 1979; Schichi *et al.*, 1980). There is another site at asn200 but from peptide analysis that position does not have any attached carbohydrate moieties (Fukuda *et al.*, 1979). The

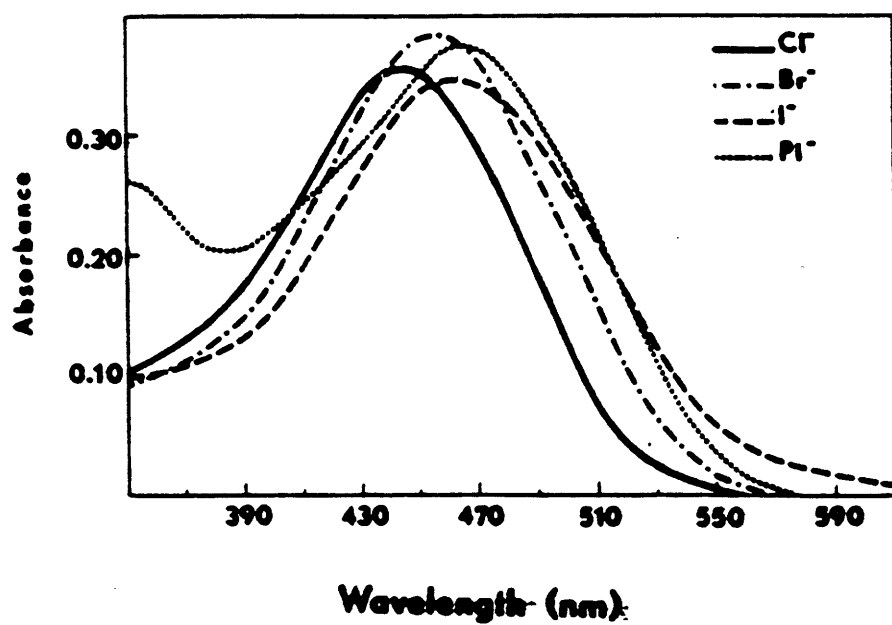


Figure 1-6 UV / visible absorption spectra of a model Schiff base, N-retinylidene-n-butylammonium chloride, bromide, iodide and picarate in CCl_4 .

mutagenesis and biochemical studies on the role of glycosylation are presented as part of this thesis. There is a fourth covalent modification of rhodopsin, phosphorylation, that occurs after photoexcitation. It is part of the mechanism by which the receptor is inactivated (Kuhn *et al.*, 1984).

1.4.5 The Three Regions of Rhodopsin

From prior biochemical and mutagenesis studies much has been learned about the structure and function rhodopsin. The intradiscal region is important for correct folding and assembly (Doi *et al.*, 1990). It is on this face of the molecule the single disulfide bond is found. For example, deletion mutants at the N-terminus or in the conserved portion of the DE loop regenerate poorly or do not regenerate at all i.e they do not form the characteristic 500 nm absorbing species in the presence of 11-*cis* retinal. These mutants are retained in the ER of COS-1 cells, consistent with them being misfolded. They also do not acquire the distinctive glycosylation smear found with wild type rhodopsin. It is unknown if these intradiscal mutants form the disulfide bond.

The transmembrane region contains within it the retinal binding pocket, lys296 and the counterion to the protonated Schiff base, glu113. Lys296 is not necessary for the formation of the 500 nm chromophore. In a series of elegant experiments Zhukovsky and Oprian (1992) demonstrated when this residue is mutated to glycine it can bind a propylamine derivative of 11-*cis* retinal and form a 500

nm chromophore which upon photoactivation can trigger transducin. The behavior of this mutant is similar to other G-protein coupled receptors that bind ligands non-covalently.

From cross-linking experiments with a photoactivatable analog of retinal and mutagenesis studies Nakayama and Khorana (1990, 1991) established some of the residues forming the retinal binding pocket. These include phe115, ala117, glu122, trp126 and ser127 in helix C along with trp265 and pro267 in helix F. Interestingly the mutants E122Q and W265F had blue-shifted absorption maxima at 480 nm.

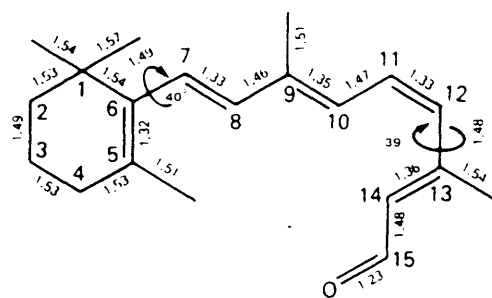
The role of glu113 as the Schiff base counterion has also been established by mutagenesis (Sakmar *et al.*, 1989; Zhukovsky and Oprian, 1989; Nathans, 1990). When this residue is changed to gln the mutant rhodopsin has a $\lambda_{\text{max}} = 380$ nm. This species exists in a pH-dependent equilibrium with a 500 nm chromophore. This mutant can also bind to all-*trans* retinal in the dark and trigger transducin in the absence of light.

The cytoplasmic loops are critical for the interaction of photoactivated rhodopsin with transducin (Konig *et al.*, 1989; Franke *et al.*, 1990; Franke *et al.*, 1992). From both mutagenesis and peptide competition studies loops CD, EF and the one extending from helix G to the palmitoylated cysteines 322, 323 are important for transducin binding and activation. It appears there is a synergistic effect between these loops.

1.4.6 Light Activation of Rhodopsin

The primary event of visual transduction is the isomerization of 11-*cis* retinal to all-*trans* retinal (Figure 1-7). This photochemical conversion occurs in approximately 2-5 nanoseconds (Schoenlein *et al.*, 1991). The activation energy for this reaction is nearly 50 kcal/mole (Cooper, 1979). Immediately upon isomerization there is no detectable conformational change in the protein itself. The photoexcited species then decays, within a millisecond, through a series of photointermediates to metarhodopsin I (mI) (Figure 1-8). These intermediates can be trapped and studied at low temperatures. Metarhodopsin I exists in a pH and temperature-dependent equilibrium with metarhodopsin II (mII)- the species that interacts with transducin (Kibelbek *et al.*, 1991). It also is the substrate for phosphorylation and subsequent inactivation. Certain detergents like digitonin favor the formation of mI whereas others like dodecyl maltoside (DM) favor mII. All-*trans* retinal is covalently bound to the protein in metarhodopsin II (Cooper *et al.*, 1987). In vitro it can decay to either free all-*trans* retinal and opsin or metarhodopsin III (mIII). This intermediate absorbs at 465 nm and can also decay to all-*trans* retinal and opsin. *In vivo* the free all-*trans* retinal is shuttled via a carrier protein to the RPE cells where it is reisomerized to 11-*cis* retinal (Rando, 1991). This is then transported back to the rod cell inner segment, where it can combine with newly synthesized opsin.

1.5 The Visual Cascade



6*S*-cis, 11-cis, 12*S*-cis-Retinal

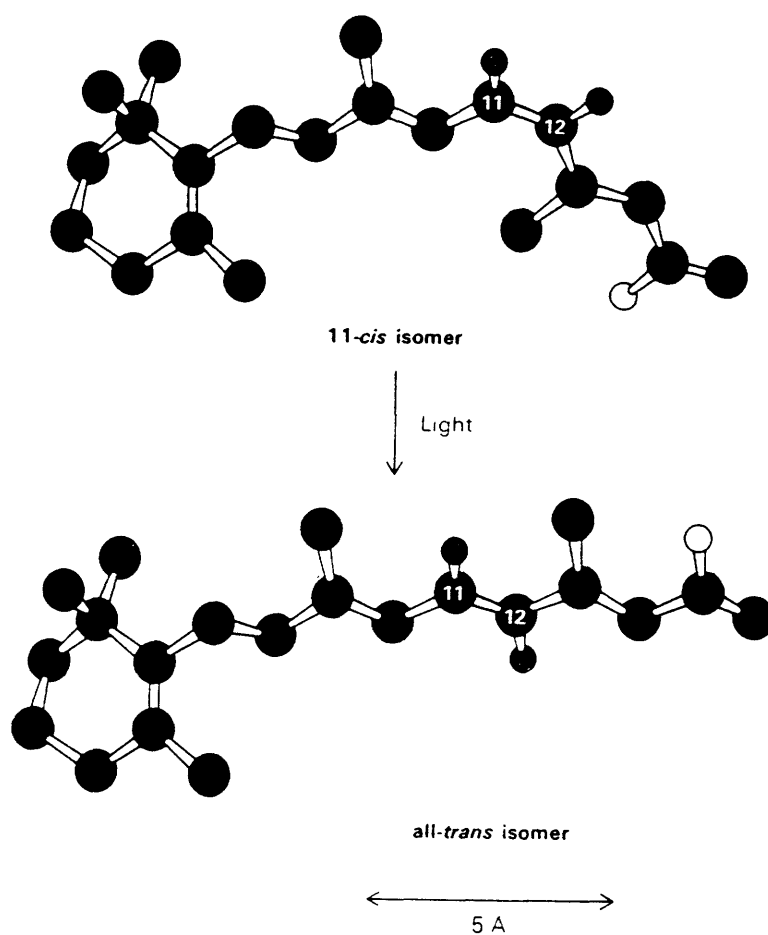


Figure 1-7 The chemical structure of 11-cis retinal and its photoconversion to the all-trans form.

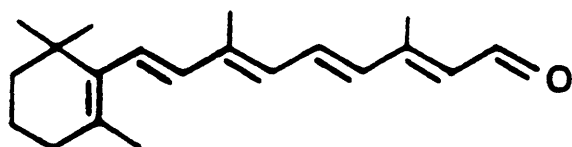
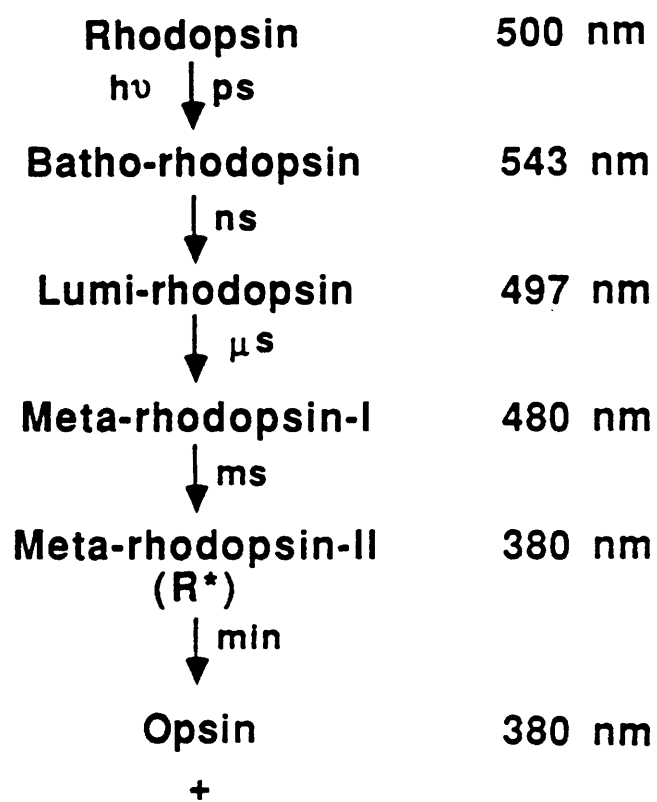
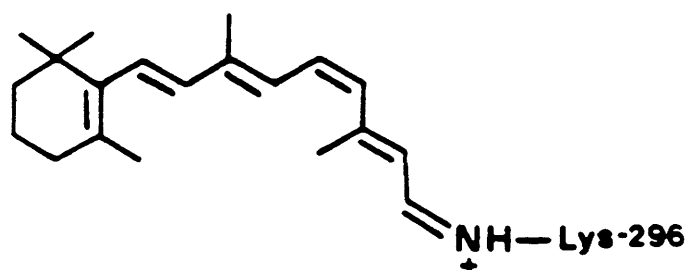


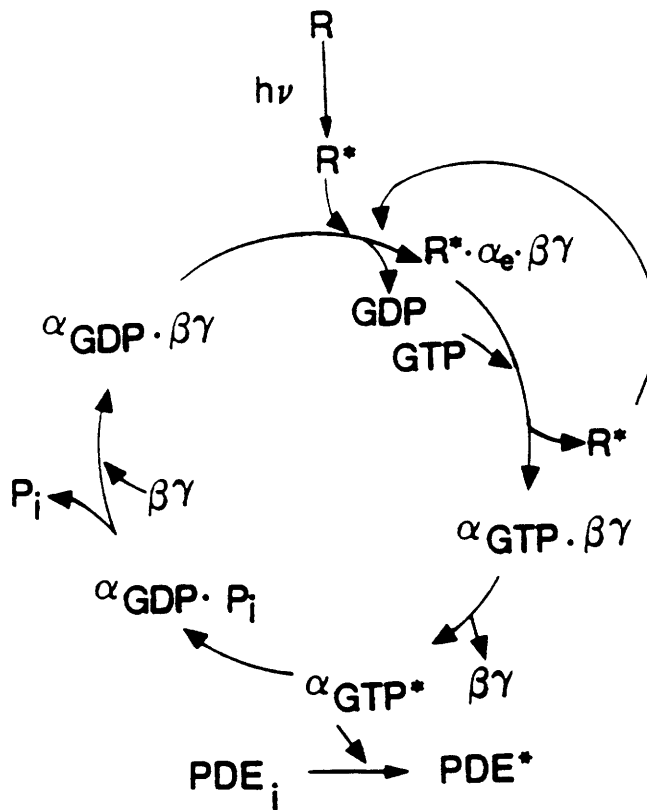
Figure 1-8 The photobleaching sequence of rhodopsin.

As mentioned mII is the photointermediate which triggers multiple transducin molecules, the rod cell G-protein, also abbreviated as G_T . The activated G_T 's then turn on many cGMP phosphodiesterase (PDE) molecules resulting in a decreased concentration of cGMP. This in turn leads to the closing of cGMP-gated channels and rod cell hyperpolarization (Figure 1-9) (Fung *et al.*, 1981; Chabre and Deterre, 1989). Finally an electrical signal is generated in the retina which is transmitted via the optic nerve to the visual cortex where visual information is integrated and recorded.

Visual transduction is an exquisitely sensitive system resulting in tremendous signal amplification. There are two distinct stages of amplification. First, each activated rhodopsin molecule i.e. mII is able to bind and trigger about 500 molecules of transducin, resulting in a 500-fold increase in signal (Liebman and Pugh, 1980; Fung *et al.*, 1981). Second, each molecule of PDE can hydrolyze over 1000 cGMP molecules (Yee and Liebman, 1978). So from a single photoexcited rhodopsin molecule over 5×10^5 molecules of cGMP can be hydrolyzed.

1.6 Transducin- The G Protein of the Rod Cell

Transducin is a peripheral membrane protein which is found on the cytoplasmic side of discs (Fung *et al.*, 1981; Kuhn, 1981). It can be stripped from the membrane by urea washing. Like other heterotrimeric G proteins it is composed of three subunits, α (39 KDa), β (36 KDa) and γ (8 KDa) (Kuhn, 1981). The α subunit is myristoylated



The transducin cycle. Rhodopsin (R), upon photoexcitation (R^*), binds $\alpha_{GDP}\beta\gamma$. GDP dissociates leaving α with an empty nucleotide binding site (α_e). Binding of GTP leads to activation of α and its dissociation from $\beta\gamma$. α_{GTP^*} activates cGMP phosphodiesterase (PDE) until α 's intrinsic GTPase activity hydrolyzes bound GTP (forming $\alpha_{GDP}P_i$). Upon dissociation of P_i , α_{GDP} rebinds $\beta\gamma$, reconstituting the heterotrimer.

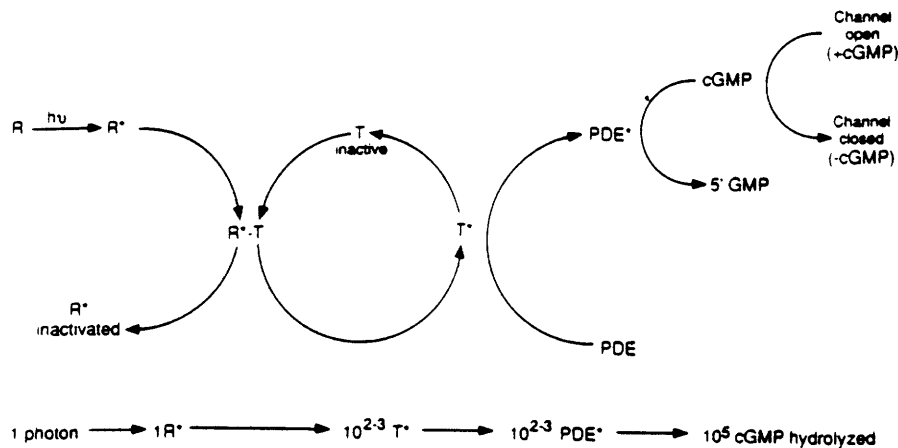


Figure 1-9 The interactions of the visual cycle. R^* in this figure is equivalent to mII.

at the N-terminus and has GDP bound to it in the dark (Stryer, 1983). When the cytoplasmic loops of mII bind to it, there is an exchange of GTP for GDP and the dissociation of $G\alpha$ from $G\beta\gamma$. The mII molecule is then able to interact with other transducin molecules (Stryer, 1983). The binding sites for mII on $G\alpha$ are unknown at this time.

1.7 Phosphodiesterase

The rod cell cGMP phosphodiesterase is also a peripheral membrane protein. It consists of an α (88 KDa), β (84 KDa) and γ (11 KDa) subunit (Baehr *et al.*, 1979). $G\alpha$ -GTP can activate PDE as can limited tryptic digestion. Activation is associated with the dissociation of two γ subunits from PDE in the former and the proteolysis of the γ subunits in the latter (Miki *et al.*, 1975; Fung *et al.*, 1981; Hurley and Stryer, 1982). PDE can hydrolyze about 2×10^3 moles cGMP per second per mole of enzyme (Baehr *et al.*, 1979).

1.8 The cGMP-Gated Channel

cGMP is the second messenger which regulates the cation channels of the rod outer segment. When there is a decrease in the cytoplasmic levels of cGMP the cGMP-gated channel is closed leading to the hyperpolarization of the rod cell. This eventually is transmitted to the synaptic region where there is neurotransmitter release.

The channel has been purified from the rod cell. It is an integral membrane protein of 66 KDa (Kaupp *et al.*, 1988). As expected the

purified molecule, when reconstituted *in vitro* with lipids, has the same properties as that found *in vivo*.

1.9 Inactivation of the Visual System

How does the visual machinery turn off the light response? At the level of transducin there is an intrinsic rate of hydrolysis of GTP when it is associated with $G\alpha$. However this activity is probably too slow to have a significant effect (Vuong and Chabre, 1990). At the level of rhodopsin there are two distinct inactivation mechanisms. First, in membranes mII has a relatively short half-life, on the order of minutes, such that it decays to free *all-trans* retinal and opsin (Matthews *et al.*, 1963). Again this is a relatively slow process. Second and more significantly, it has been demonstrated by many workers that mII can be phosphorylated by a specific rhodopsin kinase at the nine serine and threonine residues found at the carboxy terminus of rhodopsin (Bownds *et al.*, 1972; Kuhn and Dryer, 1972; Frank *et al.*, 1973; Kuhn *et al.*, 1973). The phosphorylated species is the substrate for the binding of arrestin, a 48 KDa protein also known as retinal S-antigen (Kuhn, 1981; Pfister *et al.*, 1984). When this occurs there is a great reduction in the amount of mII available for transducin binding. This mechanism is currently believed to be the method to control visual inactivation.

1.10 Goals of Thesis

The goals of this thesis are threefold. First, opsin was expressed, purified and characterized in the baculovirus/insect cell expression system. Second, the role of asparagine (N)- linked glycosylation was explored by mutagenesis and by expressing opsin in the presence of a well-known inhibitor of N-linked glycosylation, tunicamycin (TM). Third, the structure and function of the opsin mutants associated with autosomal dominant retinitis pigmentosa (ADRP) was determined.

In order to study both biochemically and biophysically rhodopsin and its mutants it is desirable to have an expression system in which large quantities of the protein are being synthesized and it can be easily purified. Rhodopsin has been expressed and purified in our laboratory from COS-1 cells- a monkey kidney cell in which multiple copies of the SV40 large T antigen have been stably integrated into the genome. Expression depends on the introduction of a plasmid, by transfection, into the cell. The vector, such as pMT4, is a shuttle vector. It can be easily propagated in E.Coli thereby making it easy to isolate large quantities of it for transfection. As such it has a bacterial origin of replication and a β -lactamase gene rendering the bacteria which has this plasmid resistant to ampicillin. Furthermore this vector has the SV40 origin of replication so that it can replicate to a large copy number in the presence of large T antigen. In COS-1 cells we are able to isolate approximately 10 μ g of opsin from 1.25×10^7 cells. Although many studies can be done with this amount of protein it is a relatively little when compared to a soluble protein of the same size. It may be for an integral membrane protein the amount of folded protein that can be obtained is determined by the available

membrane. If opsin was expressed in a cell which was larger than the COS-1 cell greater expression levels could possibly be achieved.

Insect cells are not larger than COS-1 cells. They, however, are an attractive alternative expression system. Insect cells do not require any CO₂ for their survival and grow best at 27°C. This is particularly advantageous for a biotechnology firm since the costs of growing surface adherent mammalian cells like COS-1 are prohibitive. But the real benefit of insect cells is they can be grown at high density in suspension. Many biotechnology companies have exploited this property and have grown insect cells in large bioreactors for the isolation of medically useful proteins.

To express a foreign protein in insect cells the gene of interest must be incorporated into the baculovirus genome and the recombinant viral particle must be plaque purified. Opsin expression in insect cells was driven from the polyhedrin promoter of baculovirus. As is the case of any viral infection nearly all of the target cells are infected. This is particularly desirable when heterologous proteins are being expressed. The polyhedrin promoter is an extremely powerful promoter which is turned on 24-30 hours post-infection. Indeed opsin was first observed by immunoblots 24 hours after infection. A full time course of expression was then done. It was determined the maximal amount of folded opsin, i.e. that fraction of opsin which can bind to 11-*cis* retinal, was found at approximately 72 hours after infection. After that the insect cells began to die and the opsin was proteolyzed. Since rhodopsin is an integral membrane

protein, it must be solubilized in some detergent so that it can be isolated. Other workers have used many different detergents in the purification of rhodopsin. The stability and spectral kinetics can be dramatically affected by the choice of detergent. In the past, harsh cationic detergents have been used. More recently, especially in our laboratory, the mild alkyl glycosidic detergent, dodecyl maltoside (DM) also known as lauryl maltoside (LM) has been used. When wild-type rhodopsin is purified in it the spectral kinetics are similar to those of rhodopsin in membranes. However it is not known how well LM can extract opsin from COS-1 or other cells. For insect cells a variety of detergents were used to solubilize opsin in order to determine which would extract the greatest amount. Although dodecyltrimethylammonium bromide (DTAB) extracted the greatest amount of opsin, little folded opsin could be purified, suggesting this harsh detergent denatured rhodopsin. DM solubilized less opsin from whole cells but a 500 nm chromophore could be isolated. However the rhodopsin isolated was neither spectrally pure, $A_{280}/A_{500} > 1.6$, nor was it pure when analyzed by silver stained gels. A crude plasma membrane fractionation was developed whereby spectrally and gel pure rhodopsin was obtained. This rhodopsin had similar transducin activation as ROS rhodopsin. It also was glycosylated and palmitoylated.

As mentioned in the introduction the role of rhodopsin's carbohydrate moieties are unknown. To understand their function mutations were made at asn2 and asn15. Other mutations in the tripeptide consensus sequence required for N-linked glycosylation

were also made. The mutants were then analyzed for the presence of glycosylation, their spectral properties including their bleaching kinetics, their ability to trigger transducin as well as their cellular localization as determined by immunofluorescence microscopy. Collectively, these studies clearly suggest that glycosylation at asn2 has little effect on the folding and function of rhodopsin. However mutations at asn15 effect the amount of folded obtained. Furthermore these mutants are located in a perinuclear distribution consistent with an ER staining pattern. These mutants also bleach more quickly than wild type and are less efficient in triggering transducin; nearly 1/100th the levels of wild type. It may be glycosylation at asn15 is important for the structure and function of rhodopsin but it is equally possible that asn15 besides being a glycosylation site plays an important structural role.

To deconvolute these two possibilities wild type opsin was expressed in the presence of tunicamycin. Wt(TM) was not glycosylated but was palmitoylated and transported to the cell surface. It has the same spectral properties as the wild type including the mII half-life. But this molecule is 1/10th less efficient in triggering transducin as wild type suggesting that glycosylation has an effect on the photoactivated form but not the ground state. Furthermore it appears that asn15 does in fact have a structural role.

Finally the third part of this thesis explores the structure and function of nearly 40 of the opsin mutants associated with autosomal dominant retinitis pigmentosa (ADRP). These mutations had

heterogeneous effects on folding, bleaching kinetics and the triggering of transducin. However there are some interesting findings with these mutants. First, most of the mutant ADRP proteins, including those in the intradiscal and transmembrane region, are expressed at lower levels than wild type. They also regenerate more poorly (e.g. P23H, Class III) or not at all (e.g. P171L, Class II). Second, these same mutants have abnormal glycosylation and appear to be retained within the ER- indicative of proteins that are either misfolded or fold slowly. Third, of the mutants that do form chromophore nearly all bleach more quickly than wild-type rhodopsin. Consequently, these mutants are less efficient in triggering transducin.

There are a group of mutants which are similar to wild-type both structurally and functionally (e.g. L125R). How such mutants cause RP is even more intriguing.

Chapter 2

PURIFICATION AND CHARACTERIZATION OF BOVINE OPSIN EXPRESSED IN INSECT CELLS

2.1 Introduction

In hopes of expressing large quantities of opsin, the synthetic bovine opsin gene was expressed in *Spodoptera frugiperda* (Sf9) insect cells. Previously, opsin has been expressed in COS-1 cells (Oprian *et al.*, 1987), 293S cells (Nathans *et al.*, 1989), CHO cells (Weiss *et al.*, 1990), oocytes (Khorana *et al.*, 1988) and by *in vitro* methods (Zozulya *et al.*, 1990). In transient expression studies COS-1 and 293S cells synthesize similar amounts of wild-type opsin, approximately 10 $\mu\text{g}/1.25 \times 10^7$ cells. However, in cells that have the opsin gene integrated within the chromosomal DNA i.e. stable cell lines such as CHO cells, the amount of protein is significantly less, about 20 $\text{ng}/10^7$ cells. The one advantage of this expression system is the cells can easily be grown in suspension.

Insect cells have become a popular transient expression system because of the large amounts of protein that are synthesized. For soluble proteins up to 500 $\text{mg}/10^9$ cells is made (Luckow and Summers, 1988). So far, only a few integral membrane proteins have been expressed in Sf9 cells (e.g. multidrug transporter (Germann *et al.*, 1990), β -adrenergic receptor (George *et al.*, 1989)). In all cases the

levels of expression are much lower than for soluble proteins. For example, the β -adrenergic receptor, a 7 membrane-spanning segment protein like rhodopsin, is expressed at $1.5 \mu\text{g}/10^9$ cells.

In this chapter the time course of expression, quantitation, purification protocol, structural characterization and functional activity of rhodopsin expressed in Sf9 cells is described.

2.2 MATERIALS AND METHODS

2.2.1 Materials

Sf9 cells were purchased from American Tissue Type Collection. Insect cell media were purchased from JRH Scientific. Serum was from Hyclone. N-glycosidase F and endoglycosidase H were from Boehringer Mannheim. $[9, 10\text{-}^3\text{H}]$ Palmitic acid and $[\gamma\text{-}^{32}\text{P}]$ GTP were from New England Nuclear. All detergents were purchased from Sigma and then purified, except for dodecyl maltoside which was purchased from Anatrace. The mouse monoclonal antibodies, 1D4 and 4D2, and the polyclonal rabbit anti-opsin antibodies were prepared previously in our laboratory. 11-*cis* Retinal was a generous gift of Dr. Peter Sorter (Hoffman-LaRoche).

2.2.2 Construction of pVL1393-rho and Purification of Recombinant Viral Particle

From the eukaryotic expression vector pMT4 the synthetic bovine opsin gene was isolated as an EcoRI-NotI restriction fragment and cloned in the polylinker region of pVL1393- downstream from the polyhedrin promoter (Figure 2-1). This new construct, pVL1393-rho, was co-transfected with the wild type baculovirus into insect cells by the calcium phosphate method (Summers and Smith, 1987). Three days post-infection the media bathing the tranfected cells was placed into 96-well titre plates at various dilution. This media contained the recombinant virus particle in which the opsin gene as well as the polyhedrin promoter had integrated into the baculovirus genome of *Autographa californica* nuclear polyhedrosis virus (AcMNPV) by homologous recombination. A radioactive opsin probe was used to hybridize the viral DNA adherent on the nitrocellulose filter. When a positive colony was found it was further purified by successive limited dilutions (Figure 2-2). Once this was acheived the viral titre was determined by the method described by Summers and Smith (1987). Briefly, cells were plated in 96-well plates and various dilution of the recombinant virus were added to the wells. Three days post-infection the cells were scored for the presence of inclusion bodies-a non-refractile dense body found in the cytoplasm of insect cells that is easily identified by light microscopy. From the viral dilution where 50% of the cells had inclusion bodies the titre was determined by the equation: PFU (plaque forming unit)/ml= 1/dilution x number of plaques x 1/(mls of inoculum/plate). Although this method does not exactly determine the viral titre it can approximate it reasonably well. The titre of the viral supernatants was consistently between $1-5 \times 10^8$ plaque forming units (viral particles)/ml.

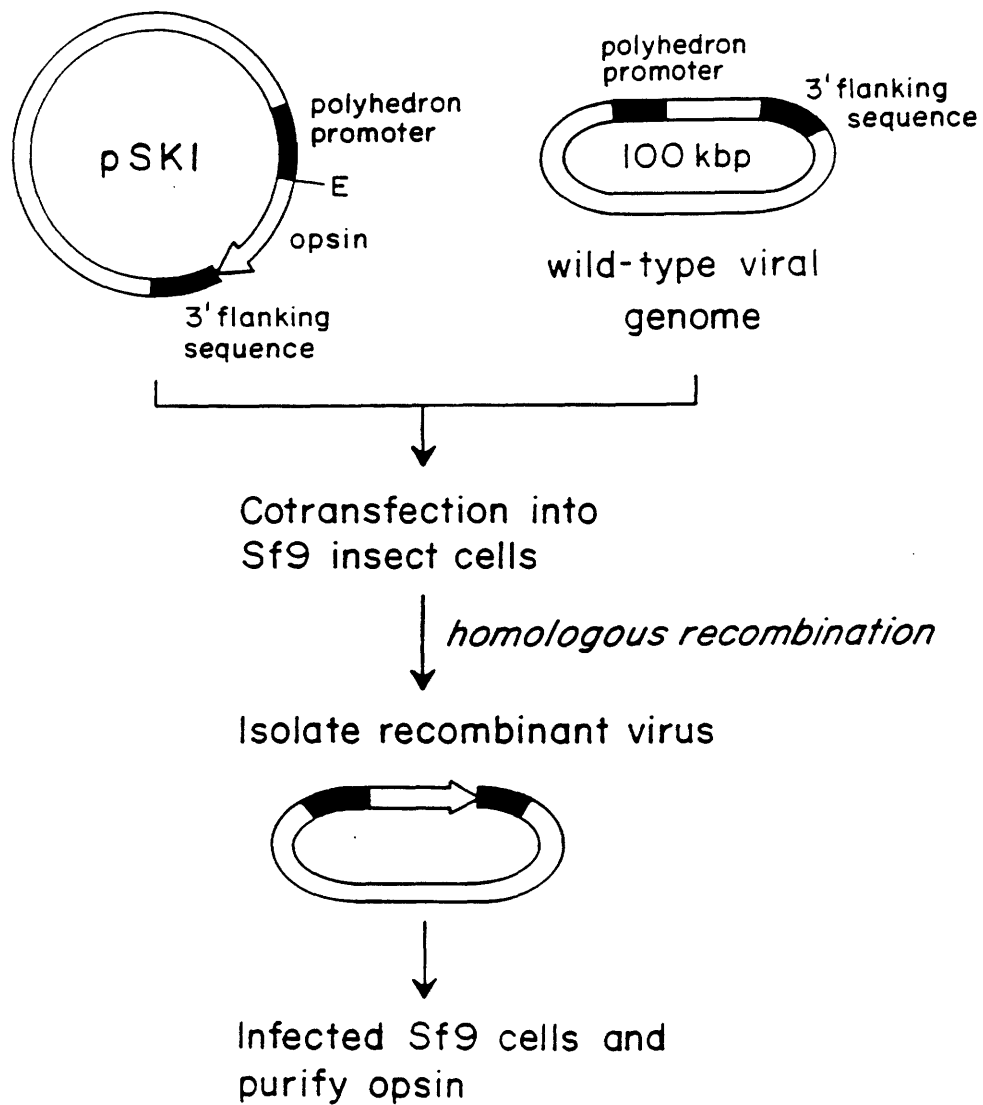
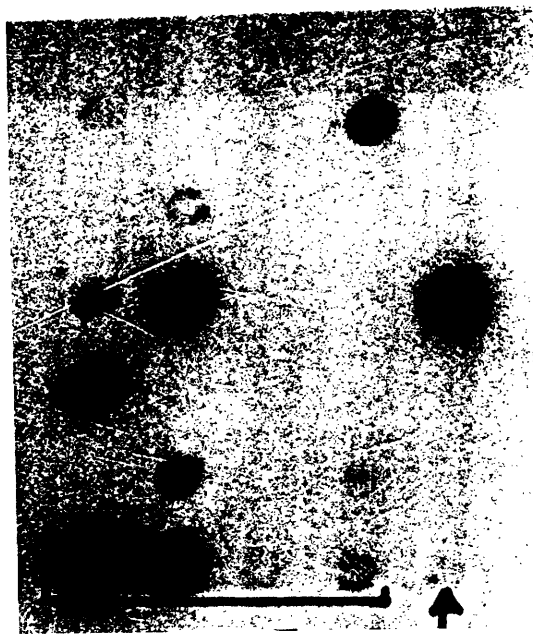


Figure 2-1 Schematic of the isolation of the recombinant baculovirus containing the synthetic bovine opsin gene.



10-5

10-6

Figure 2-2 Dot-blot hybridization of recombinant baculovirus with opsin probe. The dilutions are as shown.

2.2.3 Detergent Extraction

Seventy-two hours post-infection, 10^7 cells were spun down at 1000 rpms for 5 minutes. The cells were then solubilized in various detergents at 2% final concentration with 0.1 mM PMSF for 30 minutes at 4°C. The samples were then spun at 100000g for 30 minutes at 4°C. Equal volumes of the clarified extract was then run on SDS-polyacrylamide gels. The proteins were then transferred to nitrocellulose and immunoblotted with 1D4, a C-terminal monoclonal antibody, or 4D2, a N-terminal antibody.

2.2.4 Quantitation of Opsin from Whole Cell Extracts

1D4 was dried down on Immulon 2 polystyrene wells, 5 µg/well. The wells were then blocked with a 3% BSA solution in PBS for 2 hours at room temperature. Next the plates were washed with Buffer 1 (0.1% BSA, 0.1% LM in PBS) 3 times with 300 µls. The wells were filled with 100 µls of Buffer 1 and 100 µls of purified ROS rhodopsin (0.9 µg/100 µls) was added. 100 µls of a 1/20 dilution of clarified insect cell extract, solubilized either in 2% DTAB or 2% LM, was added to separate wells. Serial two-fold dilutions were then made. The opsin in each well was allowed to bind to 1D4 overnight at room temperature with mild agitation. The next day the wells were emptied and washed with Buffer 1 three times. A 1/7000 dilution of a rabbit anti-opsin polyclonal antibody was added and the microtitre plates were again incubated at room temperature overnight. The

wells were again emptied and washed 3 times with buffer 1. A 1/5000 dilution of a goat anti-rabbit IgG conjugated to horseradish peroxidase was added to the wells and allowed to bind at room temperature for 2 hours. The wells were decanted and washed 3 times with Buffer 1. 150 μ ls of the chromogenic substrate, 10 mM phosphate buffer pH 6.8, 0.1% aminosalisylic acid and 0.01% hydrogen peroxide was added for 45 minutes. Afterwards the reaction was stopped with 75 μ ls of 3M NaOH. The plates were then scanned with an ELISA reader at 450 nm.

2.2.5 Time Course of Expression

At various times post-infection 10^7 cells were spun down at 1000 rpms for 5 minutes. The cell pellet was solubilized with 1% dodecyl maltoside for 30 minutes at 4°C in the presence of 0.1 mM PMSF. The insoluble debris was pelleted at 100000g for 30 minutes at 4°C. The supernatant, containing solubilized opsin was loaded on 10% SDS-PAGE and immunoblotted with either 1D4 or 4D2. The multiplicity of infection (MOI), the number of viral particles to the number of insect cells, was also varied to determine if it also had any effect on expression.

2.2.6 Glycosylation of Insect Cell Opsin

Solubilized opsin, as mentioned above, was deglycosylated with N-glycosidase F in the presence of 1% SDS and 2 mM EDTA. After incubation at 37°C for 16 hours the samples were run on 10% SDS-

polyacrylamide gels and probed with 1D4 after transferring the proteins onto nitrocellulose. The solubilized opsin was also incubated with endoglycosidase H, an enzyme that recognizes high mannose structures associated with proteins retained in the ER, in 10 mM Tris pH 7.5. Finally, the Boehringer Mannheim Glycokit was used to assess the presence of mannose, sialic acid or O-linked glycosylation. This kit contains primary antibodies to the sugar moieties mentioned above. These antibodies are coupled to digoxigenin. A secondary antibody which is directed against digoxigenin and is also coupled to alkaline phosphatase. After sequential incubation with the primary and secondary antibody this system can then be used for standard immunoblotting.

2.2.7 Palmitoylation of insect cell opsin

Twenty four hours post-infection 10^7 cells were spun down at 1000 rpm and the cell pellet was brought up in 1 ml of Grace's insect cell media, without 10% fetal bovine serum, for one hour. [^3H]-Palmitic acid, 150 μCi , was added for an additional 30 minutes. Thereafter the cells were immediately pelleted, solubilized with 1% dodecyl maltoside for 30 minutes at 4°C in the presence of 0.1 mM PMSF. The solubilized cells were then spun at 100000g for 30 minutes and the clarified supernatant was allowed to bind to a Sepharose 4B-1D4 antibody column. After 4 hours the resin was washed 5 times (150 column volumes) with 10 mls of 0.1% LM in PBS. Opsin was then eluted with 35 μM of a peptide consisting of the carboxy terminal 18 amino acid of bovine rhodopsin. The purified opsin was run on 10%

SDS-polyacrylamide gels and subsequently flurographed. To cleave the thioester linkage between the palmitoyl group and cysteine the labelled opsin was incubated for 30 minutes at room temeperature with 100 mM NH₂OH pH 7.0. This protein was also run on SDS-polyacrylamide gels as well.

2.2.8 Whole Cell Regeneration and Purification of Rhodopsin

Three days post-infection 10⁹ cells (approximately 1 litre), were pelleted at 5000 rpms for 20 minutes at 4°C. The pellet was brought up in 9 mls of ice-cold PBS. In the dark, 11-*cis* retinal was added to a final concentration of 2.5 µM and incubated with the cells for 3 hours. The cells were then solubilized with 1% LM in the presence of 0.1 mM PMSF and immunoaffinity purified as detailed above. The UV/visible spectra of the purified samples were then taken on a Perkin Elmer dual beam spectrophotometer.

2.2.9 Membrane Isolation and Purification of Rhodopsin

After harvesting and pelleting the infected insect cells at 3 days post-infection they were resuspended in ice-cold hypotonic lysis buffer, consisting of 5 mM phosphate buffer pH 6.8. The cells were further disrupted with a teflon douncer, 10 strokes per sample. The homogenate was spun at 100000g for 30 minutes at 4°C. The resulting membrane pellet was resuspended in cold PBS and layered on top of a 20/50% (w/v) discontinuous sucrose gradient. The

gradients were spun at 100000g for 30 minutes at 4°C. The white fluffy band at the interface was removed with a 16 gauge needle. The membranes were washed with 12X volume of PBS and spun at 100000g for 30 minutes at 4°C. The membrane pellet was brought up in 9 mls of ice-cold PBS and regenerated for 2 hours at 4°C with 11-*cis* retinal at a final concentration of 5 µM. The membranes were then solubilized and purified as previously described. The UV/visible spectrum was then taken.

2.2.9 Purification of Rhodopsin and Transducin from Bovine Retinas

Rod outer segments were prepared from retinas by the method of Hong and Hubbell (1973) as modified by Fung *et al.* (1981). Rhodopsin and transducin were purified by the well established procedures of Litman (1982) and Fung *et al.* (1981) respectively.

2.2.10 Transducin Activation

ROS and insect cell rhodopsin were assayed for their ability to activate the GTPase activity of transducin upon light activation. The assay cocktail included: 20 mM Tris pH 7.5, 100 mM NaCl, 5 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT, 0.1% LM, 1.32 µM [γ -³²P]-GTP (1-20 Ci/mmol), 145 pmol of transducin and 5 pmol of rhodopsin. The reaction mixture, excluding GTP, was illuminated for two minutes. Afterwards the assay was initiated by adding the radioactive GTP. At various times after the reaction was initiated aliquots were removed

and the inorganic phosphate was complexed with molybdenum and extracted into an organic phase.

2.3 RESULTS

2.3.1 Detergent Extraction

To determine which detergent or detergents would extract the most opsin, insect cells were solubilized with a whole battery of detergents, run on SDS-polyacrylamide gels and subsequently immunoblotted. From the intensities of the opsin bands shown in Figure 2-3 2% DTAB appears to solubilize the greatest amount of opsin. DTAB also was able to solubilize whole insect cells the most efficiently because the 100000g membrane pellet was the smallest with this detergent. However when 1% DTAB was used later on for purifying regenerated rhodopsin no chromophore was observed (Figure 2-3). This suggests rhodopsin (i.e. the Schiff base linkage) is unstable in it. Qualitatively the amount of opsin solubilized by DM is less than that with DTAB (Figure 2-3). But from our laboratory's and other workers' experience wild type rhodopsin was most stable in this detergent (DeGrip 1982, Oprian *et al.*, 1987). In all subsequent experiments this mild detergent was used in purifying rhodopsin.

2.3.2 Quantitation of Opsin

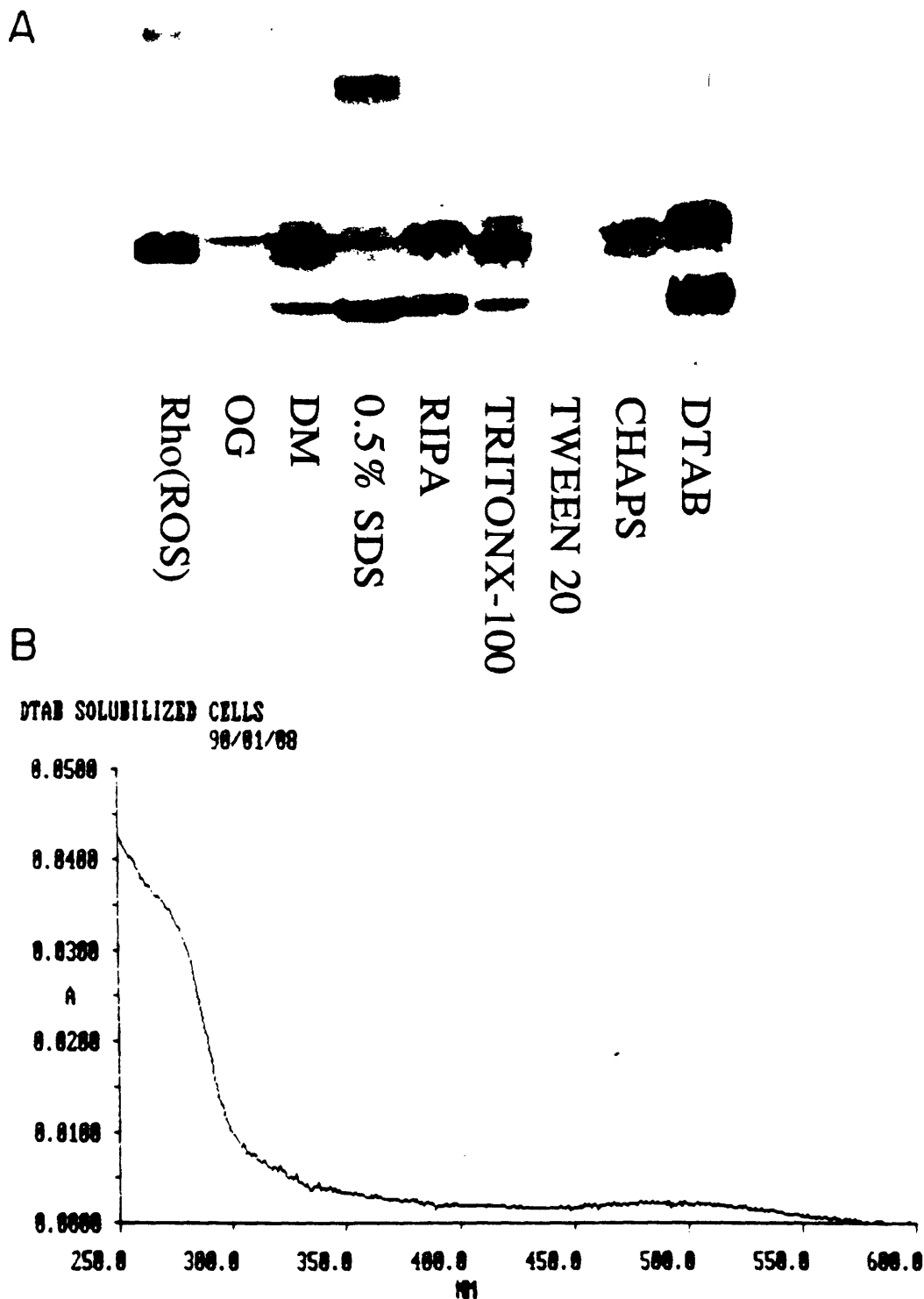


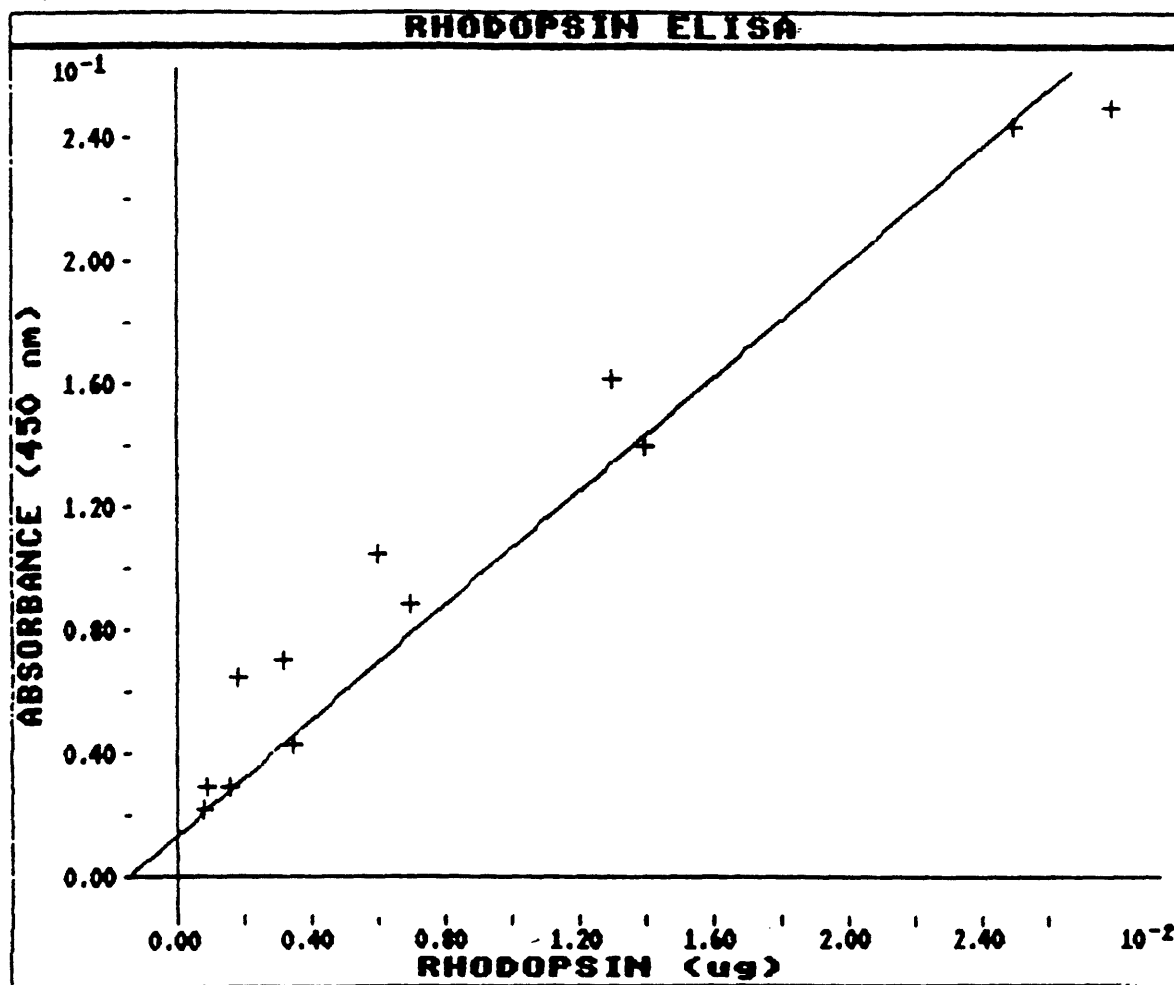
Figure 2-3 A) Detergent extraction of opsin expressed in insect cells. 10^7 were solubilized in 2% of the detergents listed and an equal volume was loaded onto a 10% SDS-polyacrylamide gel and subsequently immunoblotted with ID4, a monoclonal antibody to rhodopsin; B) UV / visible absorption spectra of opsin regenerated with 11-*cis* retinal and solubilized with 1% DTAB.

The standard curve for the rhodopsin sandwich ELISA assay is shown in the top panel of Figure 2-4. With this simple assay it is possible to detect as little as 10 ng of opsin. From this standard curve the amount of opsin produced by Sf9 cells can be easily calculated. The values shown in Table 2-1 are the average of three separate measurements. It is clear the amount of opsin solubilized by DTAB is greater than by LM. DTAB may more effectively solubilize all compartments of insect cells including the ER where partially folded opsin is most likely present. As mentioned above rhodopsin solubilized in DTAB is less stable during the whole cell purification protocol than in LM.

2.3.3 MOI and Time Course of Expression

To study the optimal MOI for expression of opsin insect cells were infected at various MOI's. The cells were then solubilized with LM or DTAB at various times post-infection and the clarified extracts were then run on 10% gels. As seen in Figure 2-5 at 24 hours at MOI's of 5, 10 or 20 only two bands are apparent. As will be discussed later the faster running band represents unglycosylated opsin while the upper band represents the fully glycosylated form. Varying the MOI's had no effect on the amount of unglycosylated opsin being expressed. At 40 and 72 hours post-infection the intensity of these bands does not appreciably change. However there are some bands at lower molecular weights. These most likely represent degradation products. When the same gels are immunoblotted with 4D2, an N-terminus monoclonal antibody, the same pattern is observed suggesting both

A



B

<u>Detergent Used</u>	<u>Days Post-Infection</u>	<u>Opsin (mg/L)</u>
2% DTAB	3	4.0
2% LM	3	0.92

Figure 2-4 A) Standard curve for opsin quantitation by ELISA; B) The amount of total opsin present in whole cells solubilized with DTAB or DM.

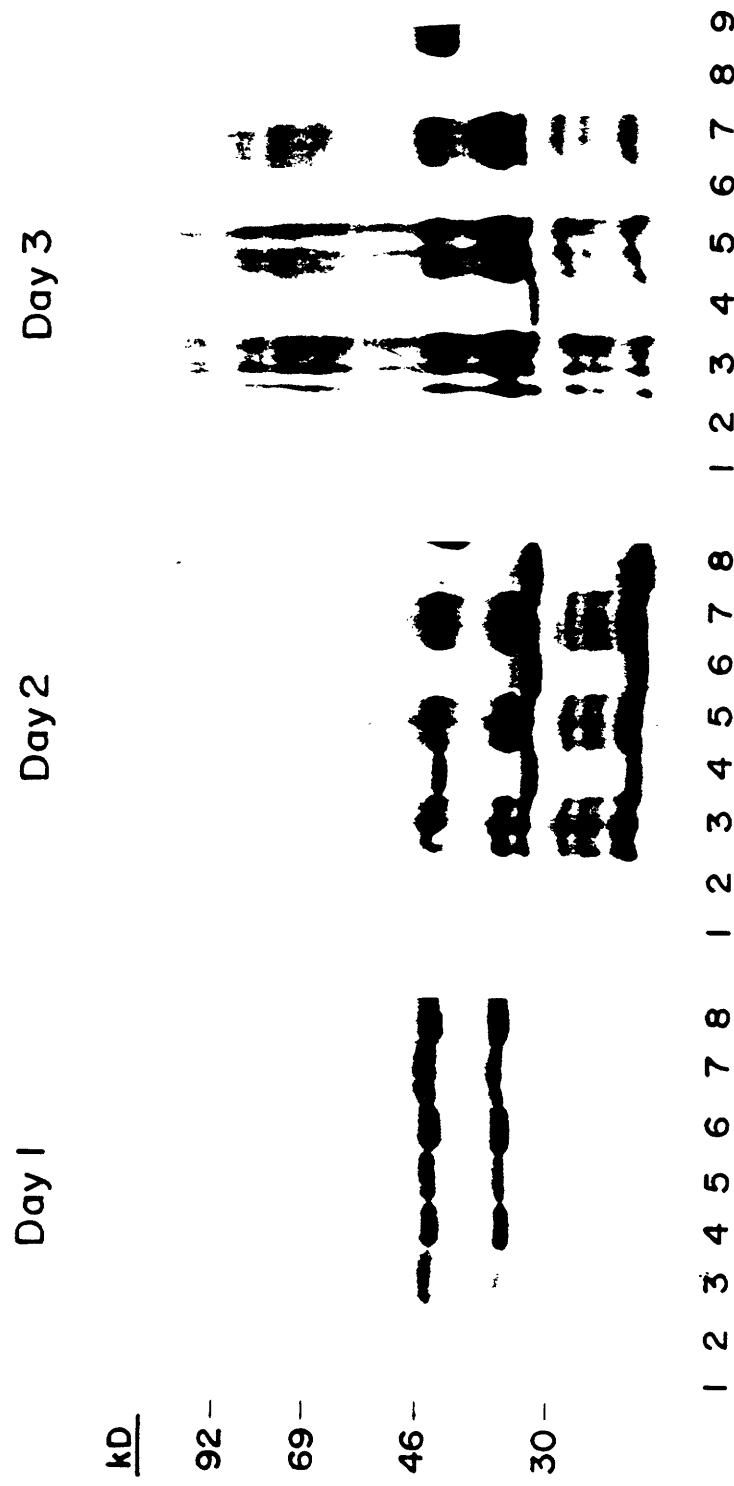


Figure 2-5 Time course of expression. At the times indicated mock infected insect cells, and cells infected with the recombinant virus containing the opsins gene at MOI's 5, 10 and 20 were solubilized with either 1% DTAB or 1% DM (1 ml/10⁷ cells). 20µls of the clarified solubilized extract was loaded on a 10% SDS-polyacrylamide gel. After transfer onto nitrocellulose, the blot was probed with ID4. Lane 1 = mock, solubilized with 1% DTAB; Lane 2 = mock, solubilized with 1% DM; Lane 3 = MOI 5, solubilized with 1% DTAB; Lane 4 = MOI 5, solubilized with 1% DM; Lane 5 = MOI 10, solubilized with 1% DTAB; Lane 6 = MOI 10, solubilized with 1% DM; Lane 7 = MOI 20, solubilized with 1% DTAB; Lane 8 = MOI 20, solubilized with 1% DM; Lane 9 = ROS rhodopsin.

the N and C- termini are intact on the glycosylated and unglycosylated forms. Cells harvested at 24 hours post-infection (infected at an MOI=5) were regenerated with 11-*cis* retinal and purified. No 500 nm chromophore was observed (Figure 2-6). The maximal amount of 500 nm species was observed at 72 hours, consistent with the expression of functional K⁺ channel in insect cells (Klaiber *et al.*, 1990).

Post Translational Modifications

2.3.4 Glycosylation

Insect cell has N-linked glycosylation like ROS rhodopsin. As shown in Figure 2-7, the band migrating with ROS rhodopsin is sensitive to N- glycosidase F. This species is also partially sensitive to endoglycosidase H. There is no evidence of galactose or sialic acid associated with insect cell opsin (Figure 2-8) although insect cells are capable of complex glycosylation (Jarvis and Summers, 1989, Davidson *et al.*, 1990).

2.3.5 Palmitoylation

ROS rhodopsin is palmitoylated at cys322 and cys323 (Ovchinnikov *et al.*, 1988, Karnik *et al.*, 1993). To examine if insect cell opsin is also palmitoylated a [³H]-palmitic acid labelling experiment was done. The fluorogram in Figure 2-9 shows the fully glycosylated species incorporates palmitic acid. The unglycosylated

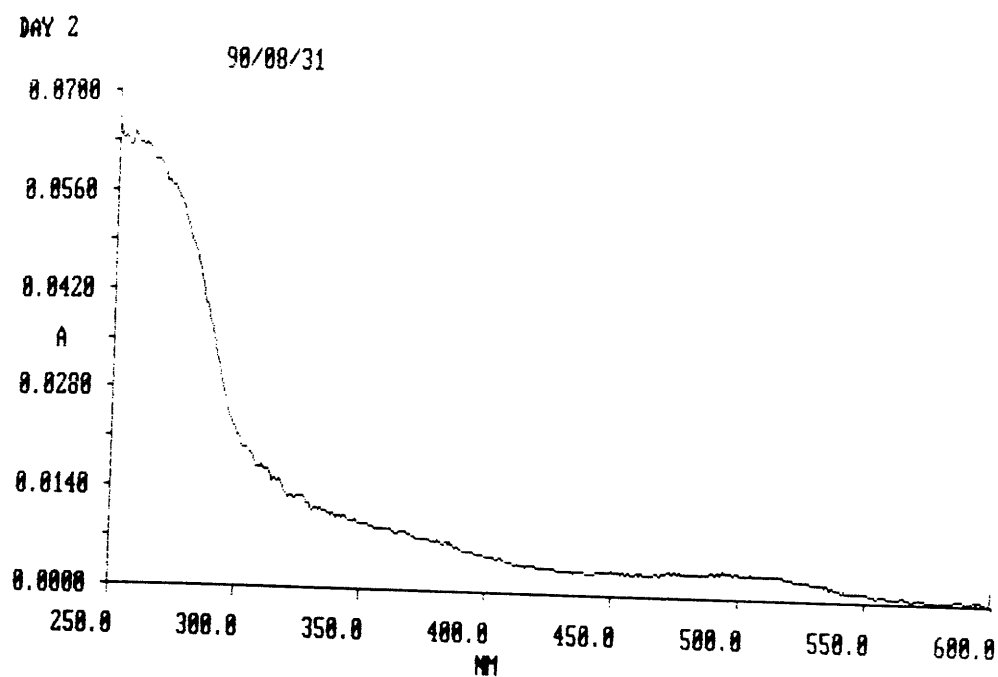
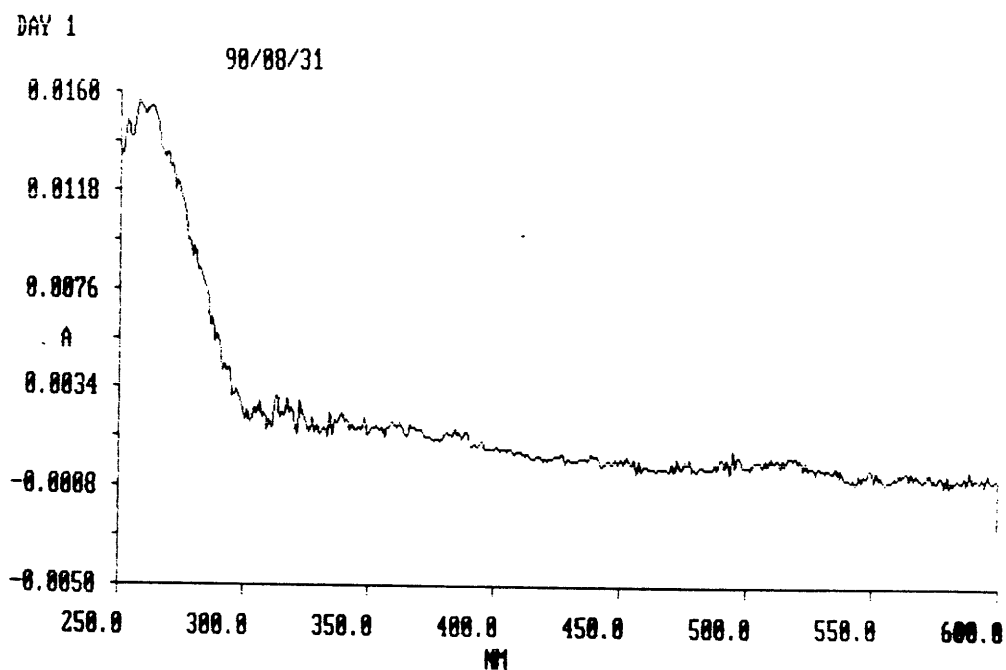


Figure 2-6 UV / visible absorption spectra of regenerated insect cell opsin 24 and 48 hrs post-infection.

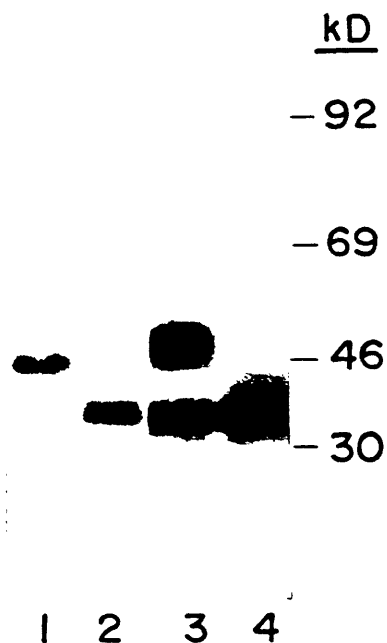
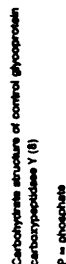
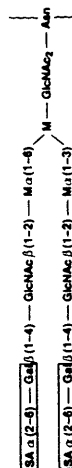


Figure 2-7 Sensitivity of insect cell opsin to N-glycosidase F. ROS rhodopsin and insect cell opsin were solubilized in 1% DM and incubated with PNGase F for 16 hrs at 37°C. The samples were then loaded onto a 10% SDS-polyacrylamide, transferred to nitrocellulose and then probed with ID4. **Lane 1** = ROS rhodopsin - PNGase F; **Lane 2** = ROS rhodopsin + PNGase F; **Lane 3** = insect cell opsin - PNGase F; **Lane 4** = insect cell opsin + PNGase F.

This reaction confirms the presence of N-glycosidically-linked "high mannose" or "hybrid"-type carbohydrate chains with the exception of yeast glycoproteins, the O-glycosidically-linked mannoses of which also react with GNA. For differentiating between N- and O-linked chains of yeast glycoproteins the Asn-linked chains may be removed with endoglycosidase H⁺, endoglycosidase F⁺, or N-glycosidase F⁺, so that only the O-glycosidically-linked mannoses remain.

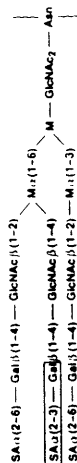


Carbohydrate structure of control glycoprotein transferrin (9)



The details given under II. also apply here.

Carbohydrate structure of control glycoprotein fetuin (10,11) which contains three N-glycosidically and three O-glycosidically linked chains.



	<u>LANE</u>	<u>SAMPLE</u>
B.	1	transferrin
	2	ROS rhodamine
	3	insect cells

C.	<u>SAMPLE</u>
1	fetuin
2	ROS rhodopsin
3	insect cell rhodopsin

Figure 2-8 Carbohydrate structure of insect cell opsin. Purified ROS rhodopsin and insect cell opsin were run on 10% SDS-polyacrylamide gels. The proteins were transferred to nitrocellulose and probed with the lectins noted above.

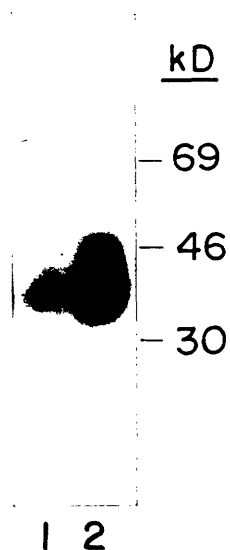


Figure 2-9 Palmitoylation of insect cell opsin. As detailed in the Materials and Methods section, [^3H]-palmitic acid labelled insect cell opsin was immunoaffinity purified, loaded onto a 10% SDS-polyacrylamide gel and subsequently fluorographed. **Lane 1** = [^3H]-palmitic acid labelled insect cell opsin incubated with 100 mM NH_2OH pH 7.0 for 1 hour at room temperature. **Lane 2** = untreated labelled insect cell opsin.

species does not. The thioester bond can be cleaved with neutral hydroxylamine. The absence of these covalent modifications suggest the unglycosylated, unpalmitoylated form is either denatured and/or selectively retained in the ER; since these covalent modifications occur in that compartment.

2.3.6 Purification

When whole cells were regenerated, solubilized and purified the best spectral ratio obtained was $A_{280}/A_{500} = 3.0$. This was done at MOI= 5 and at MOI= 10, as shown in Figure 2-10. Rhodopsin purified from the rod outer segment has a ratio of 1.60. This is considered spectrally pure rhodopsin. When the insect cell rhodopsin was run on gels two bands were seen on Western blots corresponding to the mature and unglycosylated species (Figure 2-11). Silver stained gels also revealed these bands as well as other higher molecular weight proteins (Figure 2-11). Various other methods were tried to improve the spectral ratio including a tandem two-column concanavalin A (a lectin column which recognizes glycoproteins having high mannose structures) followed by the 1D4 antibody column procedure. None of them improved the spectral ratio. However, a crude membrane preparation protocol resulted in spectrally pure rhodopsin with a ratio of 1.65 (Figure 2-10). This purification method yielded $30 \mu\text{g}/10^9$ cells; a recovery of approximately 1% of the total opsin present in whole cells. Total opsin includes both the mature and immature forms. Figure 2-12 shows an immunoblot of each step in the purification; equivalent volumes at each step were loaded into

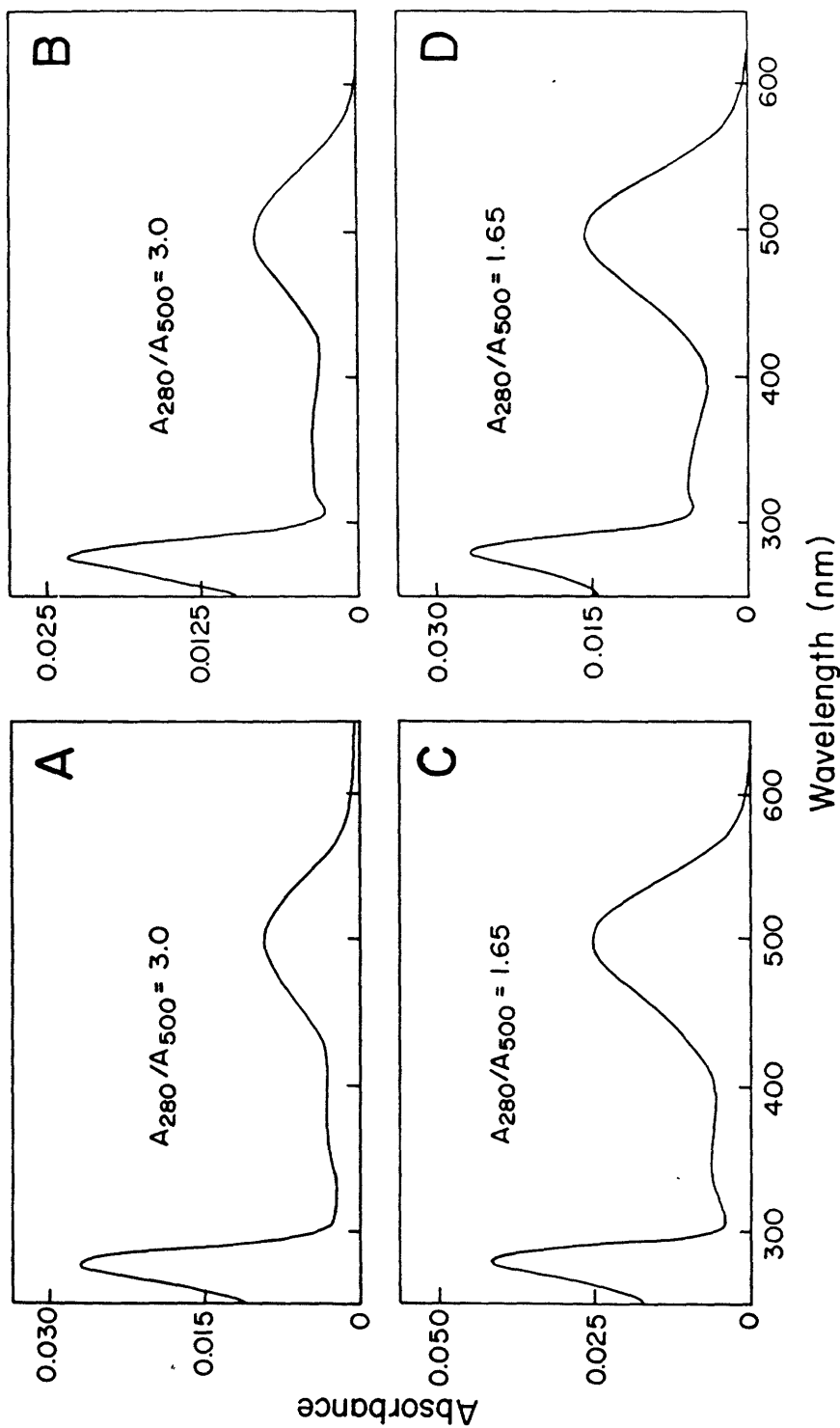


Figure 2-10 UV / visible absorption spectra of insect cell rhodopsin and ROS rhodopsin. Insect cells were infected at different MOI's and harvested at 72 hours. The opsin was either regenerated in whole cells or after preparation of a plasma membrane fraction. After solubilization with 1% DM (with 0.1 mM PMSF), clarification of the extract, the rhodopsin was immunoaffinity purified. **Panel A** = insect cell rhodopsin, cells infected at MOI=5, whole cell regeneration; **Panel B** = insect cell rhodopsin, cells infected at MOI=10, whole cell regeneration; **Panel C** = insect cell rhodopsin, cells infected at MOI=5, plasma membrane regeneration; **Panel D** = ROS rhodopsin.

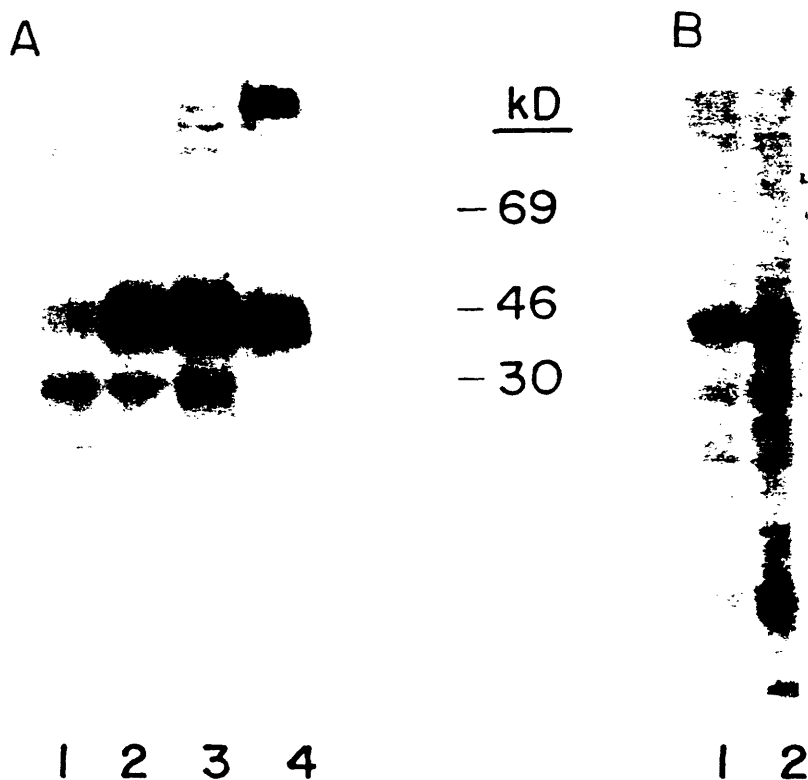


Figure 2-11 Gel characteristics of insect cell rhodopsin purified to a ratio of 3.0. A) Western blot demonstrating the presence of both fully glycosylated and unglycosylated opsin showing: **Lane 1** = 10 μ ls of eluate; **Lane 2** = 25 μ ls of eluate; **Lane 3** = 50 μ ls of eluate; **Lane 4** = ROS rhodopsin. B) Silver stain of same sample showing both the unglycosylated and glycosylated form, as well as other lower MW species as follows: **Lane 1** = 10 μ ls of eluate; **Lane 2** = 25 μ ls of eluate.

successive lanes. All of the unglycosylated opsin was separated from the glycosylated form at the sucrose gradient step. The purified, eluted protein ran as a single band on both immunoblots and silver stained gels (Figure 2-12) corresponding to the glycosylated species- no unglycosylated opsin was detected.

2.3.7 Functional Activity

As shown in Figure 2-13, insect cell rhodopsin activated transducin to about 70% of the value for ROS rhodopsin. This activity was similar to the rhodopsin isolated from COS-1 cells (Oprian *et al.*, 1985).

2.4 DISCUSSION

Rhodopsin has been expressed in Sf9 insect cells under the control of the polyhedrin promoter of baculovirus. The protein expressed has been fractionated by a crude membrane preparation, reconstituted with 11-*cis* retinal and immunoaffinity purified to a spectral ratio of 1.65. Although other membrane proteins (e.g. multidrug transporter, the β -adrenergic receptor) have been expressed in insect cells this is the first time an integral membrane protein has been completely purified from these cells. In those cases the total yield was comparable to what was observed for opsin. Opsin has been previously expressed in Sf9 cells but no purification was achieved nor any spectra shown nor any functional characterization done (Jansen *et al.*, 1988). From the present studies opsin is first synthesized between

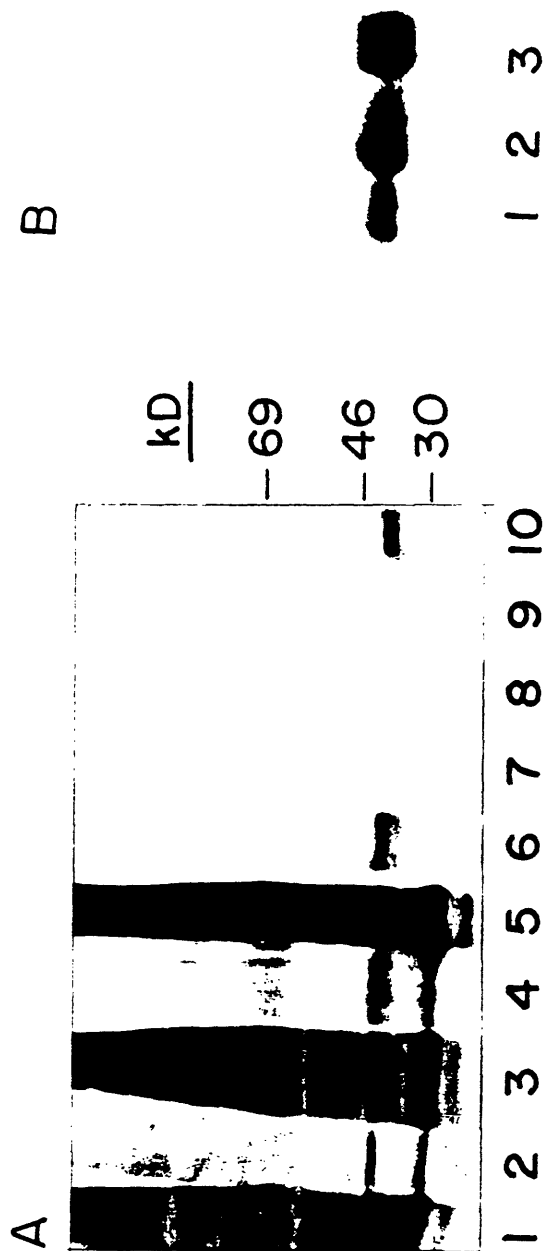


Figure 2-12 Purification of insect cell opsin. A) A fixed proportion of each fraction during the purification procedure was added to sample buffer, and loaded on a 10% SDS-polyacrylamide gel, transferred to nitrocellulose and then probed with ID4 with the results as follows: Lane 1 = cell homogenate; Lane 2 = supernatant from 100000g spin; Lane 3 = pellet from 100000g spin; Lane 4 = plasma membrane band from sucrose gradient spin; Lane 5 = pellet from sucrose gradient spin; Lane 6 = plasma membrane band solubilized with 1% DM and spun at 100000g; Lane 7 = supernatant after opsin binding to column; Lane 8 = first wash of immunoaffinity column; Lane 9 = fifth wash of immunoaffinity column; Lane 10 = purified insect cell opsin. B) Silver stained gel of purified insect rhodopsin showing: Lane 1 = 10 μ ls of eluate; Lane 2 = 25 μ ls of eluate; Lane 3 = 50 μ ls of eluate.

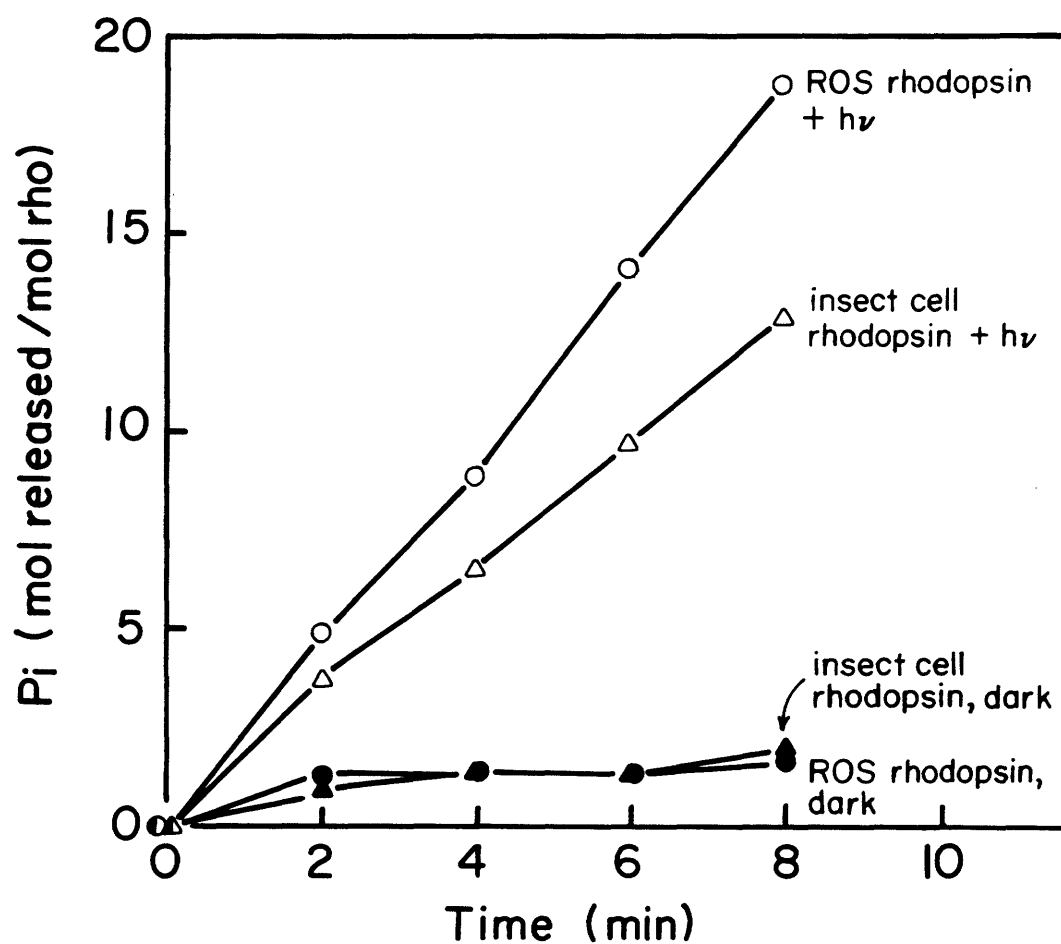


Figure 2-13 Light-dependent transducin activation of ROS and insect cell rhodopsin. As detailed in Materials and Methods section, ROS rhodopsin and insect cell rhodopsin were incubated with transducin and [^{32} P]-GTP. At various times after illumination, samples were removed and the amount of P_i released was quantitated. $\circ$ = ROS rhodopsin + $h\nu$; $\triangle$ = insect cell rhodopsin + $h\nu$; $\bullet$ = ROS rhodopsin - $h\nu$; $\blacktriangle$ = insect cell rhodopsin - $h\nu$.

18 to 24 hours post-infection at MOI's of 5, 10 and 20. Even though protein is detectable by immunoblots at these early times, the protein which is finally purified does not absorb at 500 nm. During these early times opsin may be in a partially folded state and thus can not stably bind 11-*cis* retinal. At any MOI or at any given time unglycosylated opsin was always found.

Insect cell opsin has both N-linked glycosylation and palmitoylation. ROS rhodopsin is glycosylated at asn2 and asn15 (Fukuda *et al.*, 1979, Schichi *et al.*, 1980). At both sites there are high mannose structures which are partially sensitive to endoglycosidase H suggesting that complex or hybrid carbohydrate structures are present (Kornfeld and Kornfeld, 1985). The glycosylated form of insect cell opsin is also partially sensitive to endoglycosidase H suggesting that some fraction of the that species has a high mannose structure. The remainder must have hybrid or complex structures, consistent with recent findings which demonstrated insect cells can trim and add carbohydrates like mammalian cells (Davidson *et al.*, 1991). ROS rhodopsin is also known to be palmitoylated at cys322 and cys323. The palmitoylation of insect cell opsin is the first demonstration of fatty acylation in insect cells. It is believed palmitoylation occurs in the late ER or early Golgi. Insect cell opsin must be transported at least to those organelles. From fluorescence activated cell sorting (FACS) insect cell opsin was found at the cell surface and oriented analogously to that found in COS-1 cells.

Unlike COS cell opsin, insect cell rhodopsin can not be purified by whole cell solubilization and immunoaffinity chromatography. The best spectral ratios obtained by this method was 3.0 regardless of the MOI. When run on SDS-polyacrylamide gels this sample consisted of a fully glycosylated and unglycosylated species. The high spectral ratio reflects the presence of a fraction of purified opsin that does not bind 11-*cis* retinal- the unglycosylated species. However, when a crude plasma membrane preparation was regenerated, solubilized and immunoaffinity purified a spectral ratio of 1.65 was achieved. The recovery was only 1%. This species can activate transducin at levels comparable to ROS rhodopsin. No unglycosylated opsin was seen on immunoblots or silver stained gels. The unglycosylated form was also the form that is not palmitoylated. Taken together this suggests the unglycosylated, unpalmitoylated species is partially or fully denatured such that the retinal binding pocket is poorly formed.

Insect cell rhodopsin is similar to ROS rhodopsin. Spectrally it has a ratio of 1.65 and is glycosylated and palmitoylated. It may be the polyhedrin promoter is so strong that far more opsin is translated than can be accommodated by the post translational modification machinery, as evidenced by the large amount of the unmodified, denatured protein found at even the earliest times of expression. It may be possible to express more of the folded form of the protein by driving expression from weaker promoters (Thiem and Miller, 1990).

The one major advantage of insect cells is the ease of scale up. Since Sf9 cells can survive in suspension, large amount of cells at high

density can be grown, infected and the expressed opsin can be purified by the methods described.

Chapter 3

THE ROLE OF N-LINKED GLYCOSYLATION IN RHODOPSIN STRUCTURE AND FUNCTION

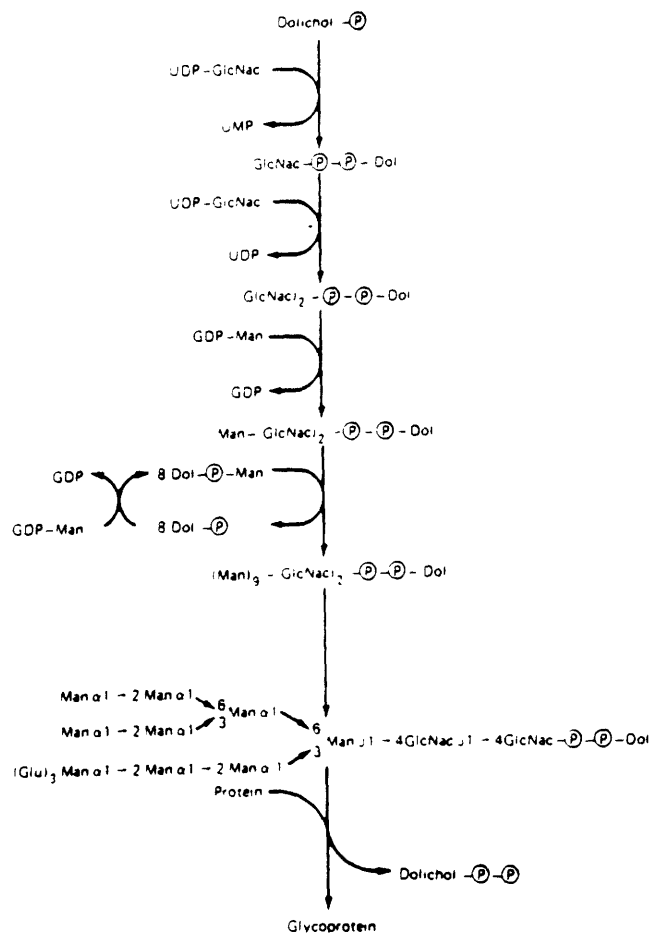
3.1 INTRODUCTION

Asparagine-linked glycosylation of membrane and secreted proteins is frequently observed, although the functions that glycosylation may serve are not clear (Hubbard and Ivatt, 1981; Kornfeld and Kornfeld, 1985). It occurs at the tripeptide sequence asn-X-ser/thr where X is any amino acid except proline (Hubbard and Ivatt, 1981). For example, Rothman and Lodish (1977) showed that for *in vitro* insertion of the VSV G-protein into microsomal membranes N-linked glycosylation was necessary. In an *in vivo* study of the same protein, Machamer *et al.* (1990) found that inhibition of glycosylation by tunicamycin (TM) prevented transport of the protein to the cell surface. Furthermore, the unglycosylated protein was associated with Bip, a resident ER-protein known to recognize and bind misfolded proteins. On the other hand Owen *et al.* (1980) found that N-linked glycosylation of the HLA A and B antigens was not required for appropriate insertion into membranes and transport to the cell surface. In another study the type A natriuretic peptide receptor has also shown the requirement of proper glycosylation (and phosphorylation) for hormone-stimulated guanylyl cyclase activity (Koller *et al.*, 1993). Further, when the β -adrenergic receptor, a member of a superfamily of G protein-coupled integral membrane

proteins, was expressed in the presence of TM, the unglycosylated protein bound to ligands but only inefficiently activated its corresponding effector phosphodiesterase (George *et al.*, 1986; Boege *et al.*, 1988; Dixon *et al.*, 1988).

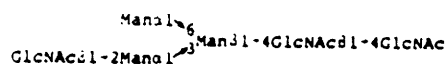
Bovine rhodopsin, as isolated from rod cell outer segments, is glycosylated at asn2 and asn15 by the heexasaccharide sequence GINAc-GINAc-Man-Man-Man-GINAc (Fukuda *et al.*, 1979; Shichi *et al.*, 1980) (Figure 3-1 and Figure 3-2). Fliester and Basinger (1985) attempted to study the role of rhodopsin glycosylation in frog retina by inhibiting glycosylation with TM. Little opsin was found in the rod outer segment. Thus, in analogy with other findings, glycosylation appeared to serve as a transport signal. In structure-function studies of rhodopsin, our laboratory is using COS-1 cells to express wild-type and site-specific mutants of rhodopsin. Using this system, the bovine rhodopsin gene was expressed in the presence of TM. Glycosylation was completely abolished. The opsin expressed was palmitoylated, transported to the cell surface and on incubation with 11-*cis* retinal, fully regenerated the characteristic chromophore ($\lambda_{\text{max}} = 500 \text{ nm}$). These properties demonstrated the retinal binding pocket was correctly assembled and glycosylation was neither required for folding nor for transport. The most significant observation was that following illumination, the TM-rhodopsin was 10% less efficient in triggering transducin, even though the mII half-life was the same as wild type. This suggests glycosylation effects the binding interaction between rhodopsin and transducin.

A

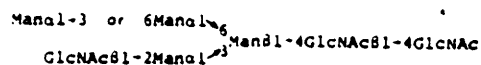


B

Oligosaccharide A



Oligosaccharide B



Oligosaccharide C

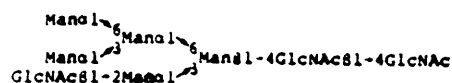


Figure 3-1 A) Synthetic pathway of dolichol diphosphooligosaccharide. B) The structure of oligosaccharides isolated from bovine rhodopsin. Oligosaccharide A is the major fraction. The amide of asparagine is linked to C-2 of the terminal *N*-acetylglucosamine.

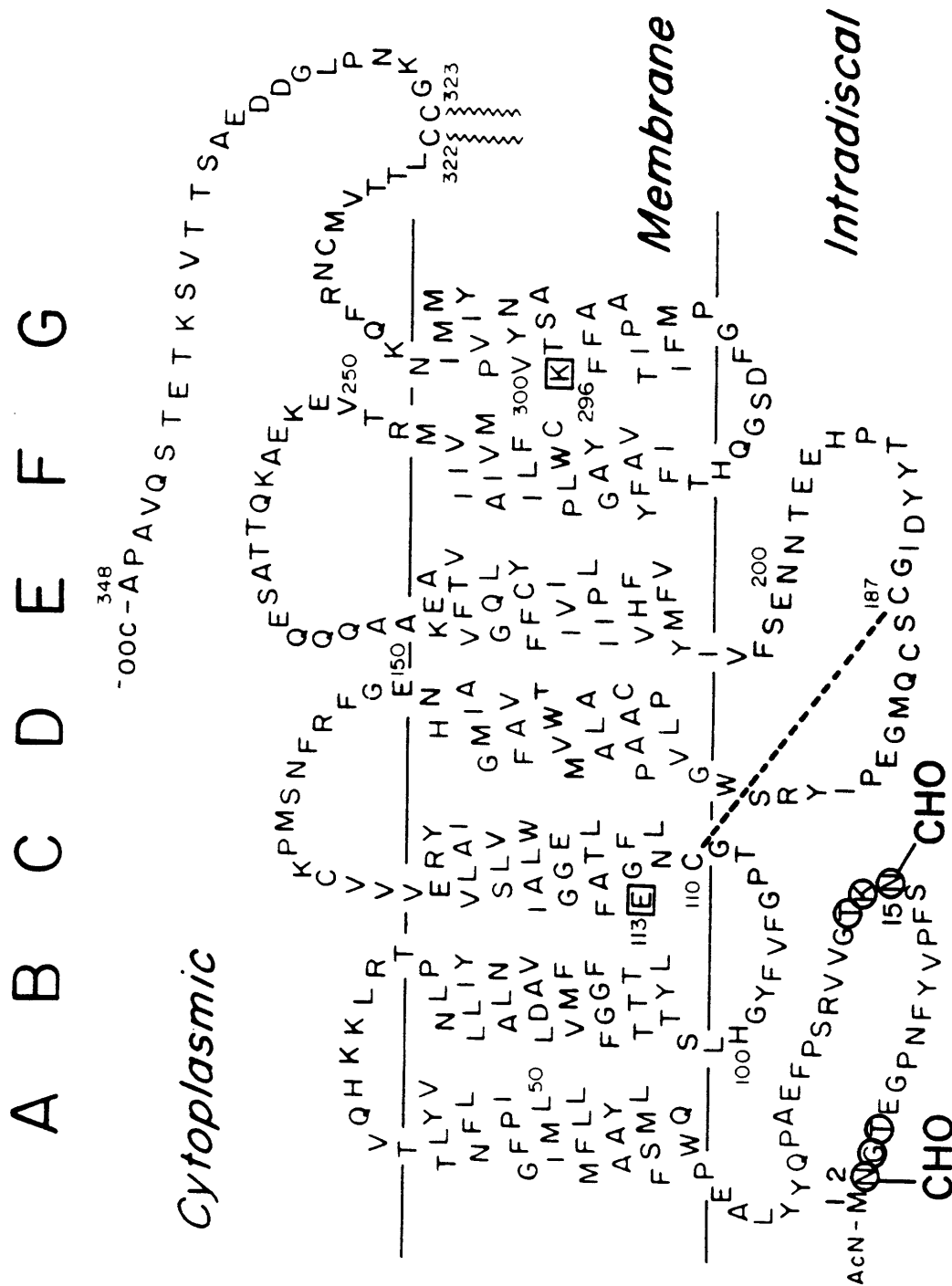


Figure 3-2 A secondary structure model of bovine rhodopsin. The amino acid substitutions investigated there are circled, and the attachment sites for the carbohydrate are designated CHO.

In other experiments the asn2 and asn15 were replaced with gln. The other amino acids involved in the tripeptide consensus sequence were also substituted to understand if they were critical for rhodopsin folding and function. It appears that asn2 and the amino acids immediately adjacent to it are not so critical. The amino acid asn15 and its neighbors play an important role both in the structure and function rhodopsin.

3.2 MATERIALS AND METHODS

3.2.1 Materials

Bovine retinas used for isolating ROS rhodopsin and transducin were from J.A. Lawson Co. (Lincoln, NE). [9,10-³H]-Palmitic acid (60 Ci/mmol), D-[2-³H]-mannose (20 Ci/mmol), deoxyadenosine 5'-[α-³⁵S]-triphosphate (500 Ci/mmol), adenosine 5'-[γ-³²P]-triphosphate and guanosine 5'-[γ-³⁵S]-triphosphate (1000 Ci/mmol) were from NEN. Tunicamycin, GTPγS and N-glycosidase F were purchased from Boehringer Mannheim. Hydroxylamine hydrochloride was from Sigma and dodecyl maltoside was from Anatrace. Sequenase (version 2.0) was from United States Biochemicals. DNA purification kits were from Qiagen. Nitrocellulose filters (HAWP25) were from Millipore. 11-*cis* Retinal was a generous gift of Dr. P. Sorter (Hoffman-La Roche) and Dr. R. Crouch (Medical University of South Carolina and the National Eye Institute).

3.2.2 Construction of Rhodopsin Mutants

All mutants were prepared by cassette (fragment) replacement in the synthetic opsin gene. All the oligonucleotides used for the construction of the mutants were synthesized on an Applied Biosystems 380B DNA synthesizer and purified as previously described (Ferretti *et al.*, 1986). The mutants at asn2 and gly3 were made by codon replacements in the EcoRI-FspI fragment of the synthetic opsin gene. The mutants at asn15, ala, cys, glu, lys, arg and at lys16, cys and pro, were made by codon replacements in the KpnI-FspI fragment in the synthetic gene. All mutants were constructed with the appropriate DNA duplex containing the altered codon. All mutations were confirmed by dideoxynucleotide sequencing of purified plasmid DNA (Sanger *et al.*, 1977).

3.2.3 Expression and Purification of Mutants

Transient transfection of COS-1 cells was done by the DEAE-dextran method (Kaufman and Murtha, 1987). Briefly, COS-1 cells were plated at a density of $1.25-1.5 \times 10^7$ cells per 150 X 25 mm culture plates. 14 hours later the cells were washed in 10 mls serum-free media (Delbecco's Modified Eagle's Media (DMEM)) twice. 12.5 μ g of purified plasmid DNA and DEAE-dextran (0.25 mg/ml final concentration) in DMEM were added to the cells for 6 hours. Thereafter the cells were washed once with DMEM before adding chloroquine (0.1 mM final) in complete media: DMEM plus 10% fetal bovine serum (Luthman and Magnusson, 1983). The cells were then washed twice with DMEM and complete media was added. The next

day the media was changed again. When tunicamycin was used it was added at 0.8 $\mu\text{g/ml}$ immediately after the chloroquine step. The cells were harvested 60-70 hours after transfection, washed with cold PBS (10 mM phosphate buffer pH 7.0, 150 mM NaCl) and incubated with 5 μM 11-*cis* retinal for 3 hours at 4°C in the dark. After solubilization in 1% DM and 0.1 mM PMSF the rhodopsin was immunoaffinity purified on 1D4-Sepharose as described previously (see previous chapter). The resin was washed (150-200 column volumes) with (i) 10 mM Tris (pH 7.0) containing 150 mM NaCl and 0.1% LM or (ii) 2 mM phosphate buffer pH 6.0 containing 0.1% LM. The rhodopsin was eluted with 35 μM with a peptide corresponding to the 18 amino acids at the carboxy terminus of bovine rhodopsin. ROS rhodopsin was purified by the same procedure. Rhodopsin preparations typically had a UV/visible absorbance ratio of A_{280}/A_{500} of 1.6-2.0. The eluted rhodopsin was analyzed by SDS/PAGE with a 5% stacking and a 10% or 12% resolving gel (Laemmli, 1970).

3.2.4 *In vivo* Labelling of Opsin in COS-1 Cells with ^3H -Mannose and ^3H -Palmitic Acid

[^3H]-Mannose incorporation was tested as follows: Forty-eight hours after transfection, the COS cells in 150 mm plates were washed twice with DMEM. The cells were then incubated in glucose-free DMEM for 1 hour. [^3H]-Mannose (100 $\mu\text{Ci/ml}$) was added and after 1.5 hours, the cells were harvested. The opsin was purified as detailed above.

For [^3H]-Palmitic acid labelling 48 hours post-transfection COS cells were starved in serum free media for 12 hours. Subsequently the cells were incubated for 30 minutes at 37°C in media containing 1% fetal calf serum and then for an additional 2 hours in the same media with [^3H]-palmitic acid (100 $\mu\text{Ci/ml}$). Thereafter the cells were harvested and the opsin was purified.

3.2.5 Molar Extinction Coefficients

Extinction coefficients were determined by acid denaturation of each mutant opsin to give a 440-nm peak. The ratio of absorption at the λ_{max} in the dark to the absorption at 440 nm after acid treatment in the dark was compared to that of rhodopsin. The extinction coefficient of rhodopsin was assumed to be 42,700 $\text{M}^{-1}\text{cm}^{-1}$ (Hong and Hubbell, 1972).

3.2.6 Binding and Activation of Transducin

Transducin was prepared by the well-established methods of Fung *et al.* (1981). The ability of wild-type, wild type(TM) and the mutant rhodopsins was measured by the GTP-GDP exchange assay (Wessling-Resnick and Johnson, 1987; Nakayama and Khorana, 1991). For this assay the reaction mixtures contained 0.5 pmoles of pH 6.0-purified wild-type, wild-type(TM) or mutant rhodopsin 4 μM transducin and 10 μM guanosine 5'-[γ - ^{35}S]thio]-triphosphate in 10 mM Tris pH 7.5/0.012% LM/100 mM NaCl/5 mM MgCl_2 /2 mM dithiothreitol. The reaction mixtures were illuminated (>495 nm) for 2

minutes at 20°C and allowed to remain in the dark for an additional 2.5 hours. Aliquots (100 µl) were removed and filtered through nitrocellulose filters. The filters were washed five times in the above-mentioned buffer without DM, dried and counted.

3.2.7 Metarhodopsin II half-life Determination

Purified wild-type rhodopsin and wild-type(TM) were illuminated with light (>495 nm) for 2 minutes. At various times thereafter an aliquot (150 µl) was acidified to pH 2.0 with H₂SO₄ (70 mM final). The spectra of each sample was then taken.

3.2.8 Phosphorylation of Rhodopsin and Mutants

Crude ROS extracts enriched for rhodopsin kinase were prepared according to Sitaramaya (1980) with the modifications of Bhattacharya *et al.* (1992). Briefly, ROS membranes were extracted with 70 mM NaH₂PO₄, pH 7.0, containing 200 mM KCl, 3mM EGTA, 3 mM DTT, and 50 µg/ml of aprotinin, leupeptin, benzamidine-HCl and pepstatin under dim red light. The membranes were centrifuged, the supernatant removed, and centrifuged to ensure all the rhodopsin was removed. The supernatant, which served as the kinase preparation, was concentrated 15-20-fold using Centricon CX30 immersable filters. To 250 pmols of purified pigments was added 200-250 units of kinase extract (1 unit=1 mol Pi incorporated/mol ROS) and a solution of 70 mM NaH₂PO₄, pH 7.0 containing [γ-³²P]-ATP, 3mM MgCl₂, 150 µM DTT, and 10 µM GTP. The final DM concentration was 0.012% (w/v)

and the reaction volume was 2 mls. Phosphorylation was initiated by illuminating the samples with a 300 W light source (>495 nm) at 20°C . At various times up to 60 minutes aliquots (200 μl s) were removed and precipitated with 5 mM phosphoric acid containing 7% (w/v) trichloroacetic acid (TCA) and 25 μg bovine serum albumin (BSA). Samples phosphorylated under dim red light for 60 minutes served as dark controls. After 2 hours at 4°C , the samples were centrifuged and the pellet washed 5 times with 1 ml of TCA. The pellets were dissolved in 0.5 mls protosol and counted. The [^{32}P] (mol) incorporated was calculated from the amount of acid-precipitable counts and the specific activity of the [γ - ^{32}P]-ATP.

3.3 RESULTS

3.3.1 Replacement of Asn by Gln at Glycosylation Sites in Rhodopsin

The first mutants to be constructed and expressed were N2Q, N15Q and N2,15Q. The glutamine substitutions represent the most conservative substitutions for asn. The mutant N2Q yielded nearly comparable amounts of chromophore to wild type rhodopsin (Figure 3-3). However, the expression of the mutants N15Q and N2,15Q gave much less opsin. Upon regeneration and purification at pH 7.0 in the presence of 100 mM NaCl they showed poor spectral ratios, $A_{280}/A_{500} > 5.0$.

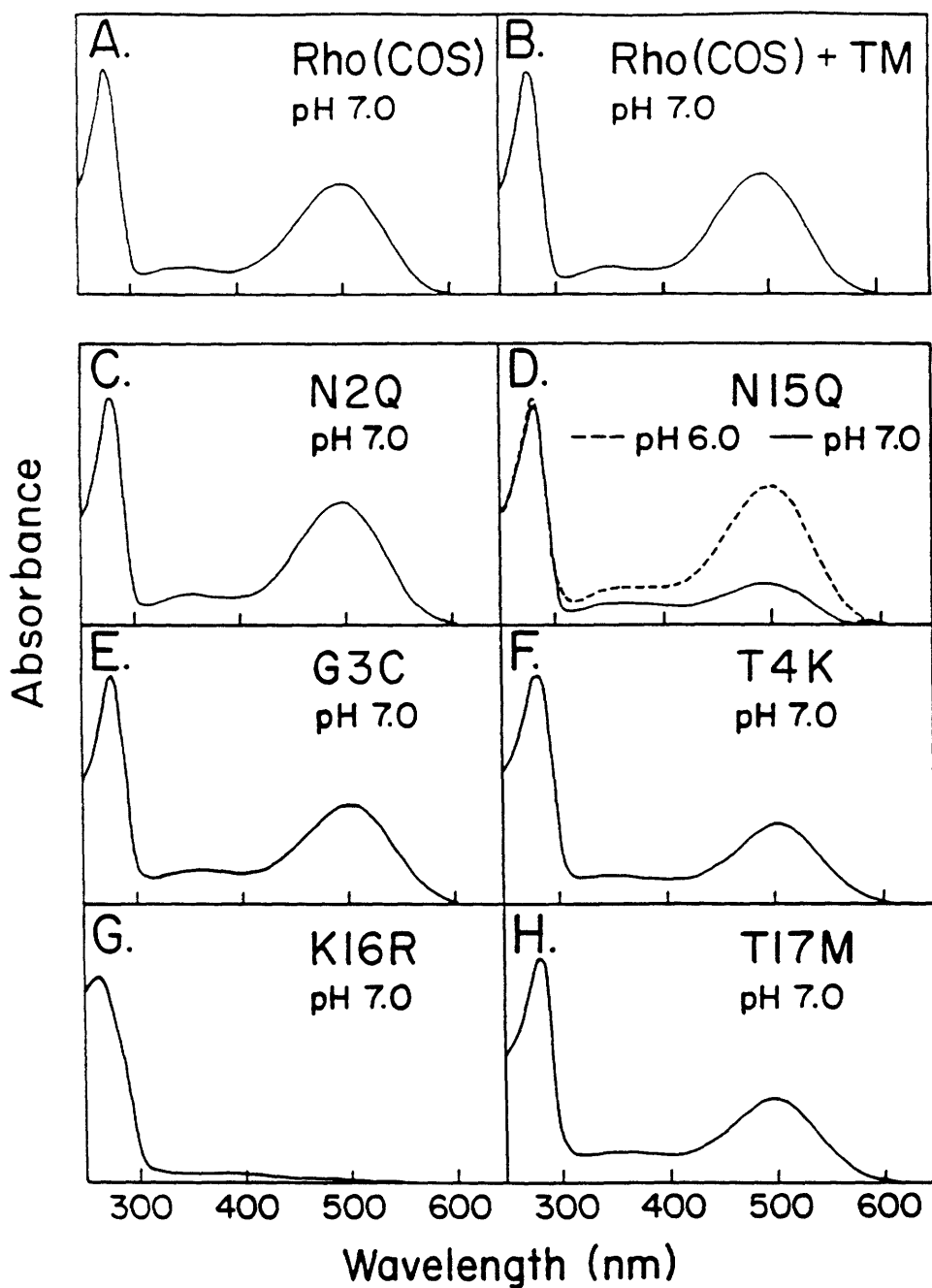


Figure 3-3 UV / vis absorption spectra of nonglycosylated wild-type rhodopsin and glycosylation-site mutants. Opsin and opsin mutants expressed in transiently transfected COS-1 cells were reconstituted with 11-*cis*-retinal, solubilized in DM, and immunopurified at pH 7.0 or pH 6.0 as indicated. Spectra recorded at 20°C in the dark are shown. The absolute A_{280} values varied from 0.1 for glycosylated wild-type to 0.04 K16R mutant.

Figure 3-4a shows an immunoblot of wild-type rhodopsin, the gln mutants and their sensitivity to N-glycosidase F. The mutant N2Q shows a glycosylation smear which migrates slightly faster than wild-type. This is consistent with glycosylation at one site (asn15) and not the other (asn2) since the consensus sequence had been altered thereby abolishing N-linked glycosylation at the latter site. The other mutants lack a smear and have a distinct intermediate band between the mature form (highest MW band) and the unglycosylated form (lowest MW form). This particular band may represent a partially folded and/or partially glycosylated species. Except for N2,15Q the other mutants collapse to a single band after incubation with N-glycosidase F.

3.3.2 Sugar Labelling of Glycosylation Mutants

To demonstrate if these mutants can incorporate mannose, [^3H]-mannose was added to the transfected cells prior to purification. As seen in the fluorogram in the of Figure 3-4b, wt, N2Q, N15Q, were labelled whereas N2,15Q was not. This is consistent with there being only two glycosylation sites in rhodopsin.

3.3.3 Cellular Localization of Mutants

As a qualitative measure of transport Figure 3-5 shows the immunofluorescence staining pattern of these mutants. When expressed, wt and N2Q were found mostly at the cell surface; the mutants N15Q and N2,15Q were found in the perinuclear region.

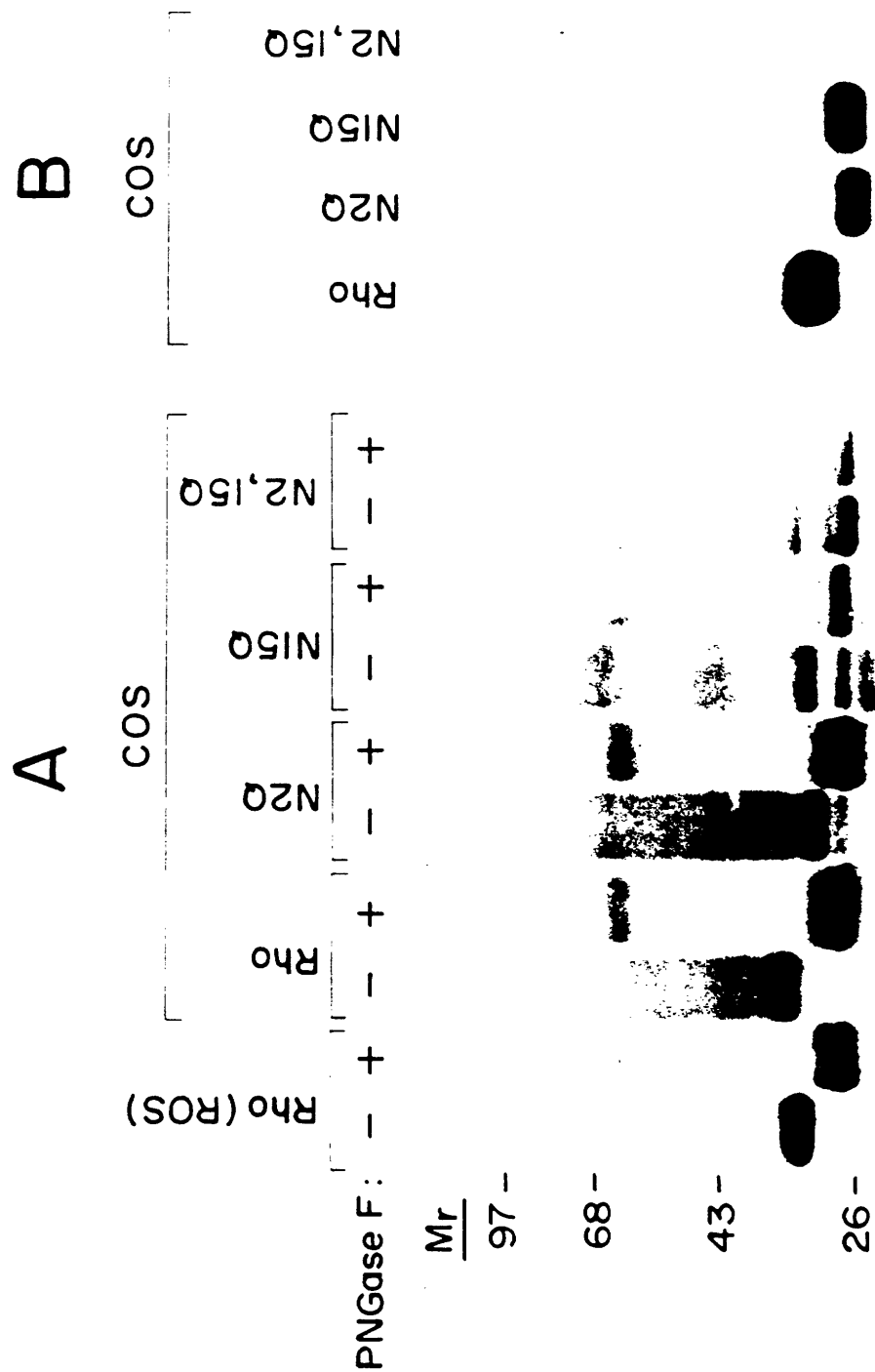


Figure 3-4 Characterization of glycosylation-site mutants of opsin. A) Sensitivity of ROS rhodopsin [Rho(ROS)], wild-type COS-1 cell opsin [Rho (COS)], and glycosylation-site mutant opsins N2Q, N15Q, and N2, 15Q to PNGase F. Equivalent amounts of protein were analyzed by SDS/PAGE and immunoblotting before and after treatment with PNGase F. B) Fluorograph of [³H]mannose labeling of wild-type COS-1 cell opsin [Rho (COS)] and glycosylation-site mutant opsins N2Q, N15Q, and N2,15Q. Equivalent amounts of protein were analyzed by SDS/PAGE and fluorography.

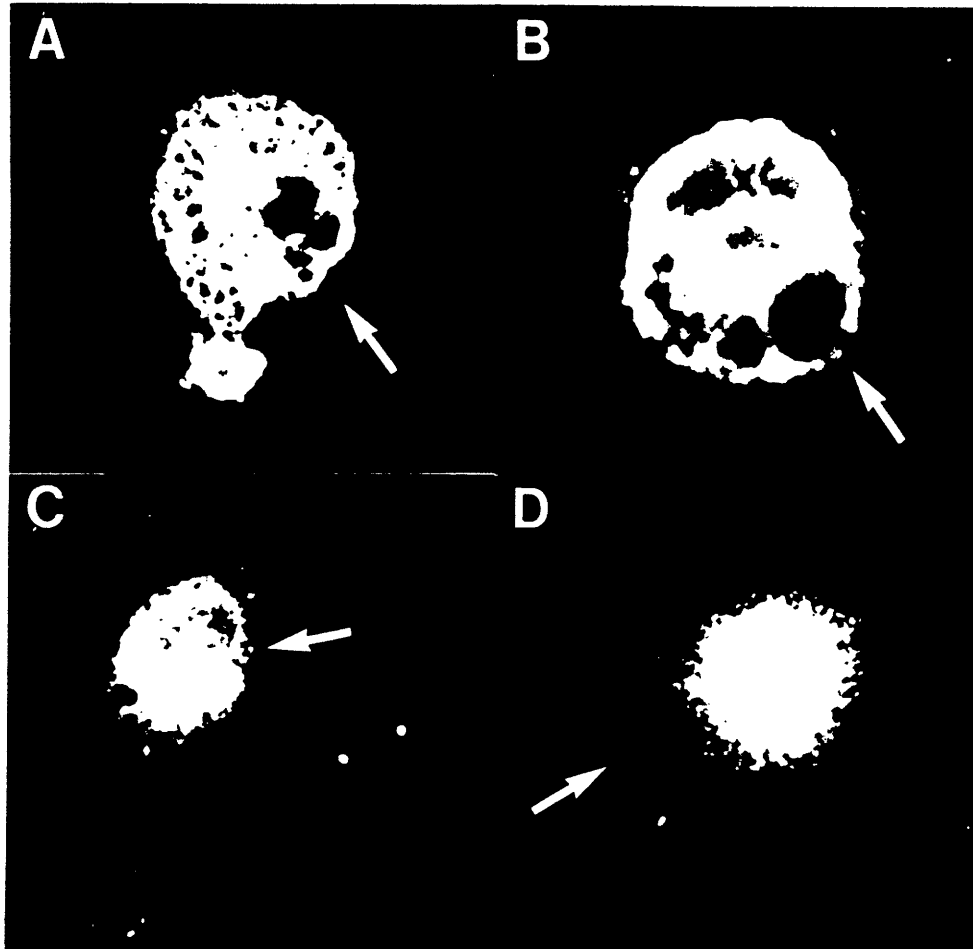


Figure 3-5 Cellular localization of glycosylated and nonglycosylated opsins by immunofluorescence. A) Wild-type COS-1 cell opsin. B) Wild-type COS-1 cell opsin expressed in the presence of TM. C) Mutant opsin N15Q. D) Mutant opsin N2,15Q. Rhodamine-conjugated goat anti-mouse antibody was used to probe for the mouse rho 4D2 rhodopsin monoclonal antibody. Arrows indicate surface of the COS-1 cells.

Presumably they were retained in the ER. It seems when opsin is translocated out of the ER its subsequent glycosylation causes a characteristic smear. The smear represents microheterogeneity in carbohydrate processing in the Golgi (Goochee *et al.*, 1991). Mutants which are retained do not acquire this smear and furthermore these proteins show a distinct multi-band pattern on immunoblots.

3.3.4 Hydroxylamine Sensitivity of Mutants

To probe the stability of the 500 nm chromophore we examined its sensitivity to hydroxylamine in the dark. It is known the color pigments and certain rhodopsin mutants react with hydroxylamine in the dark (Zhukovsky *et al.*, 1992). Wild-type rhodopsin itself is not sensitive to rhodopsin in the dark. As shown in Appendix 3 neither N2Q, N15Q nor N2,15Q react with neutral hydroxylamine (100 mM final) in the dark, suggesting that the ground (dark) state of these mutants is equally unreactive as the wild-type. The photobleaching behavior of the mutants N15Q and N2,15Q was however abnormal. They appear to release all-*trans* retinal more quickly after a 10 second illumination, indicating the mII species has a shorter half-life than wild-type (Appendix 2). It appears glycosylation at asn15 stabilizes the photoactivated species mII.

3.3.5 Characterization of COS Cell Rhodopsin Expressed in the Presence of Tunicamycin

Tunicamycin has been used extensively by others to inhibit the transfer of dolichol pyrophosphate-(GluNAc)₂-Man₉ to an asparagine residue which is part of the consensus tripeptide sequence for N-linked glycosylation (Elbein, 1987). To study the effects of TM on wild-type opsin, the concentration needed to inhibit glycosylation without affecting the overall yield was first determined. At 0.8 µg/ml COS-1 cell opsin migrated as a single band of MW ≈33,000 daltons, similar to N-glycosidase F-treated ROS rhodopsin. As shown in Figure 3-6a, the mobility of this species does not shift after treatment with N-glycosidase F, unlike wild-type rhodopsin in the absence of TM. To further demonstrate TM treated opsin was unglycosylated an attempt was made to label it *in vivo* with [³H]-mannose. The fluorogram in Figure 3-6b shows wt(TM) does not incorporate the labelled sugar even though the protein is observable by immunoblot. It had been previously shown that wild-type COS opsin is palmitoylated and transported to the cell surface. Figure 3-6c shows a fluorogram of wt and wt(TM) labelled with [³H]-palmitic acid, both are labelled. Although the actual cellular compartment of palmitoylation is not known with certainty, it is believed to occur in the "late" ER or early Golgi. Figure 3-5 demonstrates by immunofluorescence that both wt and wt(TM) reach the cell surface.

The UV/visible absorption spectra of regenerated, purified unglycosylated opsin and glycosylated wild-type rhodopsin are shown in the top two panels of Figure 3-3. Clearly the unglycosylated form is able to form a 500 nm chromophore with a spectral ratio (1.7) similar to the glycosylated form. Both species are resistant to hydroxylamine

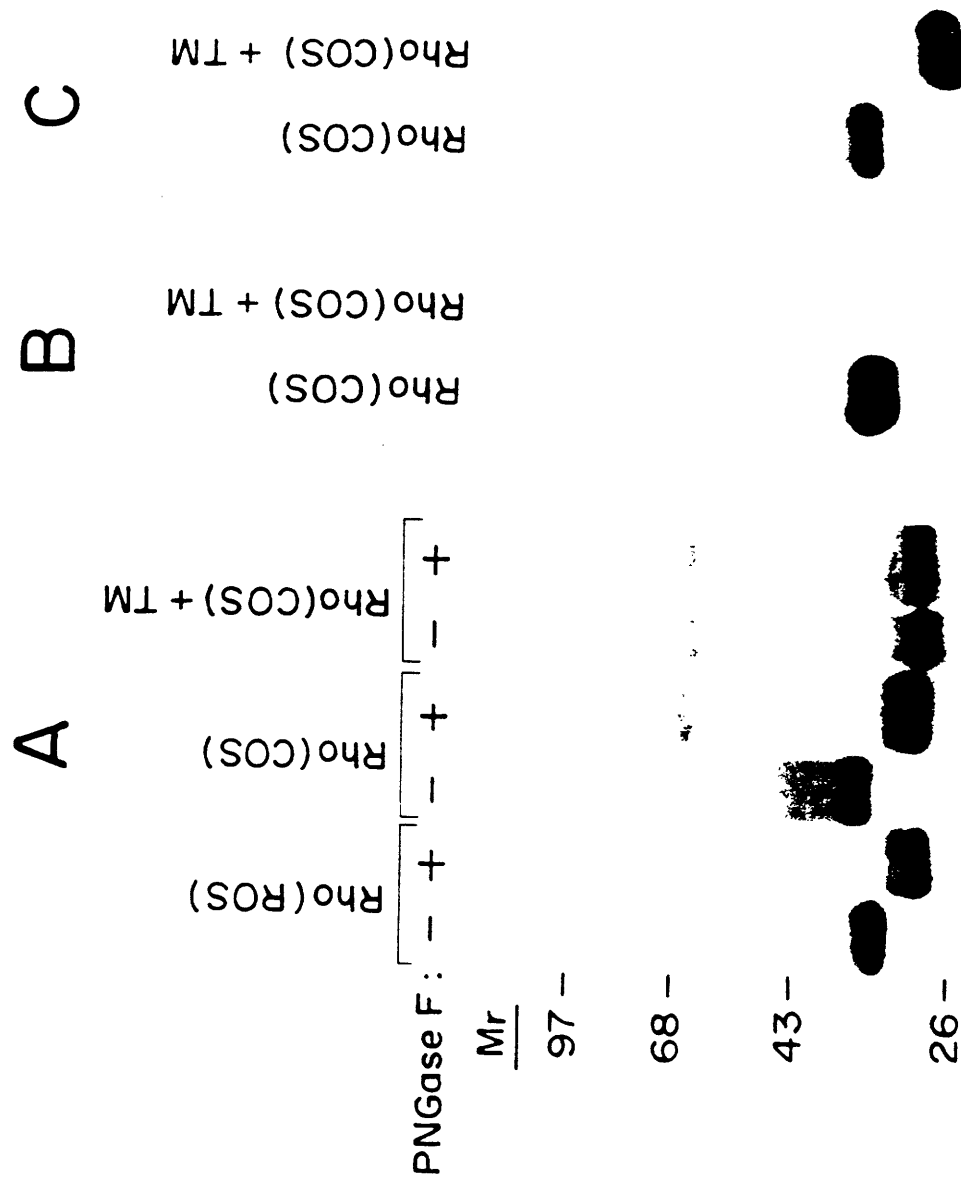


Figure 3-6 Characterization of wild-type opsin expressed in the absence and presence of TM. **A**) Sensitivity of ROS rhodopsin [Rho (ROS)], wild-type COS-1 cell opsin [Rho (COS)], and wild-type COS-1 cell opsin expressed in the presence of TM[RHO (COS) + TM] to PNGase F. Equivalent amounts of protein were analyzed by SDS/PAGE and immunoblotting before and after treatment with PNGase F. fluorograph of [^3H]mannose. **B**) and [^3H]palmitic acid-labeled wild-type COS-1 cell opsin [Rho (COS)] expressed with and without TM **C**). Equivalent amounts of protein were analyzed by SDS/PAGE and fluorography.

bleaching in the dark suggesting both chromophores are stable in the absence of light (Appendix 3).

We tested the functional competence of unglycosylated rhodopsin by examining its ability to bind and activate transducin by using a GTP γ S exchange assay. As seen in Figure 3-7 unglycosylated opsin is less efficient in coupling to transducin than the glycosylated form.

This difference is not due to a shorter mII half-life since the $t_{1/2}$'s of unglycosylated rhodopsin is the same as the glycosylated form as shown in Figure 3-8. Acid denaturation of rhodopsin upon illumination results in an absorption spectrum with a maxima at 440 nm. This is typically what is found for a protonated Schiff base that has no significant protein interactions. The acid denaturation assay therefore monitors the rate of Schiff base hydrolysis, reflecting the decay of mII. The mII half-life for both chromophores was approximately 18 minutes.

3.3.6 Characterization of Other Glycosylation Site Mutants

To further understand the role of the two glycosylation sites of rhodopsin additional mutants were constructed at other amino acids in the tripeptide consensus sequence. Gly3 was replaced with proline since it is not tolerated in the second position of the consensus tripeptide sequence. A cysteine mutant was also made at that site for

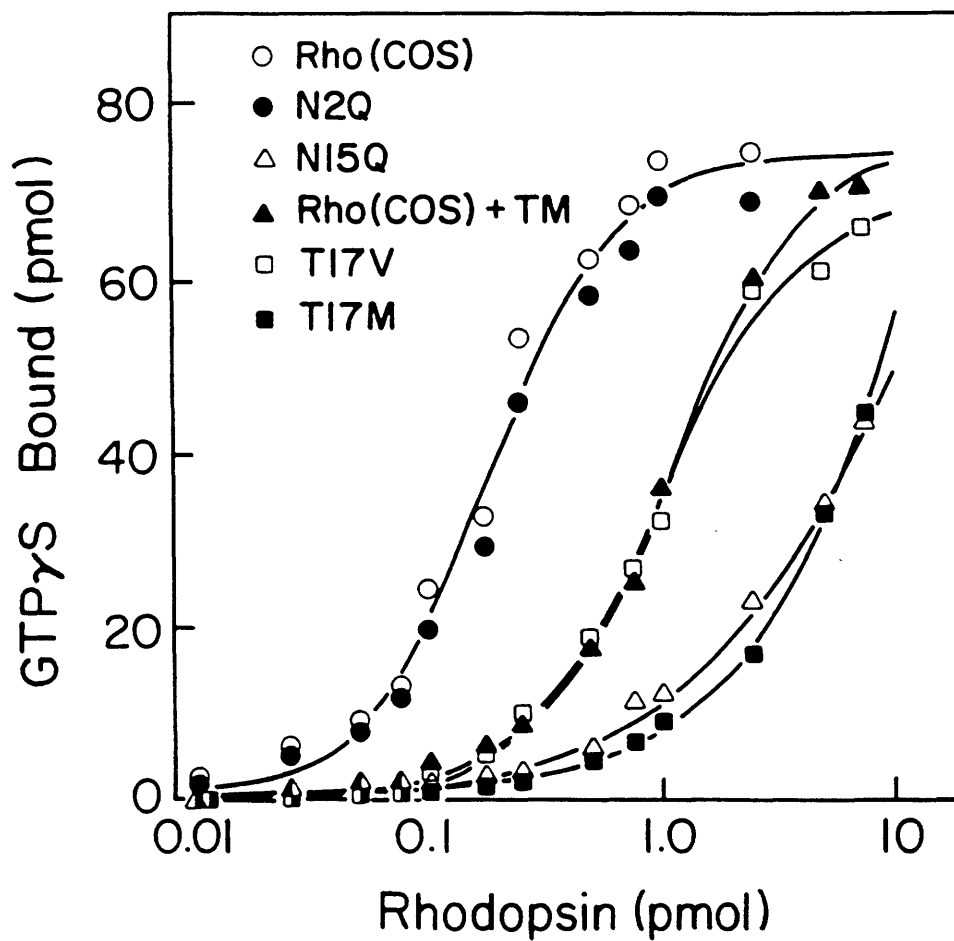


Figure 3-7 Transducin activation by nonglycosylated wild-type rhodopsin and glycosylation-site mutants. The activation of transducin as a function of rhodopsin concentration was measured by using a GTP[γ S] filter-binding assay. The amount of pigment used in the assays was based on the molar extinction coefficient. Wild-type COS-1 cell rhodopsin [Rho (COS)] expressed in the absence (○) and presence of TM (▲); N2Q (●); N15Q (△); T17M (■); and T17V (□).

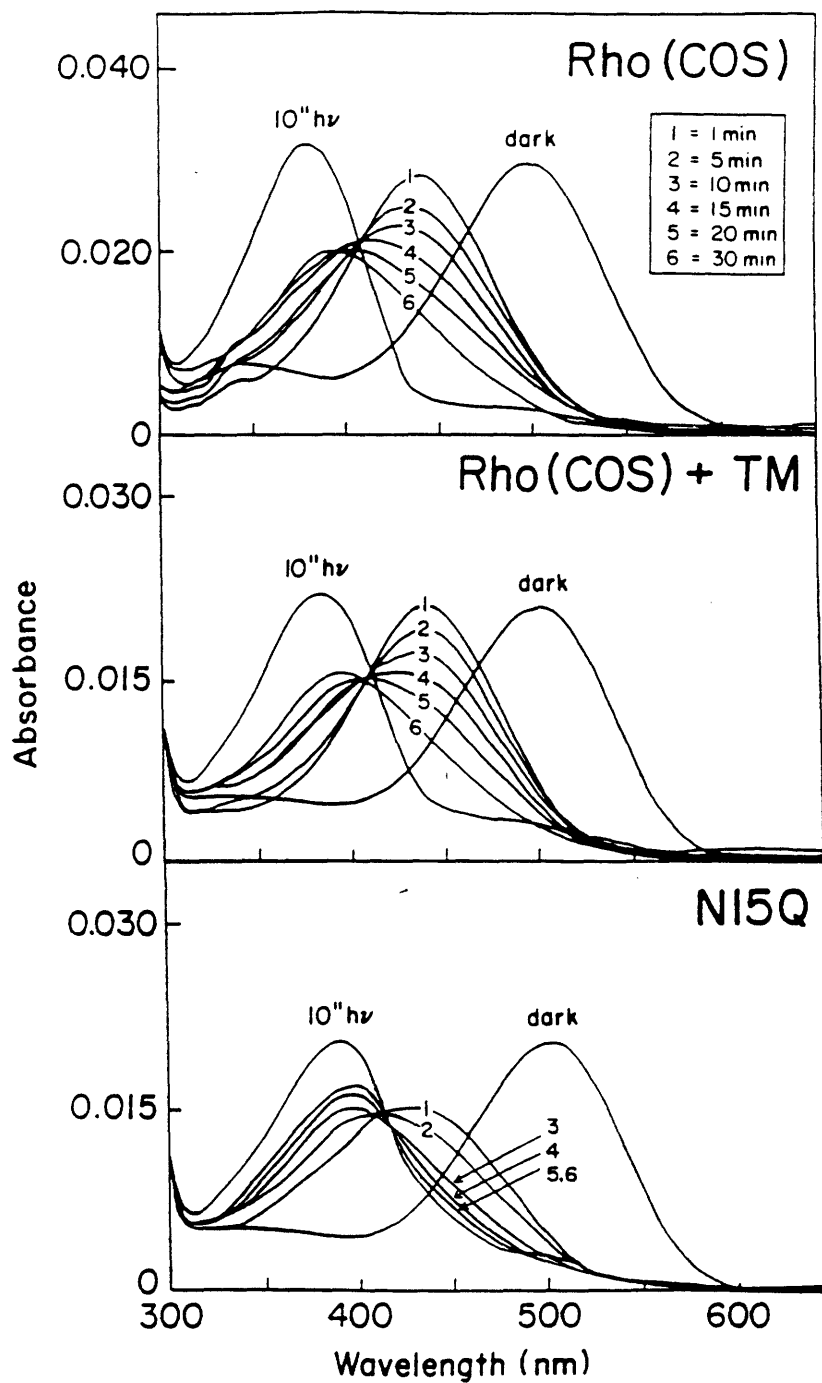


Figure 3-8 UV / vis absorption spectra of glycosylated and nonglycosylated wild-type and N15Q rhodopsins upon illumination. The pH 6.0-purified pigments were illuminated (> 495 nm) for 10 s at 20°C and subsequently denatured at the indicated time periods by the addition of 2 M H_2SO_4 to a final pH of 1.9.

future crosslinking and derivatization studies. Additional substitutions at asn15 were made to examine if residues other than glutamine would be tolerated at those positions. N15A, N15C, K16C were constructed and expressed to see if a smaller residue would allow for correct and efficient folding of opsin. The mutants N15E, N15K, N15R and K16R were made to see if charge substitutions would be tolerated.

These additional mutants can be classified by the amount of A500 and their spectral ratio. Those mutants near the N-terminus N2Q, G3C, G3P were most similar to wild-type in both yield and spectral ratio (Figure 3-3 and Appendix 1).

In contrast, those mutants at the second glycosylation site showed quite different spectral characteristics. All the mutants at N15 were similar to N15Q, i.e. low chromophore yields and high spectral ratios. The two mutants at K16 did not regenerate at all. The spectra of K16C is shown in Figure 3-3. These opsins are not sensitive to hydroxylamine in the dark. Nearly all of these mutants have abnormal bleaching characteristics, similar to N15Q, indicating the mII half-life is shorter in these mutants (Appendix 2). This is consistent with these mutants being less efficient in triggering transducin (Appendix 4).

3.3.7 Phosphorylation of wild-type rhodopsin, non-glycosylated rhodopsin and glycosylation mutants

Another assay to detect the difference in the mII-like conformations of wt, wt(TM) and the mutants is in their ability to be phosphorylated. As discussed in the introduction phosphorylation of rhodopsin occurs as a mechanism by which the activated receptor is turned off and eventually returned to the ground state. Figure 3-9 illustrates that at 60 minutes ROS rhodopsin and wt(COS) incorporate about 4.5 moles of [^{32}P] per mole of protein whereas wt(TM) and N15Q incorporate only 1 mole per mole and N2Q and T17M incorporate about 2.5 moles per mole. This would suggest there is a difference in the mII-like state of wt(TM) since its mII $t_{1/2}$ is similar to wt(COS). In the case of the mutants the possibility exists there is a difference in the mII-like state since it is shorter lived. But whether the actual conformation of those intermediates is different can not be determined from these data.

3.4 DISCUSSION

As an integral membrane protein opsin must be transported from the ER to the Golgi apparatus and eventually to the cell surface, in COS cells, or to the outer segment of the rod cell. Along this transport path it is covalently modified by the glycosylation and palmitoylation enzymatic machinery. The first glycosylation step occurs in the ER, probably as a cotranslational event (Hubbard and Ivatt, 1981). It is believed that glycosylation can occur just as long the site is available i.e. the site must be accessible before that region folds (Hubbard and Ivatt, 1981). Using both site-specific mutagenesis and tunicamycin the role of N-linked glycosylation in rhodopsin

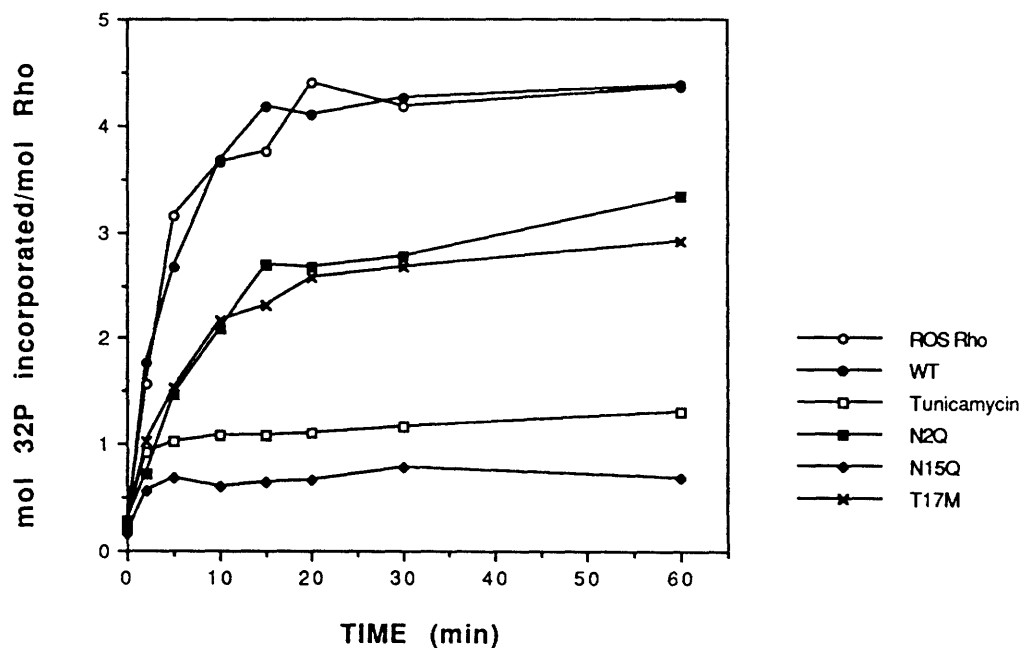


Figure 3-9 Phosphorylation of ROS rhodopsin, COS rhodopsin, wt(TM) and mutants of rhodopsin. After illumination, phosphorylation was initiated by adding rhodopsin kinase. After quenching and TCA precipitation, the extent of phosphorylation at various times was quantitated by measuring the amount of [^{32}P] incorporated.

structure and function has been studied. Conditions were established whereby wild-type opsin could be expressed in the presence of tunicamycin at a concentration that was not toxic to the cell nor that drastically altered protein synthesis. At high concentrations TM is known to block protein synthesis (Elbein, 1987).

The most important finding in this study of rhodopsin is glycosylation is not required for the *in vivo* folding, assembly and transport of opsin. Unglycosylated wild-type rhodopsin, wt(TM), is palmitoylated further demonstrating it is similar in most aspects to wild-type rhodopsin. However, unglycosylated rhodopsin is 1/10 less efficient in triggering transducin as evidenced by a rightward shift of the binding curve.

Complementing this approach, a series of mutants were constructed and expressed to separately assess the role of glycosylation at asn2 and asn15. These mutants also delineated some of the structural features of the N-terminus.

The UV/visible spectra of unglycosylated rhodopsin and the mutants is a useful way to determine the ability of the protein to form a correctly folded retinal binding pocket and its ability to stably bind 11-*cis* retinal. As such unglycosylated rhodopsin, N2Q, G3C and G3P regenerated like wild-type. It appears glycosylation at asn2 is not required for the folding or function of rhodopsin. However, all the remaining mutants regenerated moderately or poorly as reflected by the low absorbance at 500 nm and high A_{280}/A_{500} ratios. This

suggests that some fraction of the immunoaffinity purified protein is not able to bind 11-*cis* retinal via a stable Schiff base linkage. This difference may reflect how efficiently these mutants are being folded within the cell. When the mutants are purified at pH 6.0 in the absence of salt, the spectral ratio is between 1.6-1.9. The low pH elution, developed by Kevin Ridge in our laboratory, selectively releases the folded form of the mutants from the resin.

Previously in our lab Doi *et al.*, (1991) demonstrated the intradiscal region of rhodopsin is important for the proper assembly of rhodopsin. Notably N-terminal deletion mutants showed the same poor regeneration as the mutants at asn15 and lys16. These residues besides forming the glycosylation site also participate in the formation of the intradiscal tertiary structure.

On immunoblots purified ROS rhodopsin migrates as a single band. COS cell rhodopsin has a mature species comigrating with the ROS form as well as a higher molecular weight glycosylation smear. This mature form and the smear collapse to a single band when incubated with N-glycosidase F. This deglycosylated opsin comigrates with opsin expressed in the presence of TM. The mutant N2Q, as well as G3C, G3P and T4K, has a mature form and smear that has a smaller molecular weight than that observed with wild-type. This would imply that the smear is associated with asn15. Its presence is correlated with the protein being transported to the cell surface. It does not always correlate with the regenerability of a mutant but most mutants that regenerate well have an associated smear.

The other mutants do not have a glycosylation smear. They have a multi-band pattern like those found with N15Q. As shown in Figure 3-3, the intermediate and mature bands are sensitive to N-glycosidase F. It may be these species represent a pool of opsin intermediates in various compartments in the secretory pathway. Alternatively, they could be folding intermediates that have had their carbohydrates processed to varying extents. Or the multi-band pattern could be the result of overexpressing the mutants in COS cells. But the same pattern was found when these mutants were expressed in CHO cells- a mammalian expression system that expresses proteins at levels 1/10-1/100 of COS cells. The absence of multi-bands correlates with good regenerability whereas its presence is associated with mutants that regenerate poorly.

The double mutant N2,15Q has a multi-band pattern which is not sensitive to N-glycosidase F. The same pattern is found when this mutant is expressed in CHO cells. This mutant does not incorporate radioactive mannose. Clearly, it does not have N-linked glycosylation. The multiple bands may represent various conformers of the protein or even O-linked glycosylation at some accessible serine residues, such as those found at the C-terminus.

The mutants N2Q, G3C and G3P were more similar to wild-type rhodopsin in their ability to trigger transducin than the mutants at asn15. These mutants were about 1/100 less efficient in coupling to transducin. Since these mutants released all-*trans* retinal much more

readily than wild-type, directly suggesting a shorter metarhodopsin II half-life, the difference in the binding efficiency probably reflects the difference in the mII $t_{1/2}$'s of wild-type and the mutants. Taken together this data would indicate glycosylation at asn15 may be important for the stability of mII. The carbohydrate moiety at that site may provide a certain protection to the retinyladene Schiff base in the mII state.

Finally, the mutants at asn15 and lys16 demonstrated the structural requirements at the N-terminus of the intradiscal region. Even the most conservative mutation, N15Q, which differs from the asn by only one carbon atom, lead to low expression and chromophore levels. Other mutations at this site were not well tolerated as measured by A₅₀₀ and the A₂₈₀/A₅₀₀ ratio. The two mutants at lys16 were even less tolerated- these mutant proteins could not even bind 11-*cis* retinal. This would suggest the N-terminus is important for the correct formation of the intradiscal tertiary structure.

Chapter 4

STRUCTURAL AND FUNCTIONAL CHARACTERIZATION OF RHODOPSIN MUTANTS ASSOCIATED WITH AUTOSOMAL DOMINANT RETINITIS PIGMENTOSA (ADRP)

4.1 INTRODUCTION

Retinitis Pigmentosa (RP) is a type of retinal degeneration. Many different forms of this disease have been observed clinically (Heckenlively, 1988). The hallmarks of this disease include progressive nightblindness, rod cell degeneration often accompanied by cone loss as well, and progressive decrease in ERG (electroretinogram) potentials, reflecting a loss of overall retinal function (Table 4-1 and Figure 4-1) (Driyja, 1992). RP is a hereditary disease which can be transmitted as a X-linked, autosomal recessive or autosomal dominant trait. Depending on the type of RP the clinical course and severity of the disease is variable (Heckenlively, 1988). Little is known about the pathogenesis or biochemistry of this disease.

Autosomal Dominant Retinitis Pigmentosa (ADRP) is a form of RP which occurs in approximately 1/3000 live births in the United States. Driyja *et al.* (1990a) described the first mutation, P23H, in the gene encoding human rhodopsin associated with ADRP. Subsequently, this

I. CLINICAL FEATURES OF RETINITIS PIGMENTOSA

A. *Symptoms*

1. Night blindness
2. Early loss of peripheral visual field
3. Late loss of central field as well

B. *Signs*

1. Pallid optic nerve head
2. Attenuated retinal vessels
3. Bone spicule pigmentary deposits in the periphery
4. Posterior subcapsular cataract

C. *Electroretinographic abnormalities*

1. Reduced amplitude of scotopic and photopic b-wave
2. Delay in time between flash of light and peak of b-wave (delayed implicit time)

II. DISTRIBUTION OF CASES ACCORDING TO GENETIC TYPE (based on ref. 12)

- A. Autosomal dominant—43%
 - B. Autosomal recessive—20%
 - C. X-linked—8%
 - D. 'Isolate' (i.e. only one affected family member, possibly representing autosomal recessive disease, but could also be new dominant or X chromosome mutation)—23%
 - E. Undetermined (i.e. adopted, uncertain family history, etc.)—6%
-

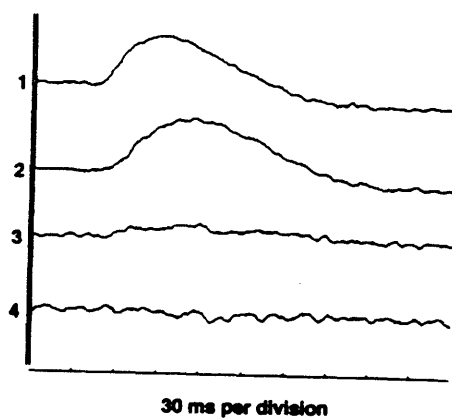
Table 4-1 The clinical and epidemiological spectrum of retinitis pigmentosa.

A



— Representative fundus photographs from four patients with autosomal dominant retinitis pigmentosa with a cytosine-to-adenine transversion in codon 23 of the rhodopsin gene to show variability with respect to the extent of intraretinal bone spicule pigmentation among patients with the same gene defect.

B



Shown here are rod responses from a normal individual and from RP patients. Tracing 1 is from a normal individual. Tracings 2 and 3 demonstrate reduced amplitude and delayed responses from RP patients. Tracing 4 shows an extinguished response from a patient with advanced RP. The horizontal time base is 30 milliseconds per division. The vertical amplitude axis is 250 μ V per division for tracing 1 and 100 μ V per division for all other tracings.

Figure 4-1 The fundusoscopic (A) and electroretinographic (B) features of RP.

group and others have discovered over 40 rhodopsin mutations in ADRP (Driyja *et al.*, 1990b; Driyja *et al.*, 1990c; Sung *et al.*, 1991; Sheffield *et al.*, 1991; Bhattacharya *et al.*, 1991; Driyja, 1992). As of now the list of mutations (Table 4-2) continues to grow somewhat reminiscent of the mutations leading to hemoglobinopathies. Three of the mutations are deletions; the remaining ones are single amino acid substitutions. Some sites have multiple substitutions (e.g. D190G, D190N, D190Y); behaving as mutational hot spots. The mutations are distributed throughout the protein in the intradiscal, transmembrane and cytoplasmic regions (Figure 4-2). Most of them are clustered in the intradiscal and transmembrane region. One mutation, C110Y (Driyja, 1992), is at the conserved cysteine residue believed to be disulfide bonded with cys187 (Karnik *et al.*, 1988; Karnik and Khorana, 1991).

There is great variability of the disease depending on the rhodopsin mutation associated with ADRP. For example patients with P23H mutations have a milder clinical course and thereby retain more useful vision than those with the P347P mutation (Driyja, 1992). But in the majority of the mutations described too few patients have been studied to make meaningful clinical correlations with the natural history and severity of the disease. As with other hereditary diseases one may expect there should be variability even among patients with a given mutations.

In this chapter the structure and function of most of these mutant proteins is described. Additional mutant opsins near the sites

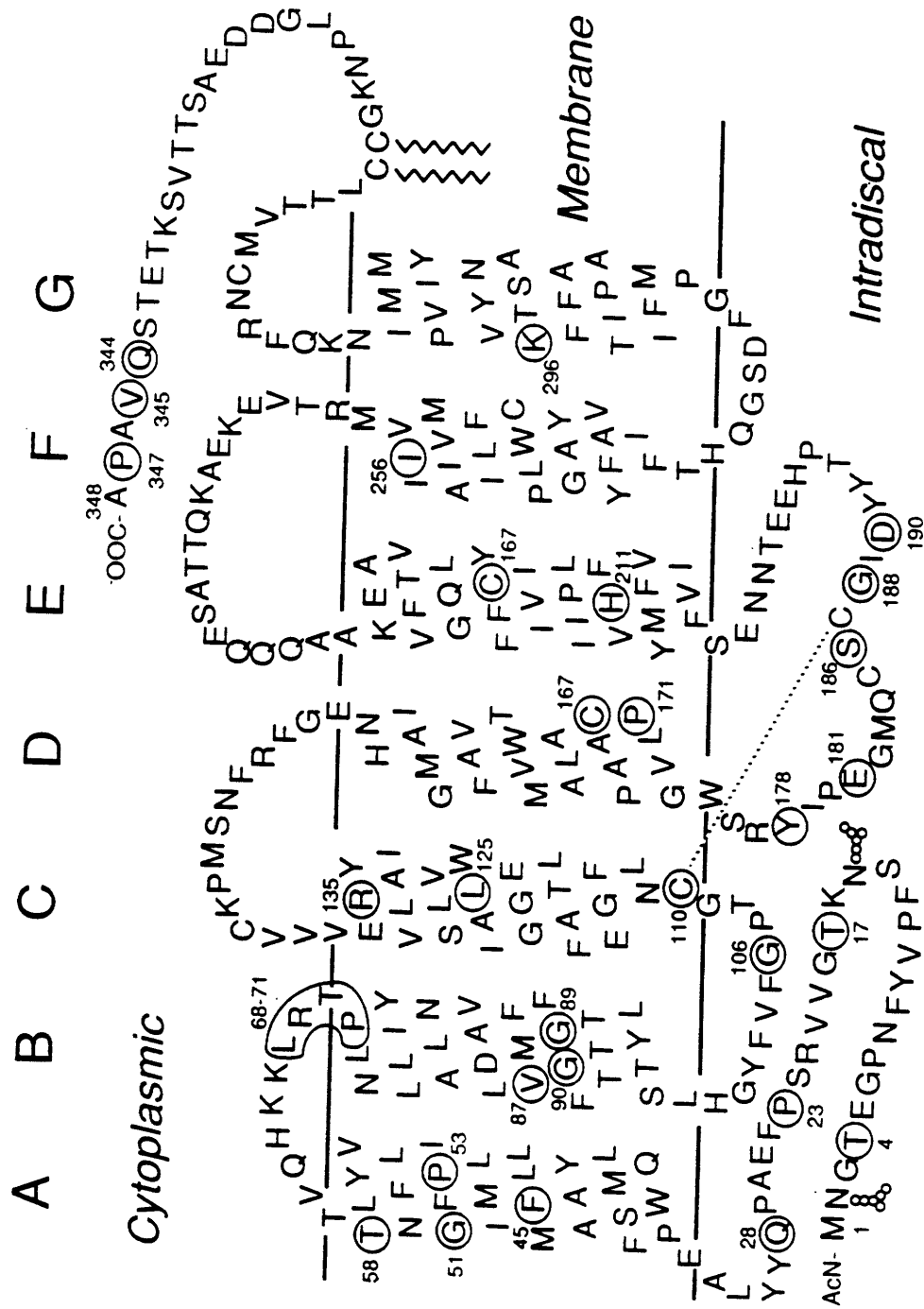


Figure 4-2 A secondary structure model of bovine rhodopsin. The putative seven membrane-embedded helical segments are indicated by the letters A to G. The rhodopsin mutants associated with ADRP are indicated by circles around the amino acids (numbered) that are mutated. The actual mutations are systematically shown in Table 4-2.

Table 4-2 The mutations of opsin associated with ADRP.

Amino Acid #	Amino Acid Chg	Amino Acid #	Amino Acid Chg
4	T→K	135	R→G,W
17	T→M	167	C→R
23	P→H,L	171	P→L
28	Q→H	178	Y→C
45	F→L	181	E→V
51	G→R,V	186	S→P
53	P→R	190	D→G,N,Y
58	T→R	211	H→P
68-71	ΔL,R,T,P	256	ΔI
87	V→D	296	K→E
89	G→D	344	Q→stop
90	G→D	345	V→M
106	G→R,W	347	P→L,S
110	C→Y		
125	L→R		

of the ADRP mutations were also constructed and expressed. From all these studies it is clear there is significant heterogeneity both structurally and functionally among the rhodopsin mutants. Some represent defects in folding as determined by their inability to bind 11-*cis* retinal. Others are mutants which regenerate poorly and have abnormal bleaching kinetics. Yet others are indistinguishable from wild-type rhodopsin. Taken collectively there are probably many different pathophysiological mechanisms by which these mutant proteins cause RP.

4.2 MATERIALS AND METHODS

4.2.1 Materials

Bovine retinas used for the isolation of rhodopsin and transducin were from J.A. Lawson Co. (Lincoln, NE). Deoxyadenosine 5'-[α -³⁵S]triphosphate (500 Ci/mmol) and guanosine 5'-[γ -³⁵S]triphosphate (1000 Ci/mmol) were from NEN. GTP γ S and TritonX-114 were from Boehringer Mannheim. Dodecyl maltoside was from Anatrace. Brefeldin A and cyanogen bromide activated Sepharose 4B were from Sigma. Sequenase (version 2.0) was from United States Biochemicals. DNA purification kits were from Qiagen. Nitrocellulose filters (HAWP25) and PVDF membranes were from Millipore. 11-*cis* Retinal was a generous gift from Dr. Peter Sorter (Hoffman-LaRoche) and Dr. R. Crouch (Medical University of South Carolina and the National Eye Institute). The antibody K16-50 was a generous gift from the laboratory of Dr. P. Hargrave.

4.2.2 Construction of Rhodopsin Mutants

All mutants were prepared by cassette (fragment) replacement in the synthetic opsin gene. All the oligonucleotides used for the construction of the mutants were synthesized on a Applied Biosystems 380B DNA synthesizer and purified as previously described. The mutants were made by codon replacements in the appropriate restriction fragments:

<u>Mutant(s)</u>	<u>Restriction Fragment</u>
T4K,T17M,T17V	KpnI-FspI
P23H,P23L	FspI-BanII
Q28H,F45L	FspI-BclI
G51R,G51V,P53R,T58R	BclI-HindIII
Δ 68-71	HindIII-BglII
V87D,G89D,G90D	BglII-NcoI*
G106R,G106W,C110Y	NcoI-XhoI [#]
L125R	XhoI-PvuI
R135L	PvuI-AhaII
C167R	AhaII-XbaI
P170L,P171L, Δ 170-171	SfiI-ClaI
Y178C,E181K,S186P,G188R	XbaI-ClaI
D190G,D190N,D190Y	ClaI-AvaII
H211P	AvaII-MscI
I256 Δ , Δ 255-256	NheI-MluI
K296D,K296E,K296R	ApaI-AatII
Q344stop,V345M,P347L,P347S	SalI-NotI

* These mutants were constructed in the expression/cloning vector pSK. The opsin gene as an EcoRI-BamHI fragment was cloned downstream from the CMV promoter in the vector pCMV5 (a gift of Dr. David Russell, Southwestern Medical Center, Dallas, Texas). Another feature of this vector is it contains the 5'-untranslated region of the Alfalfa Mosaic Virus 4 RNA. This sequence acts as a translational enhancer by decreasing the requirements for initiation factors in protein synthesis (Andersson *et al.*, 1989). The utility of pSK lies in that many more of the restriction sites found uniquely in the synthetic gene are unique in the whole vector thereby making cloning in different regions of the gene relatively easy. When the vector was constructed the first 100 bp and last 100 bp of the synthetic gene were sequenced. Many of the restriction sites found in the gene were verified by appropriate enzymatic digestion. The expression levels of wild-type opsin from pMT4 and pSK are the same.

#These mutants were first constructed and sequenced in the cloning vector pOP3. This vector was constructed by a former post-doctoral fellow in our laboratory, Dr. Barry Knox. The mutant opsin gene was excised from this vector as an EcoRI-NotI fragment and ligated into the EcoRI-NotI large fragment of pMT4. Before transfecting COS-1 cells with this vector the mutant gene was sequenced again to reverify the presence of the mutation.

All mutations were confirmed by dideoxynucleotide sequencing of plasmid DNA (Sanger *et al.*, 1977).

4.2.3 Expression and Purification of Mutants

All procedures for the culturing and transfection of COS-1 cells has been previously described. The methods of immunoaffinity purification have also been detailed as well. Wild-type rhodopsin was also expressed in the presence of Brefeldin A (BFA). It was added to the transfected cells immediately after the chloroquine incubation at a concentration of 5.0 $\mu\text{g/ml}$.

4.2.4 Binding and Activation of Transducin

Transducin was prepared by the methods of Fung *et al.* (1981). The GTP γ S exchange assays were done exactly as previously described.

4.2.5 Sodium Carbonate Extraction of Membranes

After transfecting and harvesting total cell membranes were made by first resuspending the cell pellet in hypotonic buffer, 5 mM Tris pH 7.0. The samples were then passed through a 28 gauge needle 6 times to completely disrupt the cells. The membranes were then pelleted at 100000g for 40 minutes. The membrane pellets were resuspended in 2.0 mls of 0.1 M sodium carbonate pH11.3. To insure complete resuspension of the membranes they were again passed through a 28 gauge needle 6 times. The membranes were then incubated at 4°C for 30 minutes with gentle agitation. They were pelleted at 150000g for 40 minutes. The supernatant containing the extracted proteins was saved. The membranes were then extracted once more. The two supernatants were pooled and the pH neutralized

to 7.0 by the addition of 1 N HCl. DM was added to a final concentration of 0.1% to prevent possible aggregation and the opsin was immunoaffinity purified on 1D4-Sepharose 4B resin. The remaining membrane pellet was solubilized with 1% DM and 0.1 mM PMSF. The solubilized samples were then spun at 100000g for 30 minutes at 4°C and the opsin purified. After elution from the column equal amounts of the eluate were loaded on SDS-polyacrylamide gels (Laemmli, 1970). The proteins on the gel were subsequently transferred to PVDF membranes and immunoblotted with 4D2, an N-terminal antibody.

4.2.6 Phase Separation of Proteins in TritonX-114

Solution

After transfecting and harvesting 1.25×10^7 COS-1 cells were solubilized for 30 minutes at 4°C with 2 mls of 1.0% TritonX-114 in PBS plus 0.1 mM PMSF. Afterwards the sample was placed in a 37°C waterbath for 5 minutes when a distinct oil-droplet like detergent rich (lower) phase and an aqueous (upper) phase were discernable. The samples were then spun at room temperature for 3 minutes at 500g in a clinical centrifuge. The upper phase was removed and it was extracted with 1.0% TritonX-114 as above. The two detergent-rich phases were combined. Opsin was then purified from that fraction as well as the aqueous phase. Equal volumes of the eluates were run on SDS-polyacrlamide gels; the proteins were then immunoblotted as mentioned above.

4.2.8 Immunofluorescence Microscopy and Fluorescence-Activated Cell Sorting (FACS)

COS-1 cells were transfected on microscope glass coverslips. 72 hours post-transfection the cells were washed twice with cold PBS and then incubated for 5 minutes with ice-cold methanol and then for 5 minutes with ice-cold acetone. The fixed cells were then washed twice with PBS and incubated for 2 hours with 4D2 (5 μ g/ml) and 1% BSA in PBS at room temperature. The cells were then washed with 1% BSA in PBS twice and incubated for 1 hour with a 1:200 dilution of rhodamine conjugated anti-mouse antibody. The unbound antibody was then washed away with PBS and coverslips were mounted on glass slides.

For FACS studies exactly the same procedure was used except the cells were harvested and the antibody binding were done in suspension. The live cells, those that excluded the vital dye propidium iodide, were sorted at the core facility at the MIT Cancer Center.

4.3 RESULTS

A summary of the phenotypic characteristics of all the ADRP mutants mentioned is shown in Table 4-3. The class I mutants are those that are most similar to wild-type COS rhodopsin in their level of expression, amount of chromophore regenerated, cell surface targeting and glycosylation pattern. The class II mutants are those which are expressed at low levels, do not form chromophore, are retained in the endoplasmic reticulum and are abnormally glycosylated. The class III

Table 4-3 ADRP Mutants Classified According to Their Phenotypes

<u>Class I</u>	<u>Class II</u>	<u>Class III</u>
F45L	C167R	T4K
T58R	P171L	T17M
L125R	Y178C	Q28H
R135G	E181K	G51R
D190G	S186P	G51V
D190N	D190Y	P53R
Q344stop	H211P	Δ68-71
V345M	K296E	V87D
P347L		G89D
P347S		G90D
		G106R
		G106W
		C110Y
		I256Δ

Legend: Class I closely resembles wild-type rhodopsin expressed in COS cells. Thus, these mutants fold correctly to bind 11-*cis*-retinal and form the characteristic rhodopsin chromophore. Class II mutants are defective in folding and stay in endoplasmic reticulum. They do not bind 11-*cis*-retinal and regenerate the 500 nm-absorbing chromophore only partially.

mutants are expressed at intermediate levels, form some chromophore, are also retained in the endoplasmic reticulum and also abnormally glycosylated. These mutants have abnormal bleaching kinetics and also trigger transducin inefficiently. The mutants found in each class are not specifically located to one region of the protein. Below the mutants are discussed in detail by the region in which they occur.

4.3.1 Mutants at the N-Terminus:

T4K,T17M,P23H,P23L,Q28H

Little is known about the length or structure of the N-terminal tail of rhodopsin. It is believed to be exposed to the aqueous environment (McDowell and Hargrave, 1992). All the mutants found in this region of the protein yielded less chromophore than wild-type after immunoaffinity purification (Figure 4-3). T4K, T17M and Q28H all gave similar amounts of the protein relative to one another. The mutants T4K and T17M are found at the two separate sites of rhodopsin glycosylation. Both of them disrupt the consensus tripeptide sequence required for N-linked glycosylation (asn-X-thr/ser, where X is any amino acid except proline). As expected the mature opsin of both mutants have slightly smaller molecular weights ($M_r \approx 37,000$) than wild-type COS opsin. Another mutant, T17V, was also made and expressed. It also regenerated like T17M (Figure 4-3). All of these mutants when purified at pH 7.0 gave A_{280}/A_{500} ratios of 2.5-4.0. This strongly suggests that there is a fraction of the purified mutant which is unable to bind 11-*cis* retinal, presumably because it is misfolded or it folds more slowly than wild-type. When

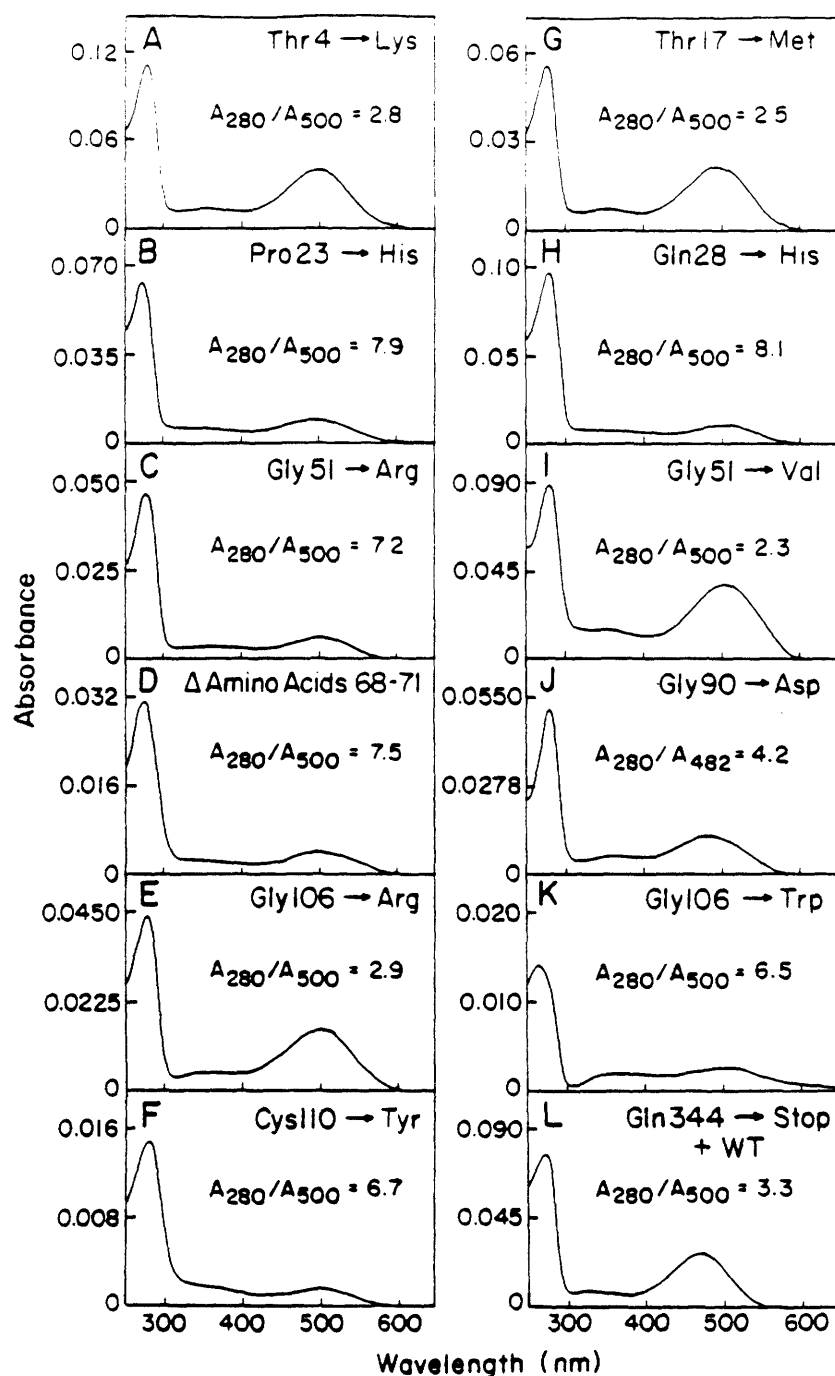


Figure 4-3 UV / vis absorption spectra in the dark of ADRP mutants (Class III) that show variable but low chromophore formation with 11-*cis*-retinal. Twelve examples are shown. Class III mutants showed variable 280/500 nm spectral ratios, ranging between 2.5 and 8.1. The spectra shown were from mutants eluted from ID4-Sepharose at pH 7.0 in the presence of 150 mM NaCl.

these mutants are purified at pH 6.0 the spectral ratio is between 1.6-1.8. At this pH, the non-regenerable protein is selectively retained on the 1D4-Sepharose resin. Such a spectral ratio indicates that it is possible to obtain spectrally pure (purified wild-type rhodopsin has a spectral ratio of 1.6) mutant chromophores. For the bleaching studies and transducin activation assays only the pigment eluted at pH 6.0 was used.

From immunoblots these mutants all had a mature form with an associated glycosylation smear (see class I mutants example in Figure 4-4). Such glycosylation is correlated with opsin that is transported to the cell surface.

The mutants P23H and P23L both yielded much less chromophore than the others at the N-terminus. The amount of chromophore was about the same for both of these mutants, $A_{500} = .006$ OD (Figure 4-3). The spectral ratio of these mutants was between 6-7, suggesting that even a larger fraction of the eluted protein was unable to regenerate i.e. more of it was misfolded. These mutants showed three discrete bands on Western blots (Figure 4-4). These phenotypic characteristics are the same as those observed with some of the glycosylation mutants (e.g. N15Q) and deletion mutants (e.g. $\Delta 18-21$) found at the N-terminus (Doi *et al.*, 1990). The mutants which show this multi-band pattern have little opsin expressed at the cell surface as quantitated by FACS. In fact, most of the protein is found in the endoplasmic reticulum as seen by immunofluorescence microscopy (e.g. P23H) (Figure 4-5).

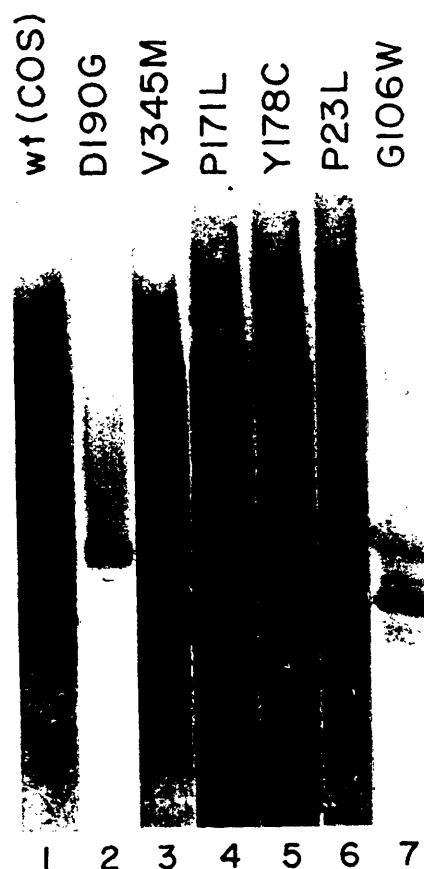


Figure 4-4 Characterization of the three types of ADRP mutants by gel electrophoresis. Examples of Class I - Class III mutants are given. Equivalent amounts of purified proteins, as determined by the absorption at 280 nm, were analyzed by SDS-PAGE (12% separating gel) and then immunoblotted with the monoclonal antibody ID4. Lane 1 represents wild type COS rhodopsin, Lanes 2 and 3 contain D190G and V345M, respectively (class I mutants). Lanes 4 and 5 show the Class II mutants, P171L and Y178C, respectively, while lanes 6 and 7 show Class III mutants, P23L and G106W, respectively.

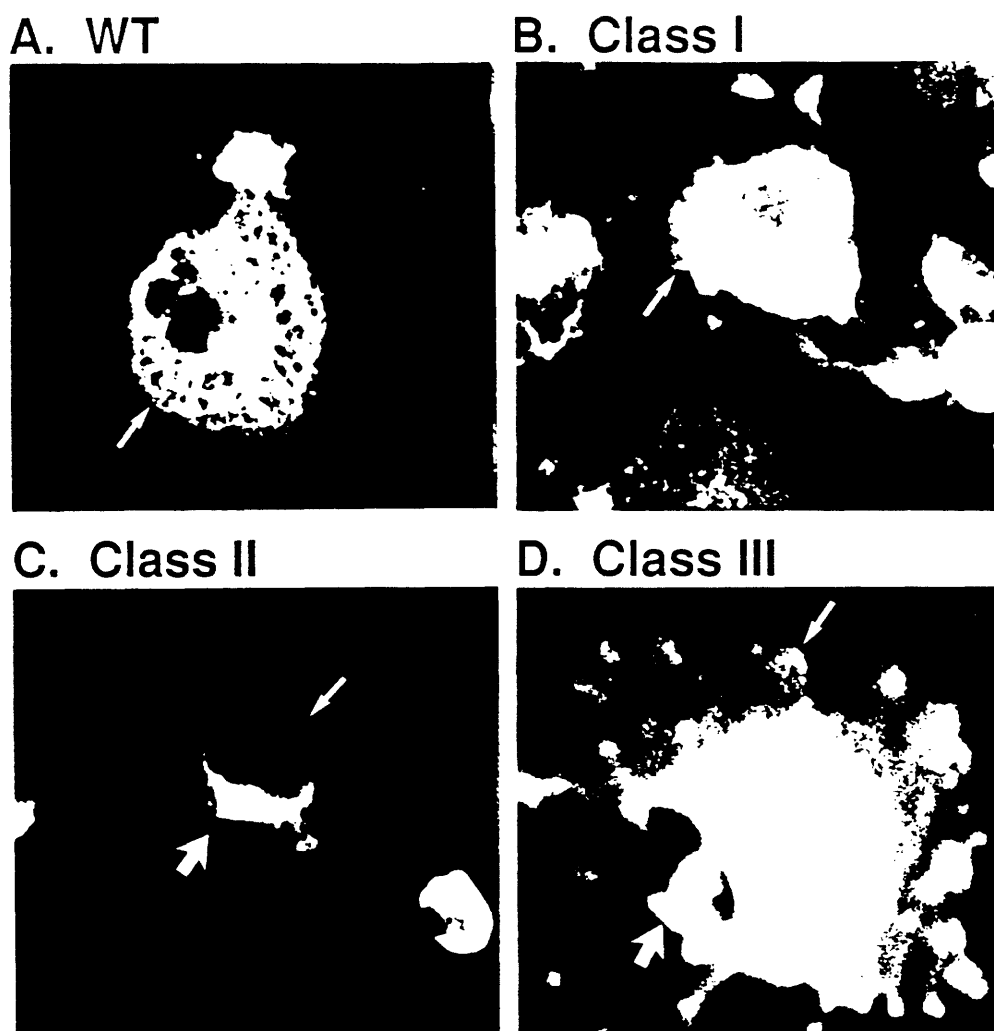


Figure 4-5 Cellular localization of ADRP mutants opsins as shown by immunofluorescence and FACS (Table 4-3). **Panel A** Wild type COS-1 cell opsin; **Panel B** the mutant P347S (Class I); **Panel C** the mutant D190Y (Class II); **Panel D** the mutant P23H (Class III). The thin arrow represents the cell surface for all four panels. The thick arrows in panels C and D represent perinuclear staining consistent with localization in the endoplasmic reticulum. Table 4-3 shows the percentage of live cells that expressed the opsin at the cell surface.

**Table 4-4 Cell Surface Expression of Opsins from ADRP Mutant Genes
as Measured by FACS**

Sample	% Positive
mock	1.48
wild-type (BFA)	0.91
wild-type	22
P23H	10
E181K	11
Q344stop	26
P347S	20

4.3.2 Mutants Found in the Intradiscal Loops:

**G106R,G106W,C110Y,P171L,Y178C,E181K,S186P,
G188R,D190G,D190N,D190Y**

These ADRP mutants are found in either the BC loop, G106R, G106W, C110Y, and in the large DE loop. So far no mutations have been found in the FG loop. The three BC loop mutants formed chromophore but to a lesser extent than wild-type and with poor spectral ratios (Figure 4-3). Both of the mutants at gly106 represent drastically different substitutions. In one case, tryptophan represents a bulky hydrophobic residue while arginine is a relatively large basic amino acid. Both substitutions disrupt the gly-pro sequence in that loop- a sequence motif that is found in β turns (Rose *et al.*, 1985). The mutation C110Y is significant since it disrupts the formation of the disulfide bond between cys110 and cys187. Prior to this study it was believed the disulfide bond was required for the correct folding and assembly of rhodopsin (Karnik *et al.*, 1988; Karnik and Khorana, 1990). This is the first example of a single mutation at cys110 that can regenerate albeit poorly, $A_{280}/A_{500} \approx 10-12$ with $A_{500} = .002$. C110Y is light-sensitive.

The ADRP mutants of the DE loop occur primarily in the conserved region among visual receptors, amino acids 171-189 (Hargrave and McDowell, 1992). The mutant proteins in this region, P171L, Y178C, E181K, S186P and G188R, did not regenerate to any significant extent (Figure 4-6). The total amount of purified opsin was

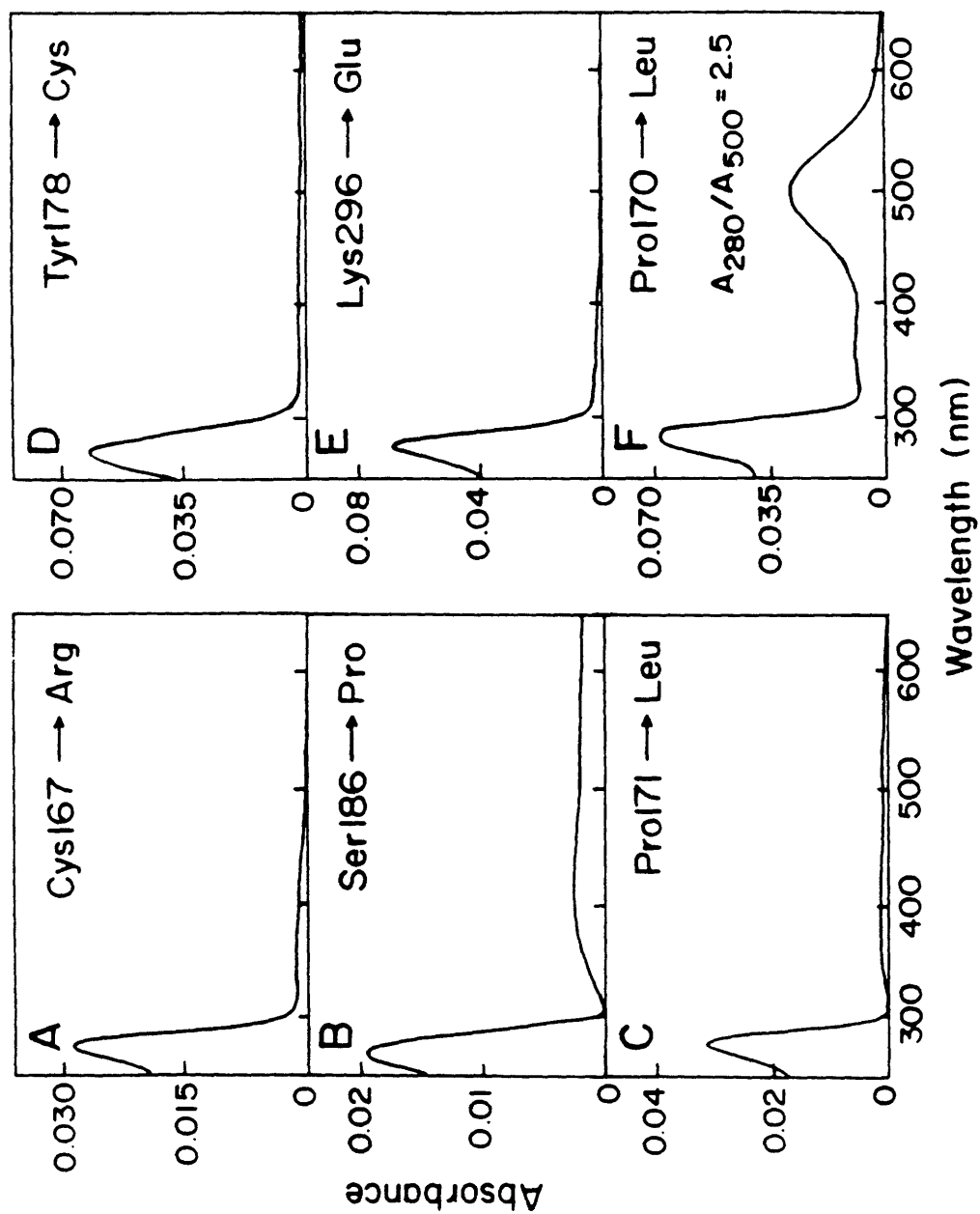


Figure 4-6 UV / vis absorption spectra of Class II ADRP mutants, these mutants failed to regenerate the chromophore. Panel F (Pro170→Leu) shows the spectrum of the non-RP mutant in which the proline at position 170 was replaced by Leu, in place of the ADRP mutation at 171 (Panel C). Methods for expression and purification of the expressed opsins are in Materials and Methods.

approximately one-third of wild-type opsin. All showed a multi-band pattern without a glycosylation smear on immunoblots (see class II mutants in Figure 4-4). This was similar to P23H. Such mutants have less protein expressed at the cell surface as observed by immunofluorescence (Figure 4-5). All of these findings are consistent with the observations of Doi *et al.*, (1990) who showed that deletion mutants of in this same region had the same phenotype. Two additional non-ADRP mutants, P170L and $\Delta 170-171$ (deletion of prolines at 170 and 171) were made to see if movement of the leucine substitution might have a different effect if it was moved by one residue and also how removal of both proline residues would effect the protein. P170L was expressed at wild-type levels and regenerated like it as well (Figure 4-6). The double mutants did not regenerate at all (Appendix 6).

The two mutants D190G and D190N, located in the non-conserved region, showed wild-type levels of expression and chromophore formation (Appendix 6). These two mutants also had a glycosylation smear as well. The mutant D190Y, on the other hand, did not regenerate at all, like the mutants in the conserved region of the DE loop (Appendix 6). It may be the introduction of a large amino acid such as tyrosine effects the packing of the intradiscal loops.

4.3.3 Mutants Found in the Transmembrane Region

Helix A: F45L,G51R,G51V,P53R,T58R

The mutants G51R and P53R represent charge substitutions within the helix while F45L and G51V are substitutions replacing one hydrophobic residue with another. It is difficult to know the exact location of thr58 in the absence of a crystal structure. From the hydropathy profile of opsin it may lie near the cytoplasmic border rather than within the membrane itself (Dratz and Hargrave, 1983). All of the mutants in this helix regenerated (Figure 4-7 and Appendix 6). F45L and T58R regenerated like wild-type whereas the others formed chromophore but at levels much less ($<1/3$) than wild-type. F45L and T58R showed a glycosylation smear similar to wild-type opsin while the other RP mutants of helix A showed the characteristic multi-band pattern of those mutants which either regenerate poorly or do not regenerate at all.

Helix B: $\Delta 68-71$, V87D, G89D, G90D

The mutant $\Delta 68-71$ is found at the top of helix B. These amino acids are probably part of the AB cytoplasmic loop. Again, in the absence of a three dimensional structure, we are uncertain about the precise location of these residues. This mutant is expressed at low levels and forms very little chromophore (Figure 4-3).

The other mutants in helix B are charge substitutions in the transmembrane region itself. G90D is not strictly an ADRP mutant. It was discovered in a Michigan family with congenital nyctalopia (nightblindness) (Sieving *et al.*, 1992). It might be expected that burying an additional lone charge would be thermodynamically

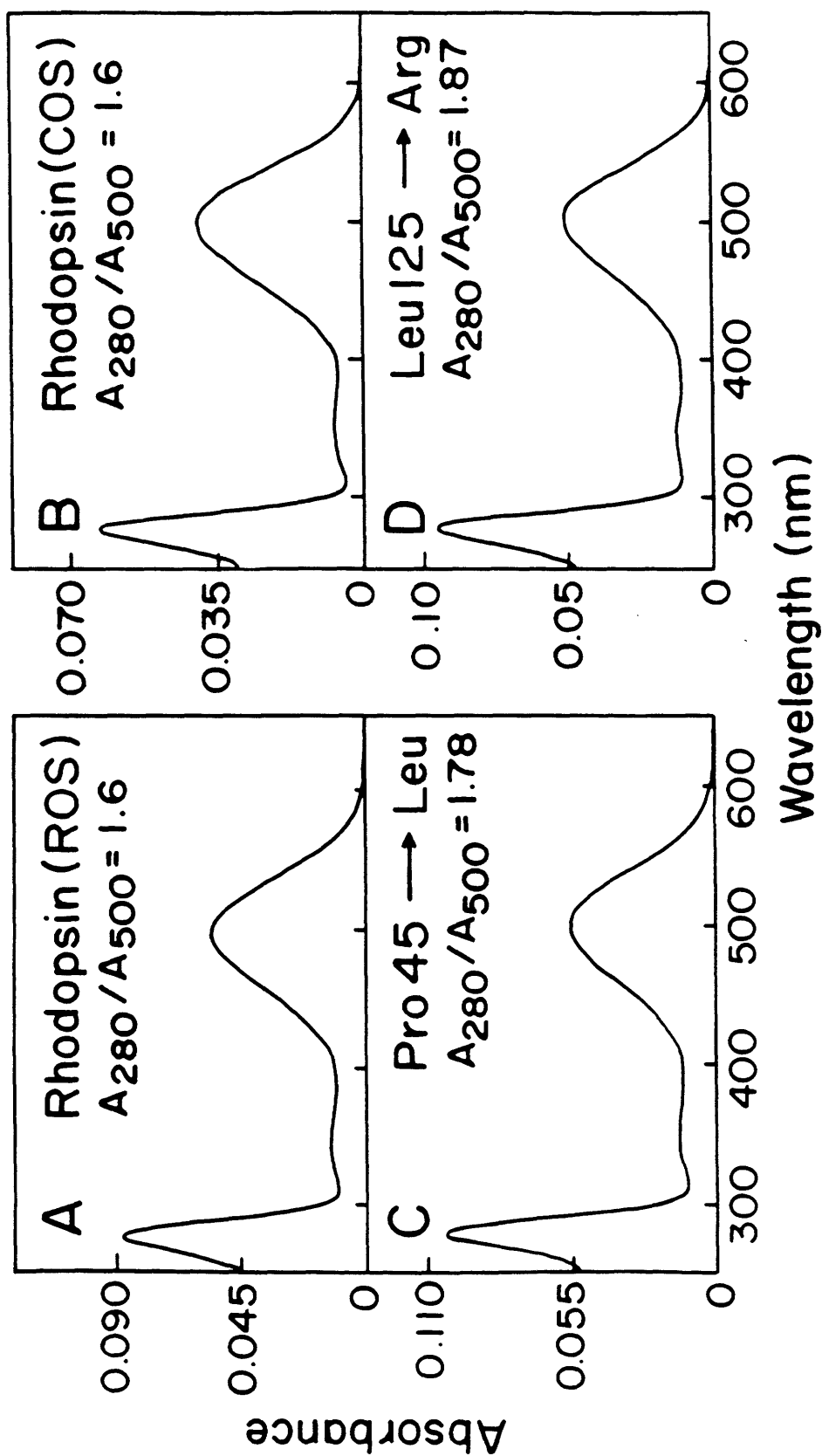


Figure 4-7 UV / vis absorption spectra in the dark of wild type and Class I ADRP mutants. The mutants in **Panels C and D** are examples of Class I of ADRP mutants which are most-like wild type rhodopsin, except for transducin activation.

prohibitive and therefore such mutants would not fold properly (Singer, 1990). All these mutants regenerated although poorly (Figure 4-2). The mutants V87D and G89D yielded less chromophore than G90D. The $\lambda_{\max}$ of G90D was blue-shifted to 480 nm. This particular residue may either form part of the retinal binding pocket or interact indirectly with 11-*cis* retinal. These mutants also showed a multi-band pattern by immunoblots.

Helix C: L125R,R135L

L125R represents a charge substitution within the helix whereas R135L is a neutral substitution at the top of the helix. It neutralizes one member of a conserved charge pair found in many mammalian pigments (Sakmar *et al.*, 1989). Both of these mutants were expressed at wild-type levels and yielded chromophore similar to wild-type (Figure 4-2). These mutants also showed a glycosylation smear (Figure 4-3).

Other Transmembrane Mutants:

C167R, H211P,I256 Δ ,K296E

C167R is a charge substitution in helix D while H211P removes a potential charge from within the membrane domain of helix E. These mutants do not regenerate at all (Figure 4-6). I256 Δ is a single amino acid deletion at the top helix E. It , along with the mutant Δ 255-256 (deletion of isoleucine residues at those positions), regenerated at very low levels (Appendix 6). K296E represents a substitution at the lysine

residue forms a Schiff base with 11-*cis* retinal. This mutant would not be expected to regenerate. This mutant as well as K296D and K296R do not regenerate at all although the amount of opsin purified is about three fourths of wild-type. On immunoblots K296E has a glycosylation smear like class I mutants. There is no multi-band pattern unlike other mutants that did not regenerate.

4.3.4 Mutations at the C-Terminus:

Q344stop,V345M,P347L,P347S

Like the N-terminus the C-terminal tail of rhodopsin is believed to be exposed to the aqueous phase. The mutant V345M was the only one which could be successfully purified on the 1D4-Sepharose 4B antibody column. It regenerated like wild-type rhodopsin and had a similar glycosylation smear (Appendix 6 and Figure 4-4). The mutant Q344stop did not bind to the resin at all. It did form chromophore like wild-type as demonstrated by taking light/dark difference spectra of the dodecyl maltoside solubilized cell extract (Appendix 6). The mutants P347L and P347P when eluted from the immunoaffinity column showed small amounts of chromophore with high spectral ratios (Appendix 6). This is because the epitope of 1D4 requires the P347 for efficient binding to rhodopsin. On immunoblots probed with 4D2, an N-terminal antibody all these mutant proteins had glycosylation smears like wild-type.

ADRP patients are heterozygous for the mutant allele i.e. they have one copy of the mutant gene and one copy of the wild-type gene.

To mimic the *in vivo* genotype COS-1 cells were co-transfected with both the wild-type and Q344stop plasmids. Since Q344stop does not bind to antibody column we would be able to study the *in vivo* effect of this mutant gene on wild-type expression. As seen in Figure 4-3, the level of expression and the amount of chromophore is not drastically effected in the presence of Q344stop but the spectral ratio is greater than for wild-type alone. This suggests that the presence of the mutant allele may effect the amount of wild-type opsin that is properly assembled and transported. Of course this model tissue culture expression system may not be completely analogous to what occurs in the rod cell's inner segment.

4.3.5 Expression of Wild-Type Opsin in the Presence of Brefeldin A (BFA)

One concern in regenerating whole cells is whether 11-*cis* retinal is able to penetrate into other membrane compartments besides the plasma membrane. Because of its hydrophobic nature 11-*cis* retinal most likely partitions into the first membrane it encounters. In this way the opsin which is in the ER and Golgi membranes may encounter it at significantly lower concentrations. This may, in part, explain the poor spectral ratio of many of the ADRP mutants like P23H which are retained in the ER.

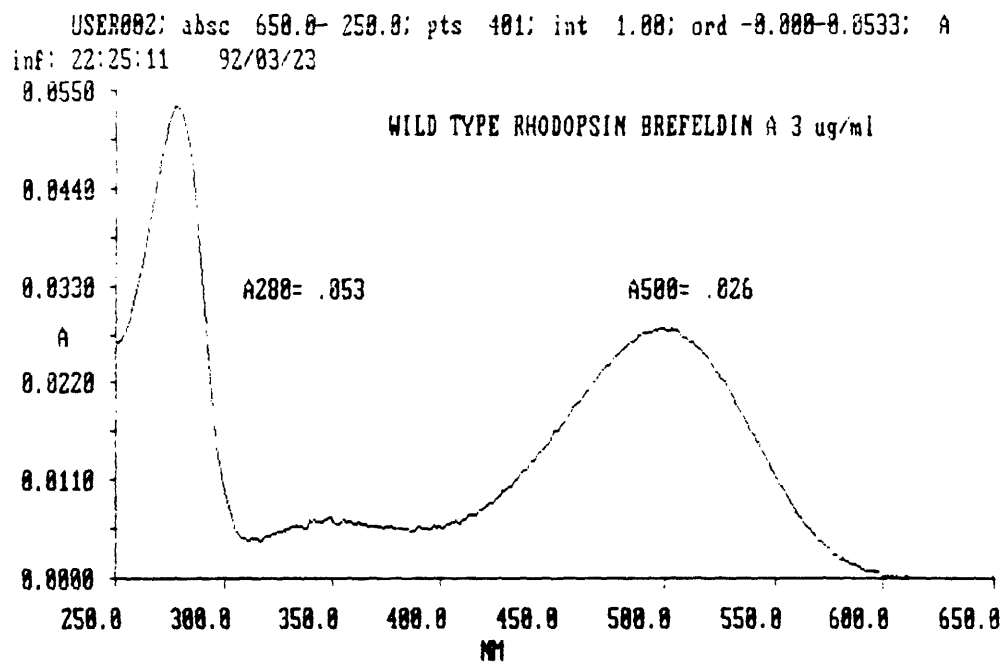
Brefeldin A is a macrocyclic lactone made from palmitate. It was initially found to have antiviral activity but was later on discovered to block protein secretion at some early step.

Subsequently it has been found to block protein transport from the ER to the Golgi by disrupting the Golgi membrane structure (Klausner *et al.*, 1992). Membrane or secreted proteins that are expressed in the presence of BFA are retained in the ER maintain only the high mannose carbohydrate moieties. When expressed in the presence of BFA (3 μ g/ml) wild-type opsin was not expressed at the cell surface as determined by FACS (Table 4-4). From immunoblots this protein ran as a single band with a molecular weight slightly larger than ROS rhodopsin ($M_r \approx 40,000$) (Figure 4-8). No glycosylation smear was present. Nearly all of this species was sensitive to endoglycosidase H, an enzyme that recognizes high mannose structures. Furthermore when this protein was regenerated and purified it gave wild-type expression and chromophore levels suggesting that in fact 11-*cis* retinal is able to penetrate internal membrane compartments (Figure 4-8).

4.3.6 Bleaching Properties of the ADRP Mutants that Regenerated

The bleaching behavior of the RP mutants that regenerated was studied. When wild-type rhodopsin is illuminated and then acidified a distinct 440 nm species is formed (Appendix 2). As mentioned previously, this species represents the covalent Schiff base linkage between all-*trans* retinal and lys296 without any contributions from chromophore-opsin interactions. This covalent linkage is preserved upto the mII intermediate ($\lambda_{max} = 380$ nm) whereafter it is hydrolyzed, releasing all-*trans* retinal ($\lambda_{max} = 380$ nm) from the

A



B

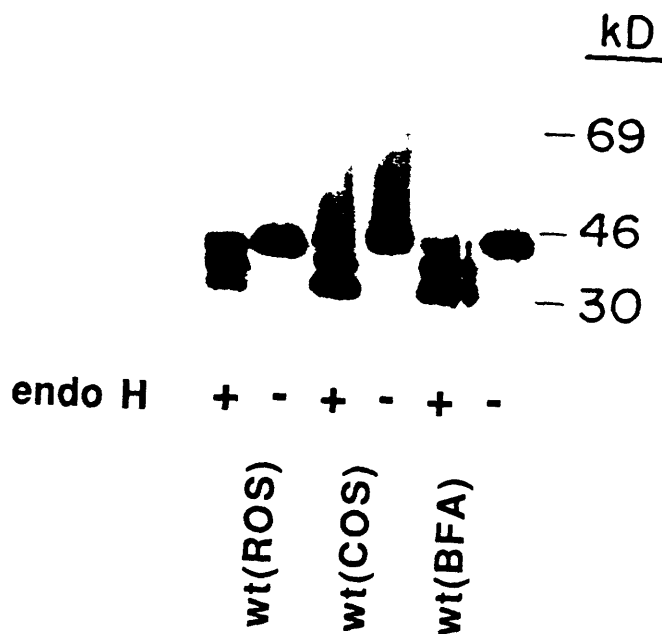


Figure 4-8 Expression of opsin in the presence of Brefeldin A (5 μ g/ml).
A) UV / visible absorption spectra of wt(BFA). B) Endoglycosidase H
sensitivity of ROS rhodopsin, COS rhodopsin and wt(BFA).



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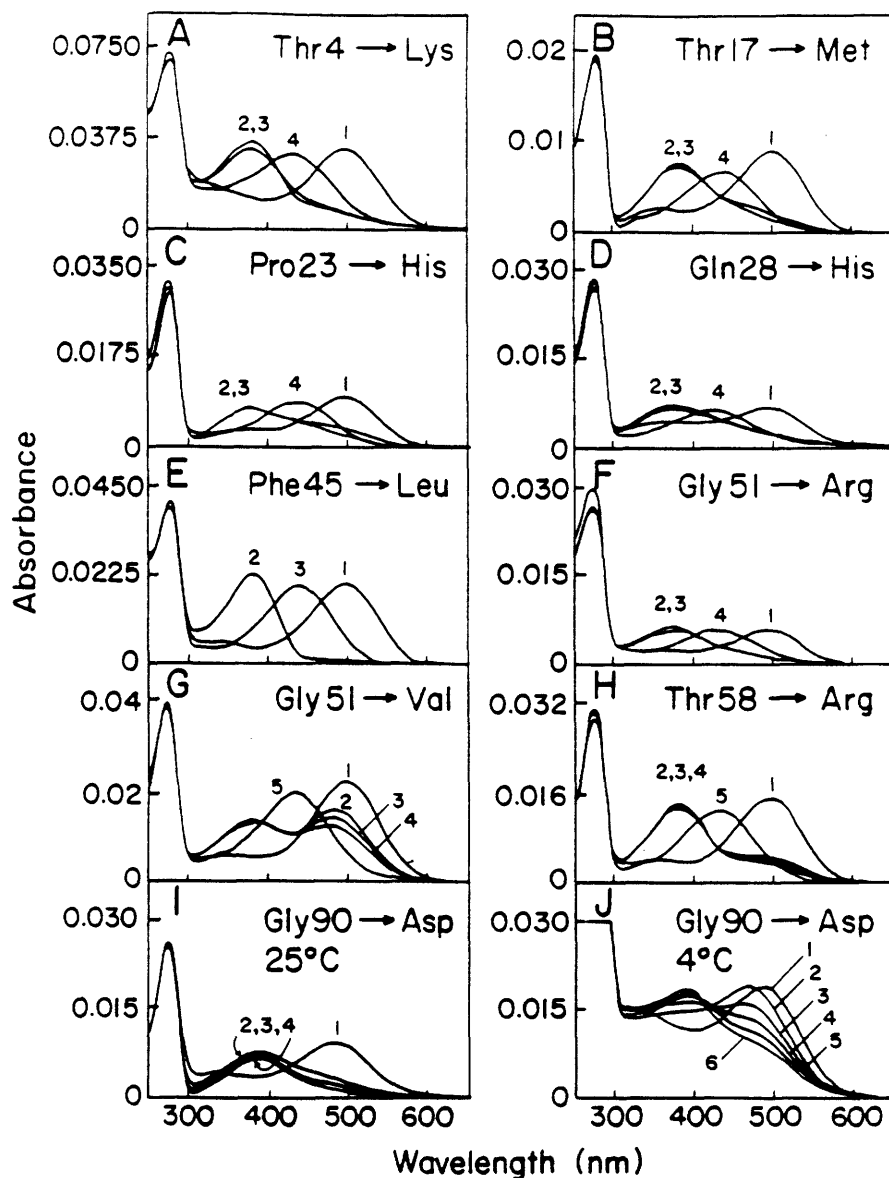


Figure 4-9 Absorption spectral shifts as observed on bleaching of different ADRP mutants. Purified ADRP mutants were illuminated for various lengths of time at 20°C and the solutions were then acidified by the addition of 2N H₂SO₄ to a final pH of 1.9. In all cases, the absorption spectra in the dark are labeled "1" and each subsequent number represents a 10 sec illumination with a fibre optic light source with a 495 nm cut off filter. In the case of Gly90→Asp no cut off filter was used. The spectrum with the largest number in each panel represents the chromophore on acidification.

once the pigment did isomerize free all-*trans* retinal was quickly released.

The mutant G90D bleached more quickly at room temperature- upon acidification there was no species absorbing between 420-440 nm, the entire 380 nm species represented free all-*trans* retinal. But when the bleaching was done at 4°C the blue shifted chromophore of 480 nm first formed an intermediate of 460 nm, probably the mI-like species for this mutant, which then slowly formed a 380 nm species upon further illumination.

4.3.7 Transducin Activation

Some of the mutants were also characterized by their ability to trigger transducin in the GTP γ S exchange assay. As expected from the bleaching studies the mutants which showed faster release of all-*trans* retinal (e.g. T4K and T17M) were less efficient in allowing nucleotide exchange of transducin (Figure 4-10). These results suggest much greater quantities of mutant pigment are required to achieve the same level of transducin activation as wild-type rhodopsin. Also shown in Figure 4-10 are the activation curves for F45L and L125R. Both of these mutants have bleaching characteristics like wild-type but nevertheless are less efficient in triggering transducin except at very high concentrations. This difference probably reflects the difference in the binding constant of the mII-like species in these mutants for transducin. The mutant K296E as purified from the 1D4 antibody column does not activate transducin.

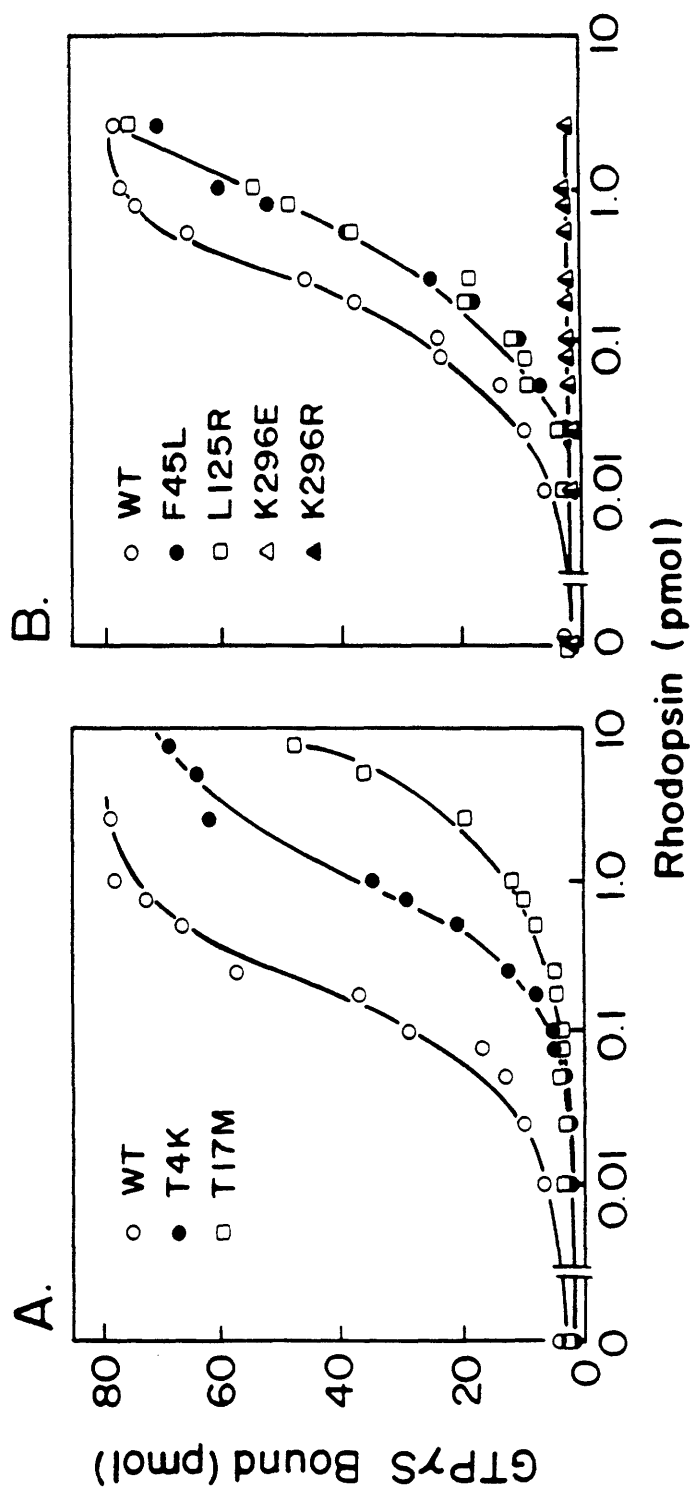


Figure 4-10 Transducin activation ADRP mutants. The binding and activation of transducin as a function of rhodopsin concentration was measured as described under Materials and Methods. The ADRP mutants used in the assay were immuno-purified using ID4-Sepharose, elutions being carried out with 2 mM NaH_2PO_4 , pH 6.0, in 0.1% LM in the absence of any salt. The amounts of the mutant rhodopsins used in the assay were based on the molar extinction coefficient.

4.3.8 Purification of Mutants on K16-50-Sepharose4B Resin

As discussed above many of the ADRP mutants have poor spectral ratios indicating that some fraction of the eluted protein does not bind 11-*cis* retinal. Most likely this fraction is misfolded.

K16-50 is a C-terminal monoclonal antibody like 1D4. However its epitope is located further up, amino acids 335-342 (Adamus *et al.*, 1991)). An antibody column with this antibody was made just as previously described and wild-type rhodopsin and some ADRP mutants were purified with it. As shown in Figure 4-11, the eluted protein, in the case of wild-type, from this immunoaffinity column does not contain any chromophore. The supernatant from the binding was applied to the 1D4 resin. All of the regenerated opsin was found in the eluate of that resin (Figure 4-11). The mutant K296E and P347S were also purified on the K16-50 column. When the eluates of these mutants and wild-type opsin were run on SDS-polyacrylamide gels and immunoblotted with 4D2, three distinct bands were observed like that seen P23H and P171L (mutants that regenerated at low levels or not at all). No glycosylation smear was noticed. Taken together this implies the K16-50 antibody column recognizes the unfolded form of opsin. Secondly, this experiment suggests that even for wild-type opsin there is a fraction which is either in an unfolded or denatured state. Finally it appears the fraction eluted from this column has three separate non-regenerable species.

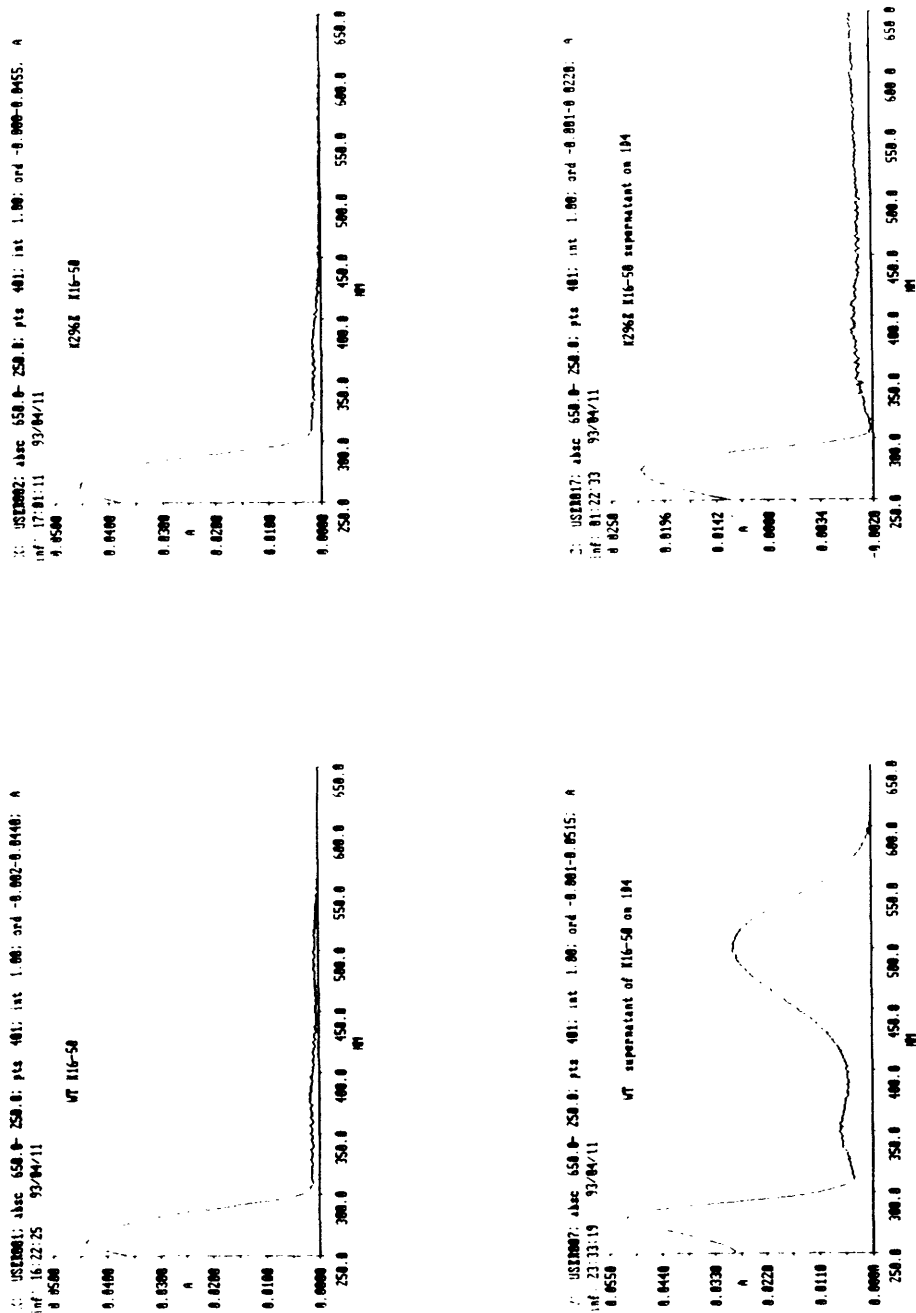


Figure 4-11 UV / visible absorption spectra of wild-type opsin and K296E purified on K16-15 and ID4.

4.3.9 Extraction of COS-1 Cell Membranes

One method to assess if a protein has been inserted completely into a membrane is to try extracting it with 0.1 M sodium carbonate pH 11.3 (Bonifacino *et al.*, 1991)). At high pH peripheral and partially inserted proteins are extracted whereas integral membrane proteins stay bound to the membrane.

COS cell membranes were prepared. The supernatant from the membrane pellet was kept to see if any soluble forms of opsin were present. Equal volumes of purified opsin from the 0.1 Na₂CO₃ extract, the solubilized membrane pellet remaining from the extraction procedure and the supernatant from the membrane pellet were run on SDS-polyacrylamide gels and then immunoblotted with 4D2. As seen in Figure 4-12, for wild-type P23H, P171L and V345M two fractions were extracted from the membranes. The slower moving form, so-called mature form had a $M_r \approx 40,000$, similar to ROS rhodopsin. The faster migrating form had a $M_r \approx 35,000$ which co-migrated with unglycosylated opsin. For wild-type and V345M there is some opsin which has an associated glycosylation smear. The relative fraction of opsin that was extracted with sodium carbonate was 10% for wild-type, 25% for P23H (class III), 30% for P171L (class II) and 15% for V345M (class I). The greatest fraction of sodium carbonate extractable opsin was from the mutants which had the poorest regeneration characteristics. In all four samples the largest fraction of opsin was still in the membrane. There was also a significant amount of opsin in the supernatant of the membrane preparation. This

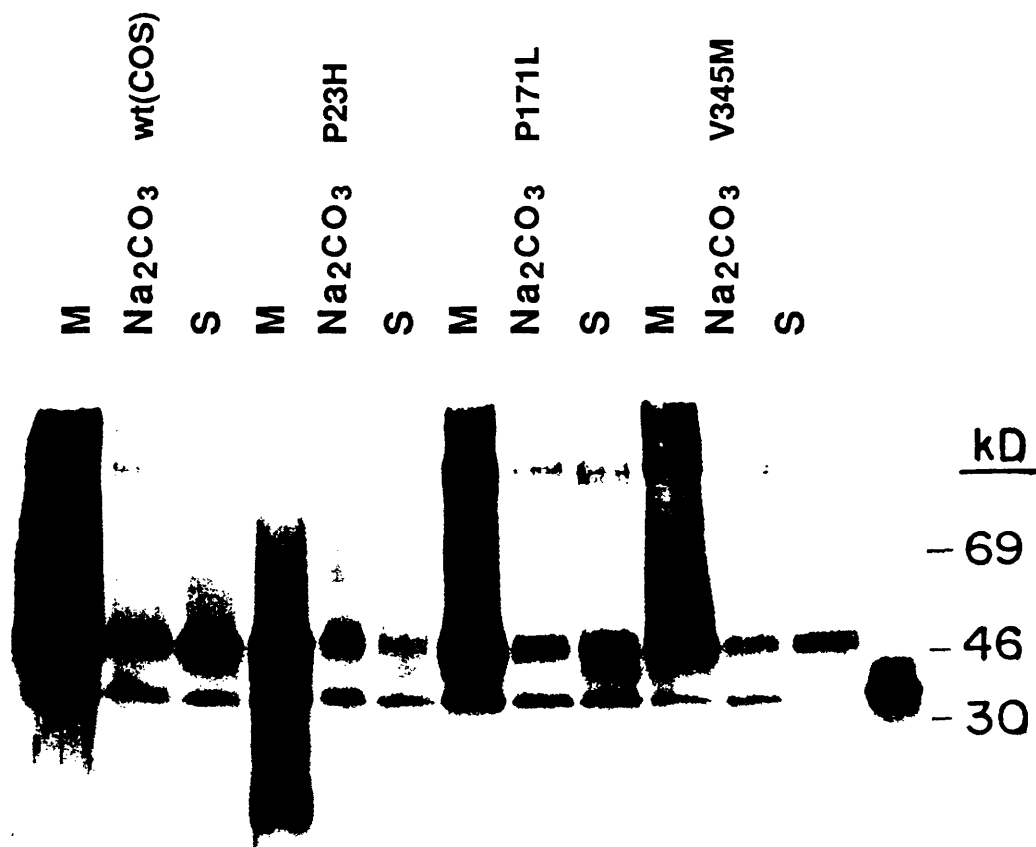


Figure 4-12 Extraction of opsin with 0.1 M sodium carbonate. Wt(COS), P23H (Class III), P171L (Class II), and V345M (Class I). M= membrane pellet remaining after carbonate extraction; Na₂CO₃= opsin extracted with Na₂CO₃; S= supernatant of membrane preparation prior to extraction with Na₂CO₃.

represents a soluble fraction of opsin. For both P23H and P171L besides the two molecular weight species discussed above there is an intermediate band, $M_r \approx 37,000$, that was also present. It could be a form which has only glycosylation at one of the N-linked glycosylation sites.

4.3.10 Phase Separation of Opsin with TritonX-114

TritonX-114 (TX114) has a cloud point at 30°C where the detergent forms large, dense micelles that precipitate out of solution thereby creating an aqueous and detergent-rich phase. Because of this unusual property it has been exploited for determining if a given protein is an integral membrane protein or a soluble one (Bordier, 1981). For example, when bacteriorhodopsin is solubilized with TX114 and the sample incubated at 30°C greater than 98% of this integral membrane protein partitions into the detergent phase. For hemoglobin, greater than 98% partitions into the aqueous phase.

The phase behavior of wild-type, P23H, P171L and V345M was studied using TX114. Figure 4-13 shows that for COS-1 cell wild-type and the mutants there is an equal distribution of the opsin in the detergent and aqueous phases. For wild-type and V345M there is an large aqueous phase fraction which has a glycosylation smear. This is somewhat different from what was observed by sodium carbonate extraction. TX114 does appear to solubilize only the mature opsin fraction- none of the lower molecular weight species are present. The

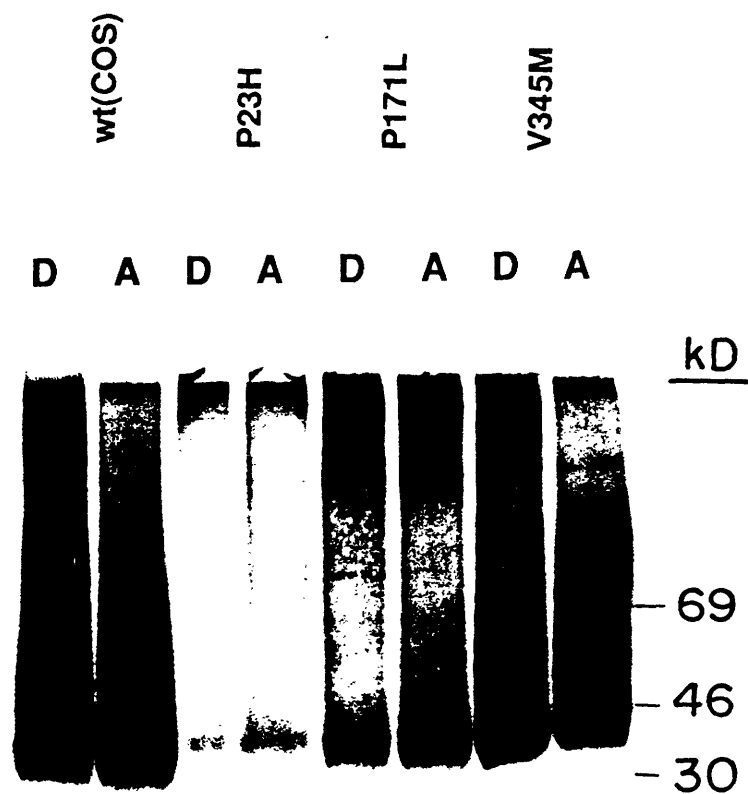


Figure 4-13 Phase partitioning of wild-type opsin and ADRP mutants with 1% Triton X-114. COS opsin, P23H (Class II), P171L (Class II), and V345M (Class I). **D** = detergent - rich phase. **A** = aqueous phase.

difference may reflect the mechanism by which these two methods interact with opsin.

4.4 DISCUSSION

Retinitis Pigmentosa (RP) is an eye disease in which the photoreceptor cells are destroyed leading to retinal degeneration and eventual blindness. The clinical hallmarks of this disease include nyctopia (nightblindness), mid-peripheral vision loss and diminished ERG's. It is the most common form of blindness among middle-aged persons, occurring in 1/3000 people (Heckenlively, 1988).

Over the past few years the groups of Driyja, Bhattacharya, Nathans, Stone and Seiving have identified a large number of mutations in the rhodopsin gene associated with autosomal dominant retinitis pigmentosa (ADRP). These mutations are believed to account for approximately 30% of all ADRP patients (S. Bhattacharya, personal communication). These mutations are distributed throughout the protein. The types of amino acid substitutions are variable. For example, some are replacements of neutral residue with a charged one (e.g. P23H, G89D), others are the introduction of bulky residue (e.g. C110Y, D190Y) while others are the removal of a charge (e.g. R135L). In a few instances there are deletion mutations such as $\Delta 68-71$ and I256 Δ . How do all these different types of mutations lead to the disease? Do they have a common pathogenetic mechanism or are there multiple mechanisms?

To begin understanding this problem our laboratory has used site-directed mutagenesis to construct, express and purify these mutants. Initially, we studied how well the mutants were able to fold by their ability to bind 11-*cis* retinal. We quantitated our operational definition of the extent of folding by the presence and amount of chromophore formed. The mutants fell into three categories (Table 4-3). The majority of the mutants were expressed at low levels and formed chromophore but at low levels. For example P23H a large fraction of the purified protein did not regenerate. Furthermore it was abnormally glycosylated and retained in the ER. Together these findings indicate the mutant did not fold efficiently or certain non-native, off-pathway, structures were favored. Indeed, it is known that proteins which fold slowly or are misfolded are retained in the ER where they may eventually be targeted for degradation (Bonifacino *et al.*, 1990; Klausner and Sitia, 1990; Wikstrom and Lodish, 1992; Shin *et al.*, 1993). A mutant like P23H may have a high rate of degradation explaining in part the low levels of expression. In fact on immunoblots of this mutant (and others like it as well) there often are protein bands which have a smaller molecular weight than unglycosylated opsin. These are probably degradation products. The abnormality in glycosylation most likely then reflects the protein's inability to leave the ER rather than a true defect in the inability of the mutant to acquire the mature glycosylation smear.

P23H is an N-terminal mutation but there are many mutations within the transmembrane region (e.g. P53R, V87D) and the intradiscal loops (e.g. G106R) which also had a similar phenotype. One mutant

was a deletion on the cytoplasmic face, $\Delta 68-71$. The expression level, amount of chromophore and spectral ratio of the mutants in this class varied. In general, the spectral ratio reflects the extent of regeneration i.e. the higher the A_{280}/A_{500} ratio the greater the unregenerated fraction. Q28H had a low spectral ratio of 3.0 while P23H had a high ratio, 6.5. Although there was some variability from transfection-to-transfection these results are reproducible. Increasing the time of regeneration did not increase the amount of chromophore. Such variation would imply the rate of folding in COS-1 cells is different for the members of this mutant class.

Since these mutants were retained in the ER one reason why they could not completely regenerate was their inaccessibility to 11-*cis* retinal. However when wild-type rhodopsin was artificially retained in the ER by expressing it in the presence of Brefeldin A this protein fully regenerated indicating that 11-*cis* retinal can penetrate the internal membrane compartments of COS-1 cells.

Many of the mutations in this class occur within the transmembrane region, clustered in helix A and B. It was surprising some of the charge substitution mutants within this domain like G51R and G89D regenerated because of the unfavorable energetics of burying charges in a hydrophobic environment. We do not know the interaction of the native amino acids which are sites of the ADRP mutations. It could be these two helices are less important in the overall assembly of the retinal binding pocket or the charge substitutions are stabilized by other intra-protein interactions. Or the

mutation effects the efficiency of folding or possibly how the protein is inserted into the membrane, during or after synthesis.

If the transmembrane segments are α -helices the amino acids gly89 and gly90 would be rotated by 100 degrees from one another. The RP mutations at these sites, G89D and G90D, are identical charge substitutions. Both regenerated but G90D was expressed at higher levels and formed greater amounts of chromophore. Furthermore it had a blue-shifted chromophore with a λ_{max} of 480 nm. Although glu113 is believed to be the primary counterion for the Schiff base in native rhodopsin the aspartic acid in the RP mutant clearly alters its spectral properties. It probably directly interacts with 11-*cis* retinal in the dark state. This mutant also bleaches more quickly on illumination suggesting it also plays a role in the conformational changes accompanying photoisomerization.

One particularly interesting mutant in this class is C110Y. Cys110 and cys187 are believed to form the single disulfide bond in rhodopsin which is critical for its assembly. There are eight other cysteine residues found throughout the protein including the transmembrane region (e.g. cys167, cys222) and cytoplasmic surface (e.g. cys140, cys316). The ADRP mutant at cys110 is the first single amino acid substitution at this site which regenerated although at extremely low levels ($A_{500} = .002$). The extent and amount of regeneration was similar to that observed for Cys X, a mutant which retained only cys110 and cys187 (Karnik *et al.*, 1989). Upon illumination C110Y did form a 380 nm species. This would imply the

disulfide bond is not strictly required for the folding and assembly of rhodopsin but may affect the rate of folding and/or the stability of folded opsin.

The class III mutants had bleached rapidly and were less efficient in triggering transducin.

The second group of mutants (class II) were those which did not form any chromophore at all. They were primarily clustered in the conserved region of the DE loop on the intradiscal surface (e.g. Y178C, S186P). However some were found in the transmembrane domain including helix D (e.g. C167R, P171L), E (e.g. H211P) and F (e.g. I256Δ). No mutant proteins from helix A, B or C fell into this class. They were expressed at low levels, had abnormal glycosylation and were also retained in the ER. These mutants probably reflect mutations which either grossly altered the structure of the retinal binding pocket thereby outrightly preventing the binding of 11-*cis* retinal or configured the retinal binding pocket in such a way the Schiff base linkage was not stable i.e. the rate of hydrolysis of the Schiff base was greater than its rate of formation. We tried to regenerate these mutants with all-*trans* retinal but no chromophore was observed.

Doi *et al.* (1990) described a similar phenotype for a set of deletion mutants in the conserved region of the DE loop. In general, one may expect such deletions to affect the folding and assembly of opsin more dramatically than a substitution since it changes the actual length of a structural element. Our laboratory has found that small

two amino acid deletion mutants of opsin in the BC loop do not fold (A. Anukanth and H.G.K. manuscript in preparation). Of course if an amino acid is critical for establishing or maintaining certain tertiary contacts within the protein than a substitution at that site could have drastic effects on folding. This is observed in the ADRP mutants in the conserved portion of the DE loop. In this case we are uncertain about its exact structure or how much of it is in the membrane since the aqueous-lipid boundaries are unknown. It is quite a large region extending from amino acids 171-189. Furthermore because it is conserved in the mammalian visual receptors one may surmise it is important for structure i.e. help establish the retinal binding pocket. Cys187 is found in this region and mutations in its vicinity may prevent the formation of the disulfide bond.

The boundary region of helix D has two vicinal prolines at positions 170 and 171. Prolines are known to introduce kinks into α -helices (MacArthur and Thornton, 1991; von Heijne, 1991; Yun *et al.*, 1991). It is probably unlikely these residues actually are part of an α -helix but may be part of a critical structural component of the retinal binding pocket. The ADRP mutant P171L does not form chromophore. As such it may destroy the retinal binding pocket. On the other hand P170L, a non-RP mutant, regenerated to wild-type levels. This result suggests this residue does not directly participate in the folding or assembly of rhodopsin. However it may be one of many residues in the membrane that faces away from the retinal pocket and interact with the surrounding lipids.

The mutant K296E is a special member of this class. It did not form any chromophore because the lys residue required for forming the Schiff base linkage was absent. This mutant when purified in 0.1% LM from the 1D4 column did not activate transducin. Oprian's group reported this mutant was constitutively active when it was present in the membrane bound form (Robinson *et al.*, 1992). Either this mutant is extremely sensitive to detergents or there is a separate fraction which has the ability to trigger transducin but is not recognized by 1D4. We found the K16-50 antibody column recognized a fraction of wild-type COS-1 cell opsin that did not regenerate. This species had a multi-band pattern by immunoblots without a glycosylation smear. A similar fraction was found for K296E.

The class I mutants regenerated like wild-type, had glycosylation smear and were transported to the cell surface. They also had bleaching properties similar to wild-type except for T58R. These mutations were found in helix A (e.g. F45L), helix C (e.g. L125R) and at the C-terminus (e.g. V345M). These mutants in the transmembrane region were also less efficient in triggering transducin. Those amino acid substitutions must effect the the conformation of the binding surface of mII that interacts with transducin.

We studied the membrane insertion of one member of each group. In the four cases studied we found there was a fraction of opsin that was extractable including wild-type. But we found for P23H and P171L a greater fraction of the opsin could be extracted with sodium carbonate suggesting a larger fraction of the mutant protein was not

embedded entirely in the membrane. Whether this represented a significant difference is uncertain. But if it is true for other members of each class it would partially explain the folding defects in those mutant classes. We also found there was a soluble fraction in all cases as well.

Using a heterologous expression system (293S human embryonic kidney cells), Sung *et al.* (1991) reported on the ability of some ADRP mutants to bind 11-*cis* retinal and their cellular localization. This group, however, did not purify the mutant opsins. Furthermore, prior to taking light/dark difference spectra hydroxylamine was added (50 mM final concentration) without actually knowing the dark reactivity of the mutant to this potent nucleophile. As mentioned in the previous chapter some rhodopsin mutants are reactive to hydroxylamine.

Driyja's group (Olsson *et al.*, 1992) reported on the characterization of transgenic mice carrying the mutant allele P23H. They demonstrated these mice developed retinal degeneration and showed dilatation of rod inner segments in at least one clone, P23H-L, suggesting that some fraction of the mutant protein may be retained in the ER. Interestingly, they also reported that wild-type human opsin, when expressed at high levels in a transgenic mouse could also lead to a phenotype very similar to that observed with P23H.

Other investigators have also shown that misfolded or abnormally glycosylated proteins are retained in the endoplasmic reticulum and stably associated with at least one resident ER chaperone, Bip (Machamer *et al.*, 1990; Suzuki *et al.*, 1991). Bip (heavy chain binding protein) is a member of the hsp70 family of heat shock proteins whose expression is induced when the cells are exposed to such stressful events as elevated temperature, exposure to certain drugs (e.g. tunicamycin, cyclohexamide) and by the ER retention of misfolded mutant proteins. Other ER chaperones are also induced as well, such as grp94 (the ER equivalent of hsp90) and the folding enzyme protein disulfide isomerase (PDI), cyclophilin B, a protein with peptidyl prolyl isomerase activity, and calreticulin (Gething and Sambrook, 1992).

In *Drosophila*, a mutation in the *nina A* gene results in the ER retention of one particular type of rhodopsin, presumably because it is misfolded (Colley *et al.*, 1991). This sequestration is believed to subsequently cause rod cell degeneration. This group believes the *nina A* gene encodes a peptidyl prolyl isomerase specific for rhodopsin (by sequence homology). Furthermore, they have found that in the presence of the *nina A* background certain mutations in rhodopsin result in a wild-type phenotype, without retinal degeneration (Charles Zuker, personal communication). This is one example of how the interaction of a foldase with rhodopsin may lead to retinal degeneration.

We did not find any convincing evidence to establish the association of these chaperones with the ADRP mutants retained in the ER.

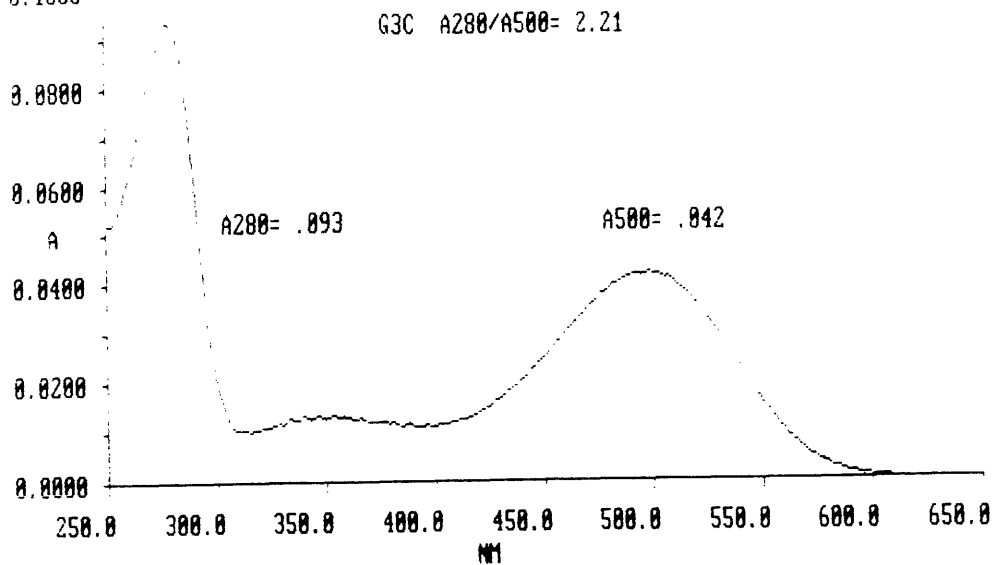
In the case of the cystic fibrosis transmembrane regulator (CFTR) the most common mutation associated with the disease, F508 Δ (deletion of phenylalanine at position 508), is a temperature-sensitive folding mutant (Denning *et al.*, 1992). When expressed at 37°C in CHO or COS cells, the non-permissive temperature, little functionally active protein is found at the cell surface. Indeed most of the mutant CFTR protein is found in the ER. At 27°C, the permissive temperature, much more of the properly folded protein is found at the cell surface and is functionally active. This study does not speculate why at 37°C, the mutant is retained in the ER. Some of the ADRP class III mutants were expressed at lower temperature in COS-1 cells but no increase in cell surface expression was detected.

The present investigation represents the largest study of all ADRP mutants. Obviously, from this study there is tremendous heterogeneity among the mutant ADRP proteins. How they all lead to retinal degeneration is not clear. From our results a vast majority of the ADRP mutants fold inefficiently or are simply misfolded. Both classes of mutants are retained in the ER and are not transported to the cell surface. This may be what is occurring in the rod inner segments of these patients. However, it is unclear how the presence of one normal opsin gene modulates the expression of the mutant.

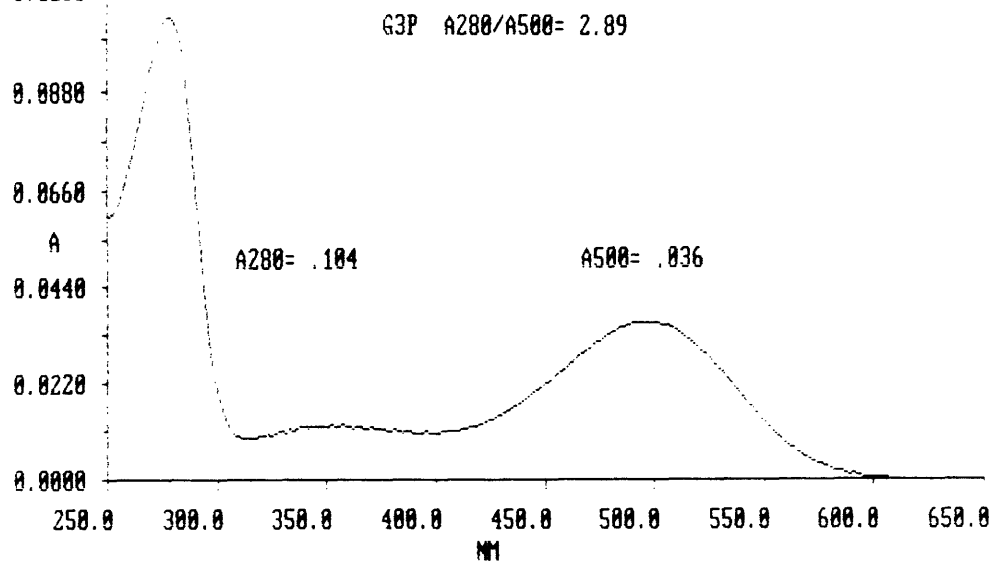
What is more puzzling is how mutants that appear like wild-type except for their inefficient triggering of transducin cause RP. Such mutants may have defects in other aspects of structure and function. Much more must be done before a clear picture of the molecular pathogenesis of ADRP emerges.

APPENDIX 1
SPECTRA OF GLYCOSYLATION MUTANTS

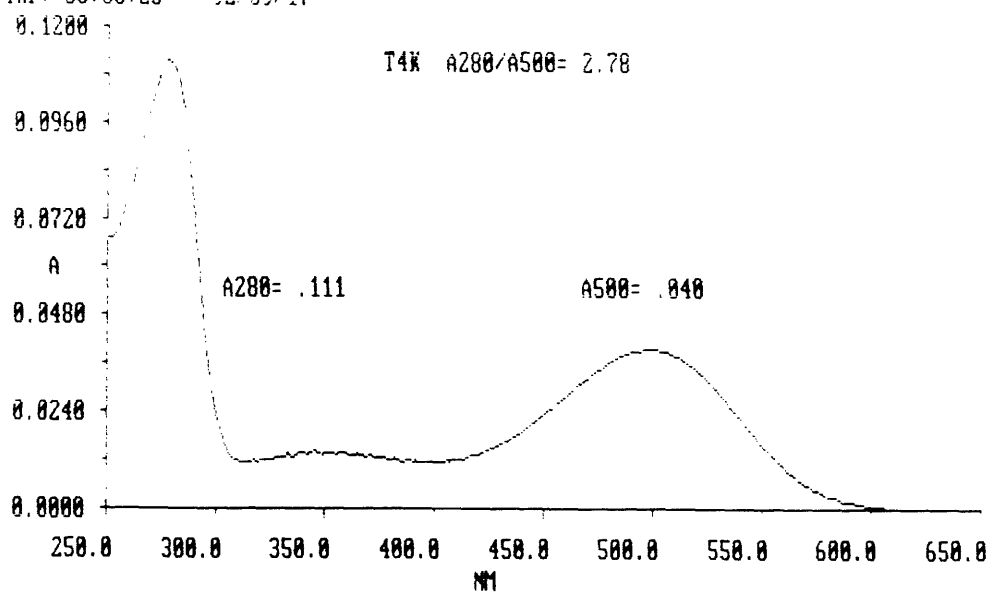
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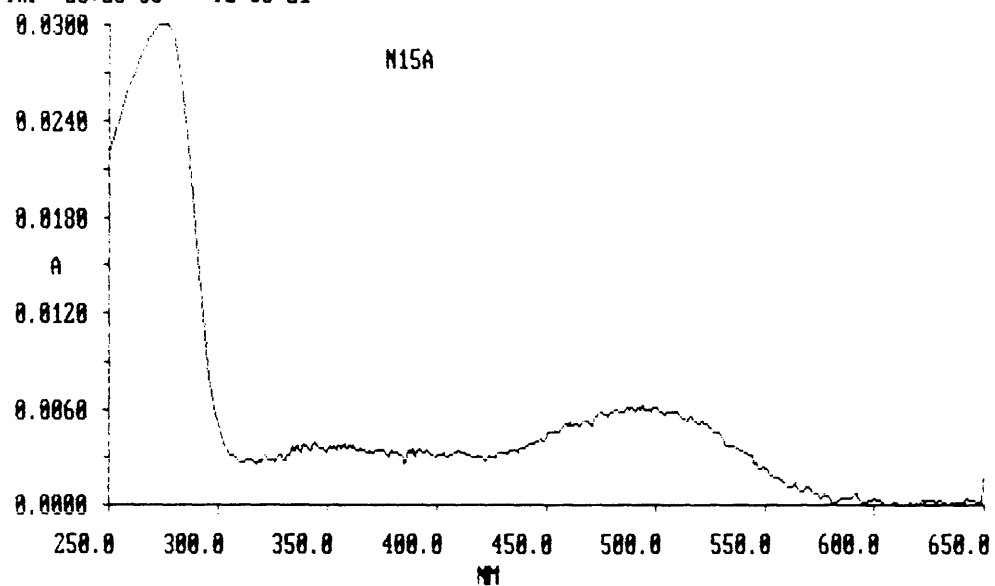
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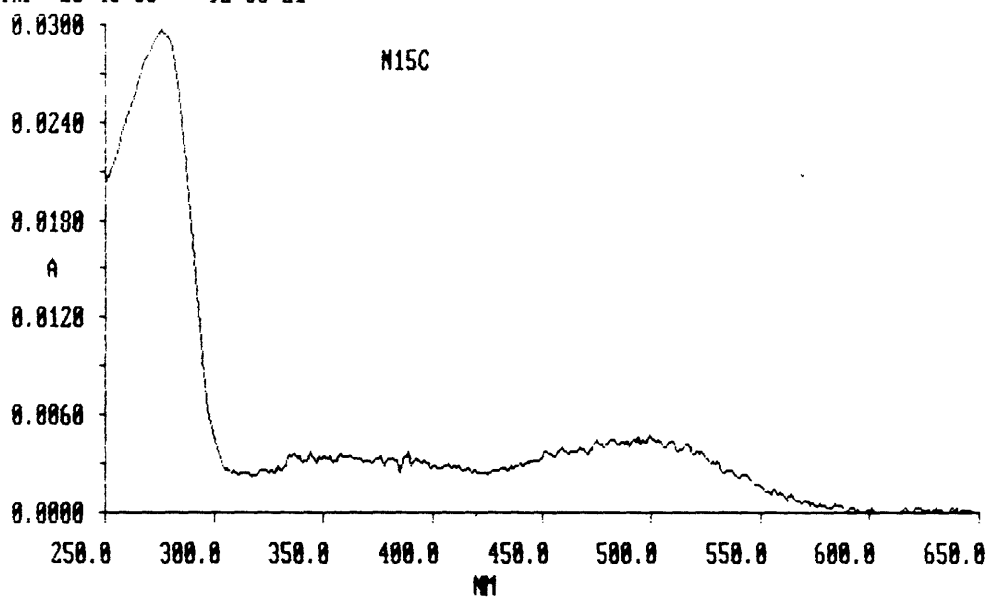
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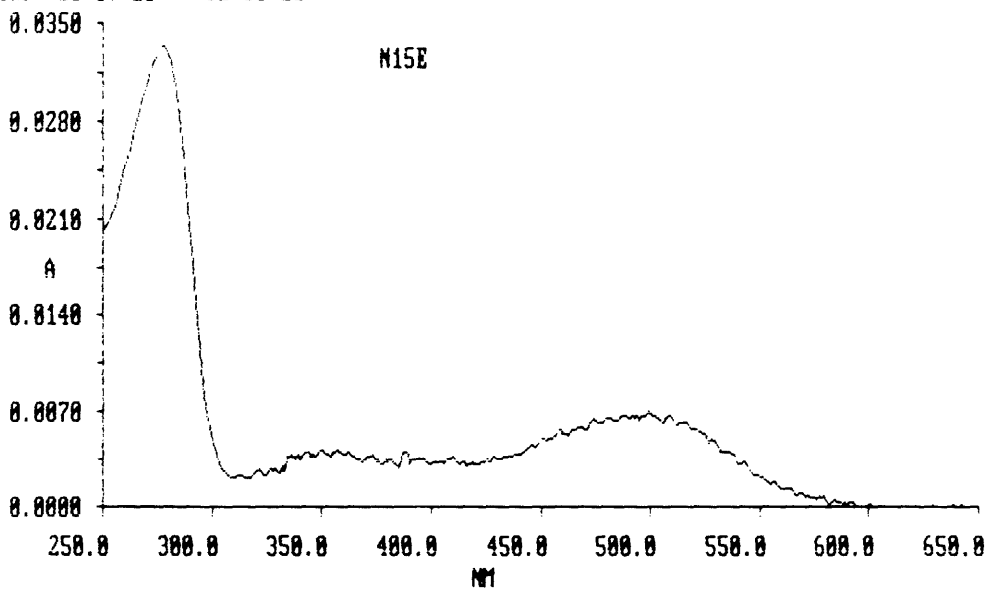
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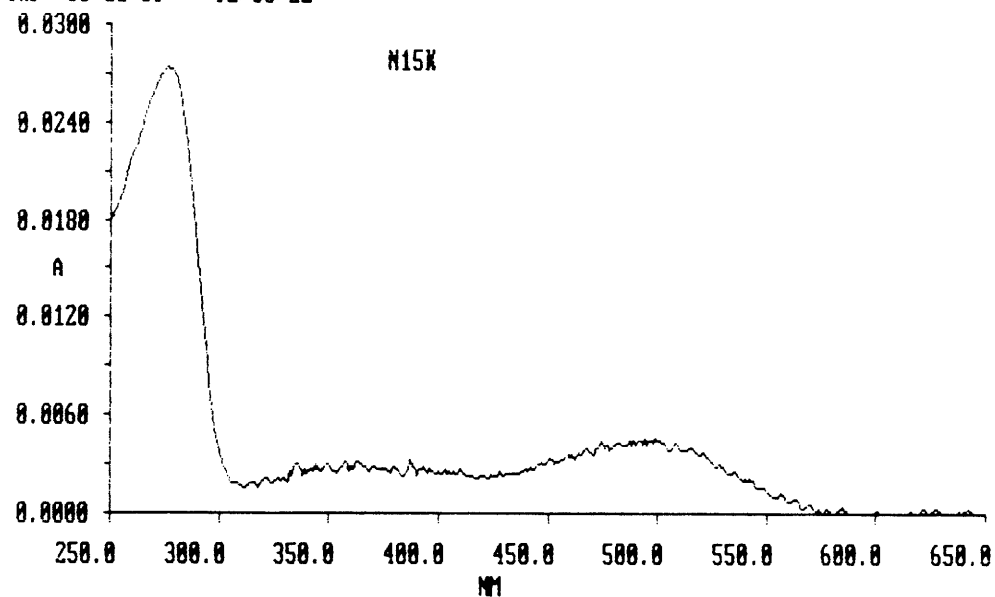
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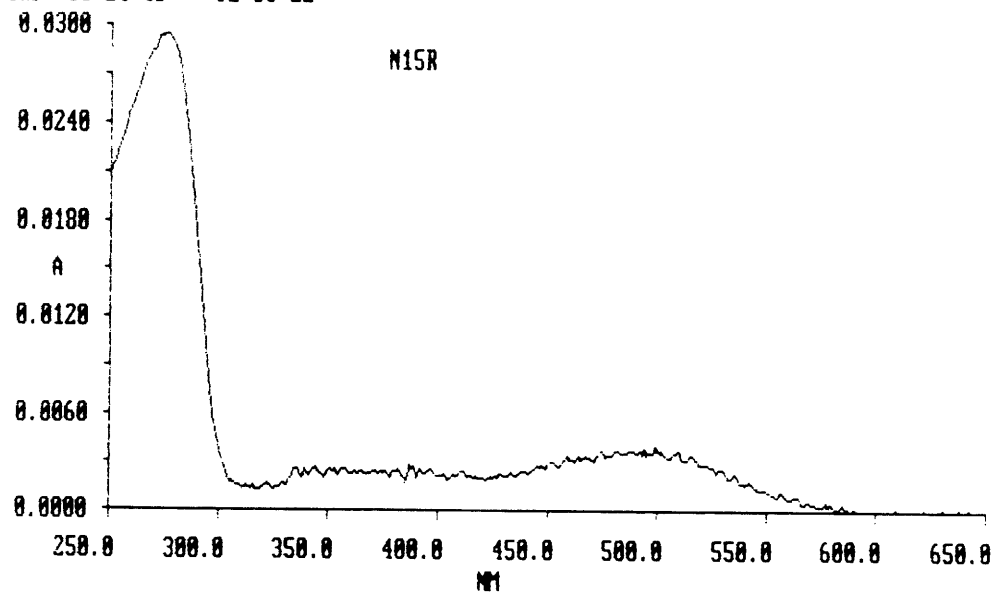
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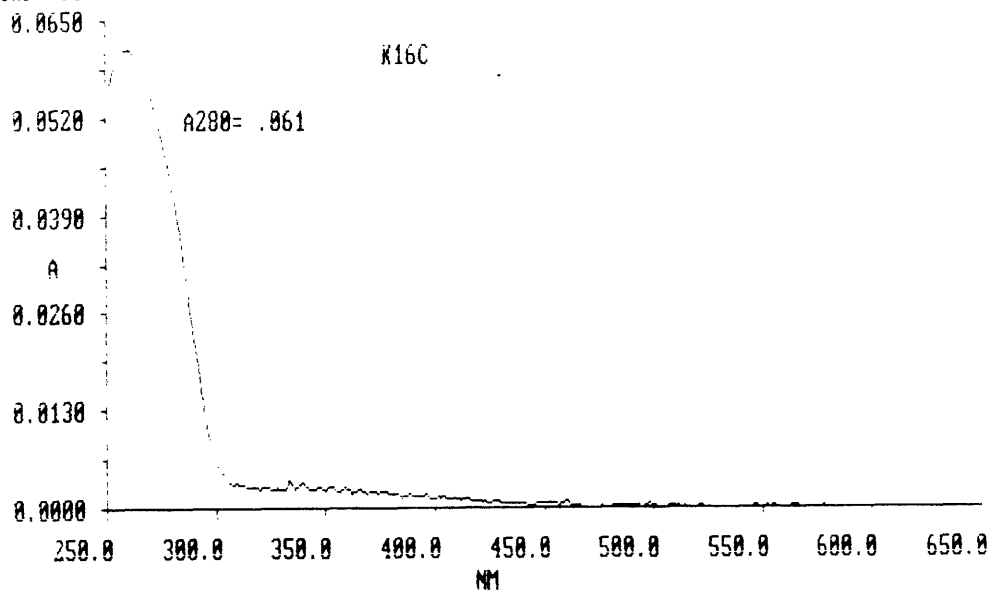
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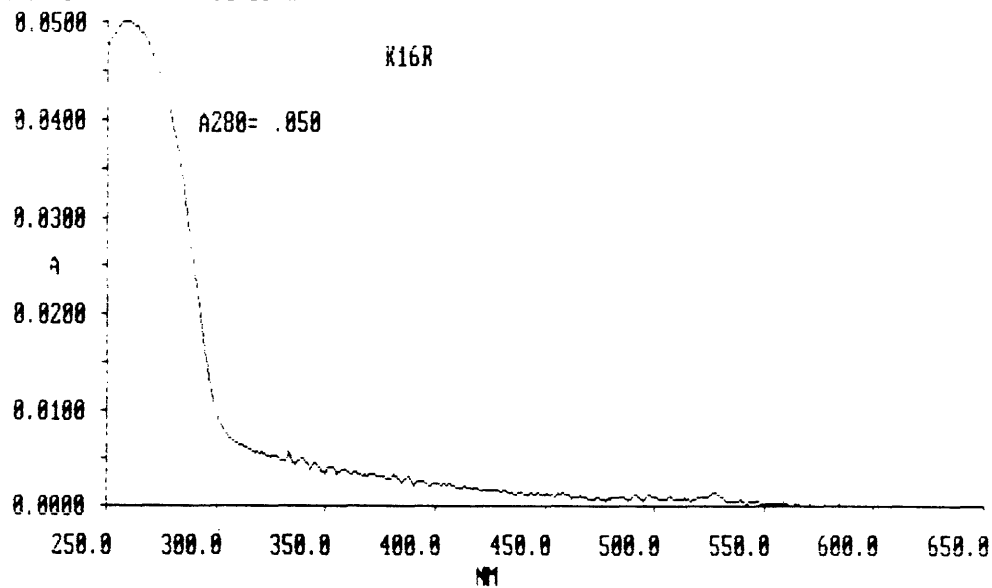
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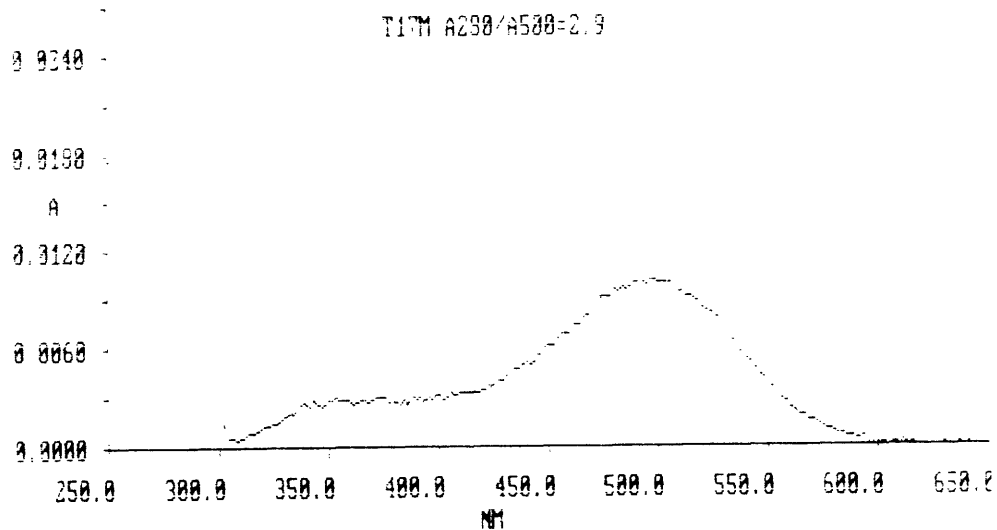
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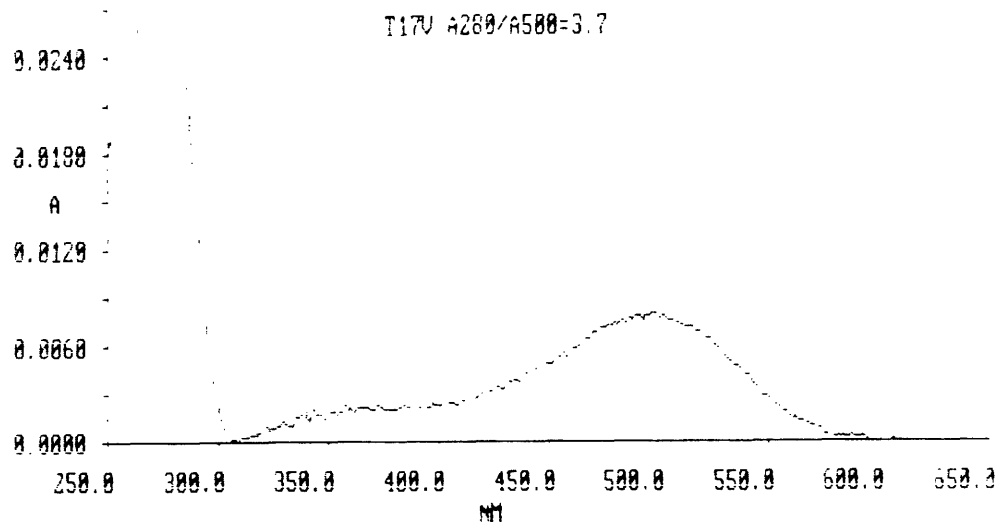
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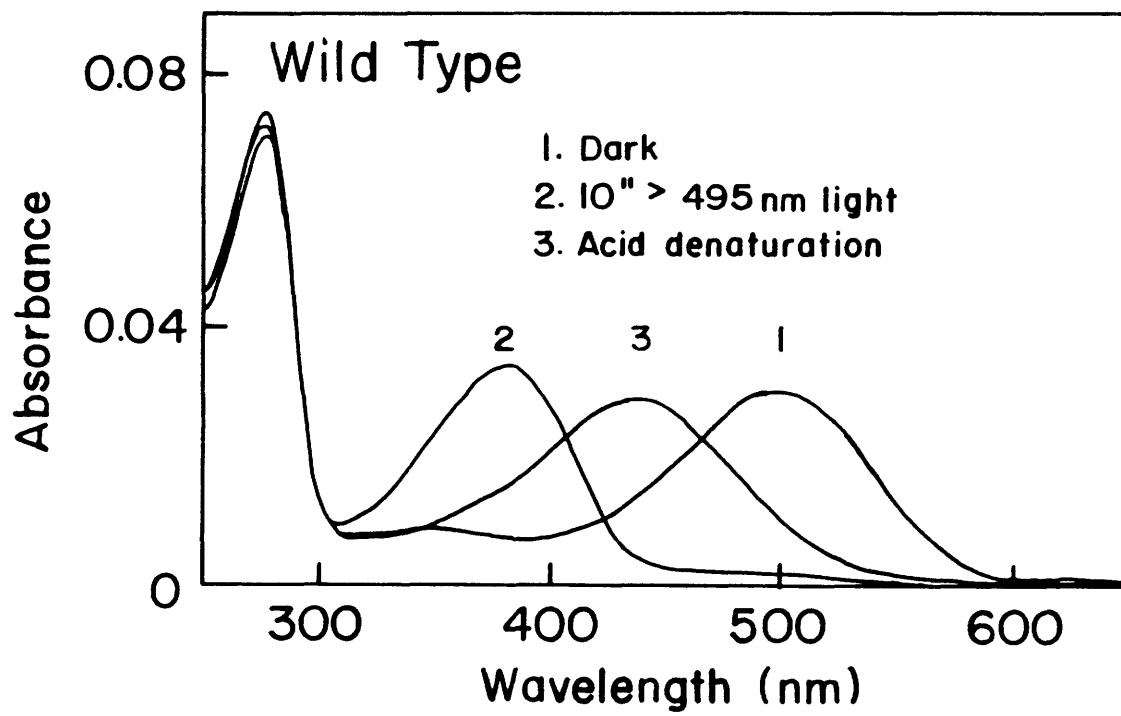


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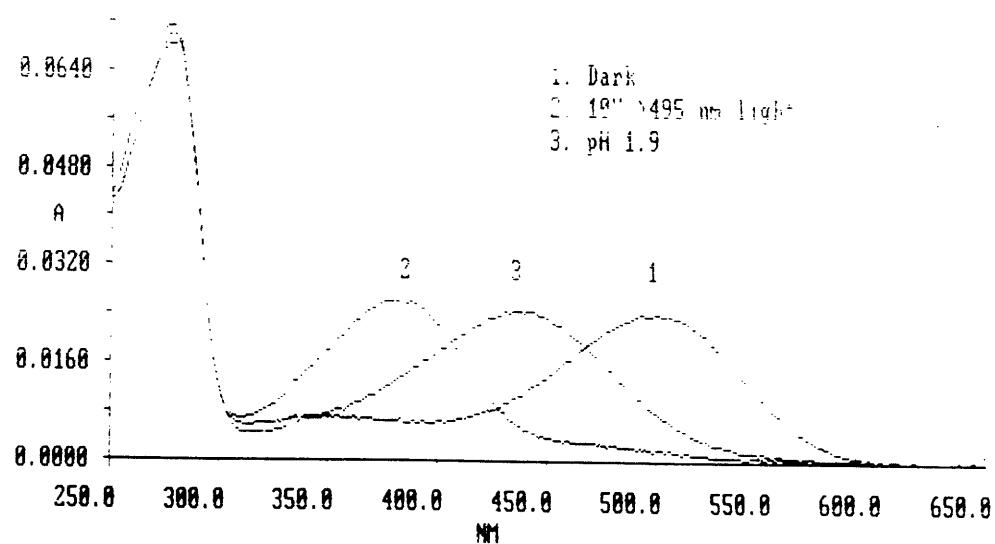


APPENDIX 2

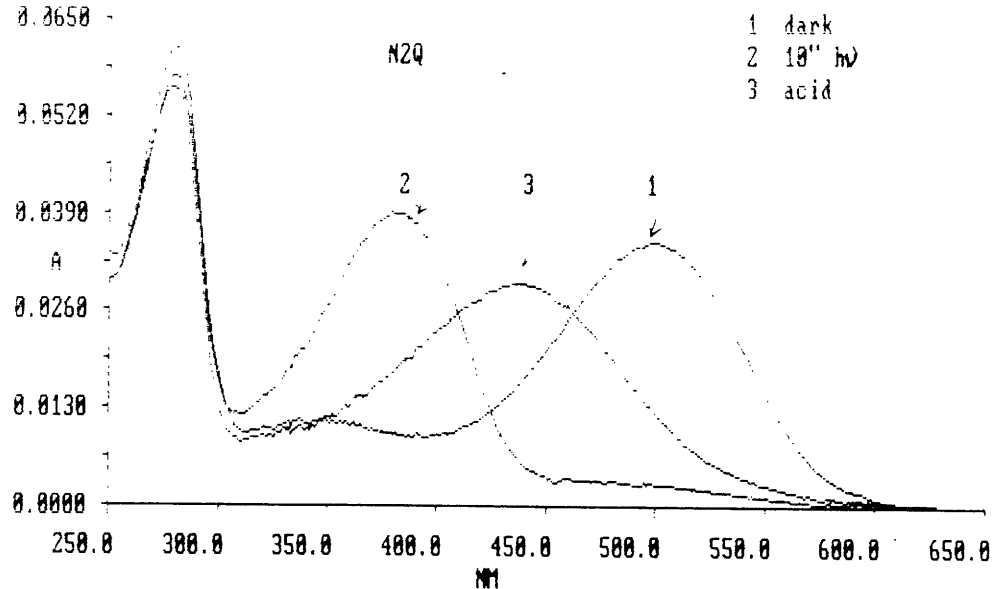
BLEACHING SPECTRA OF THE GLYCOSYLATION MUTANTS



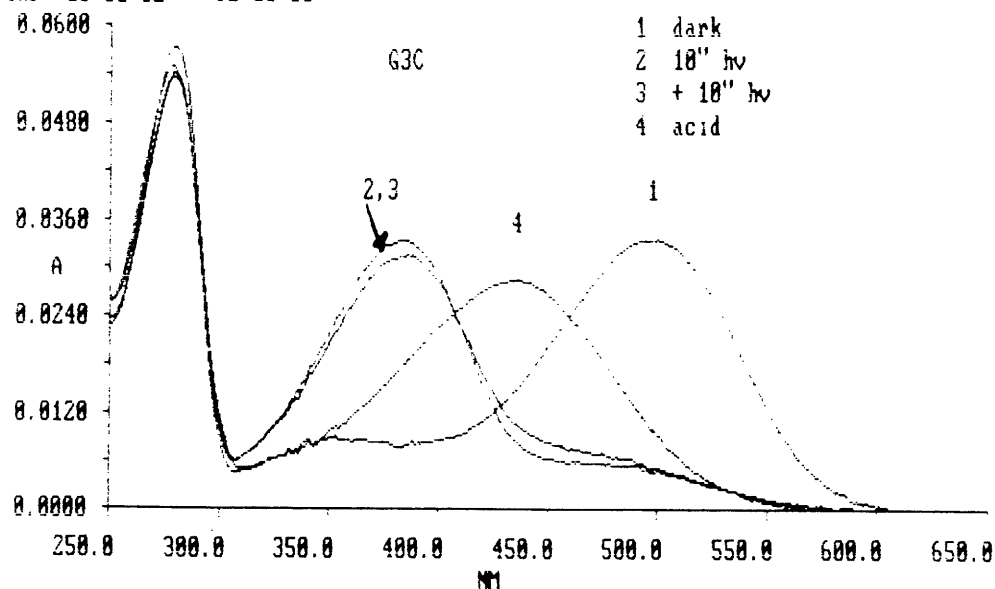
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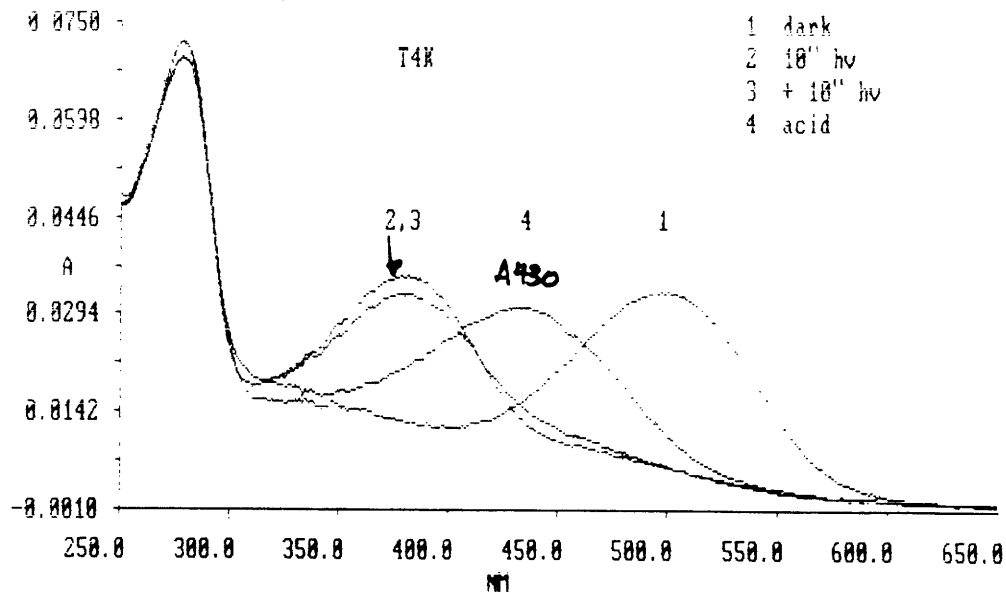
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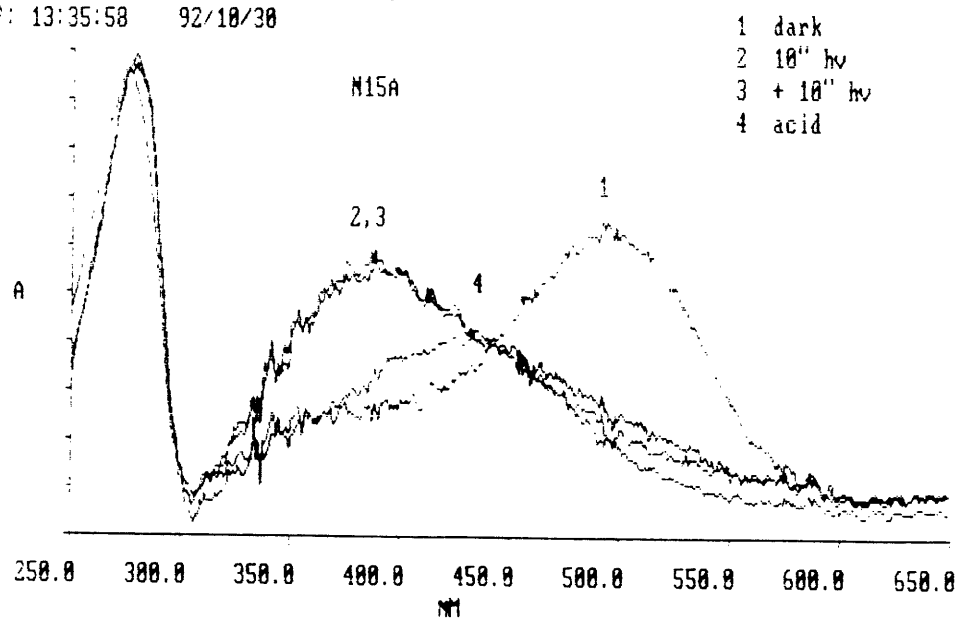
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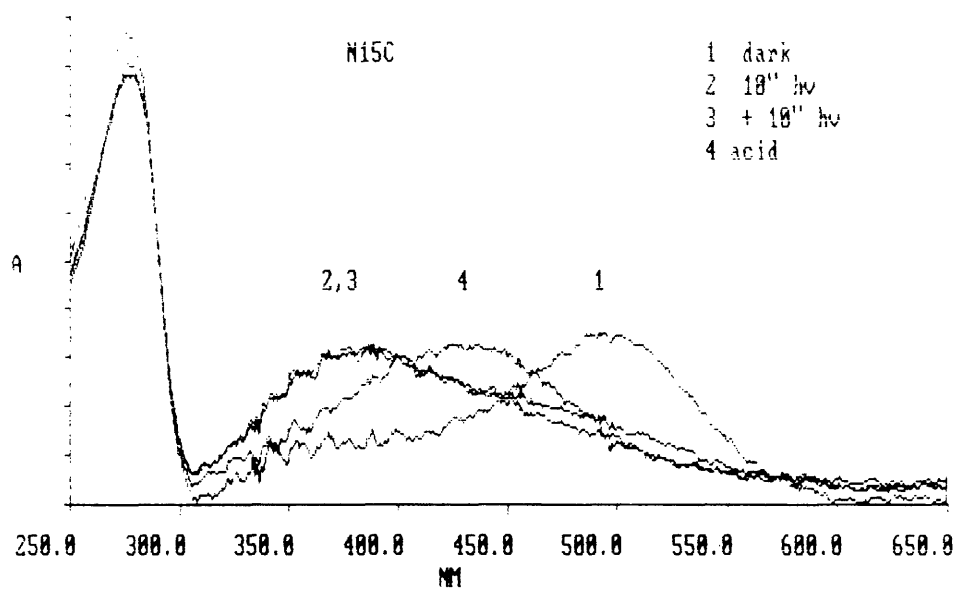
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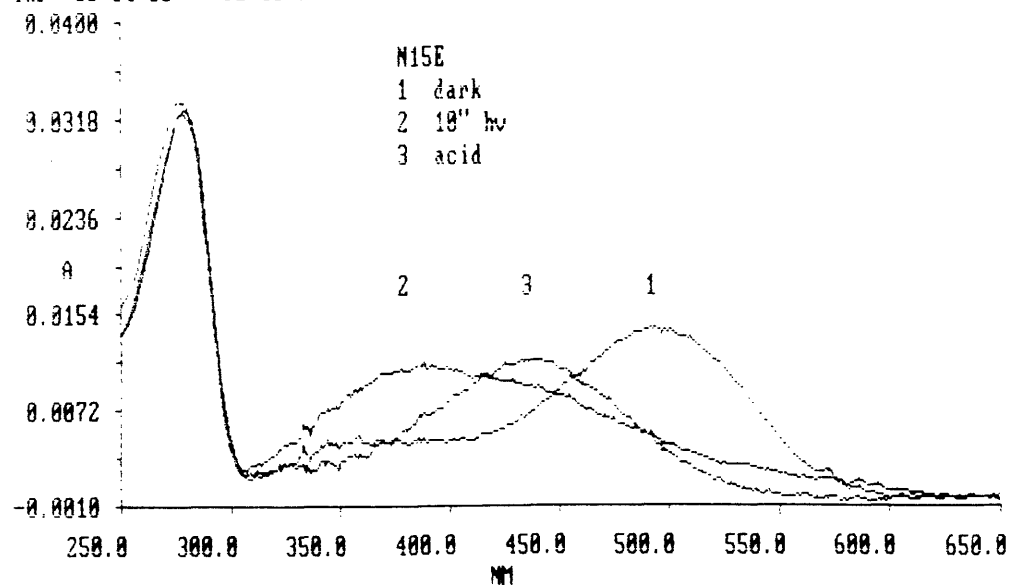
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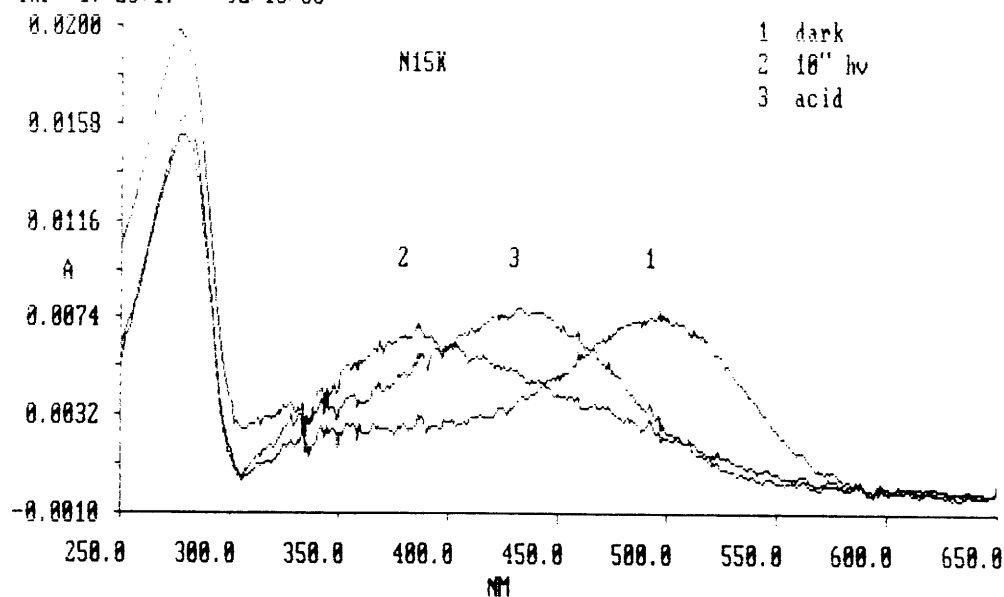
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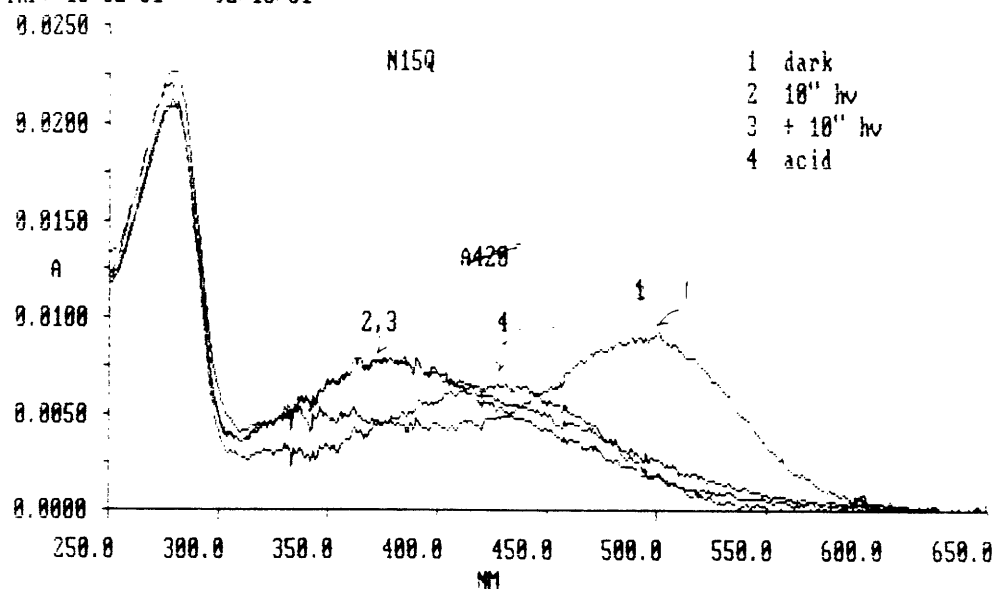
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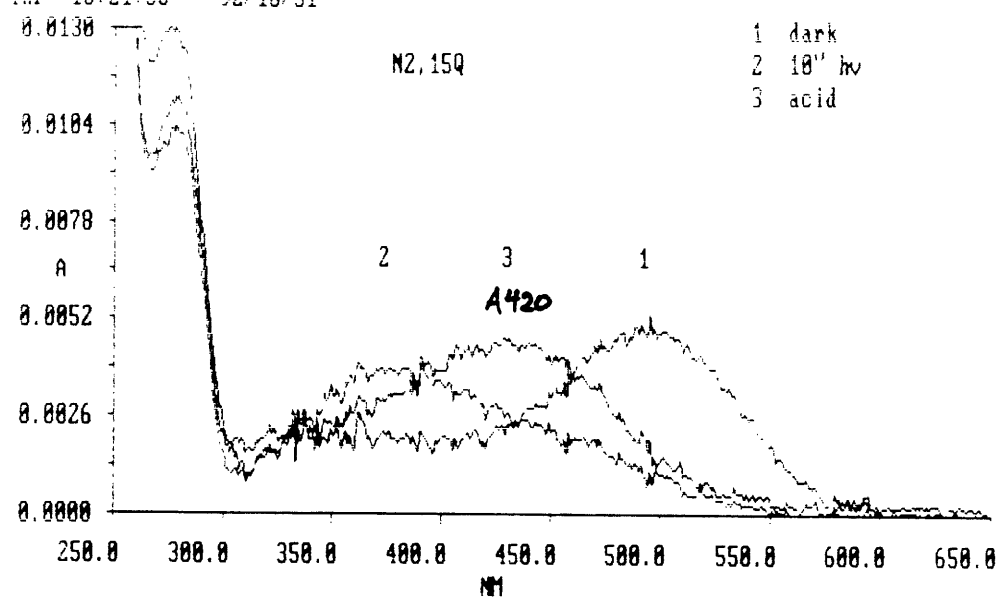
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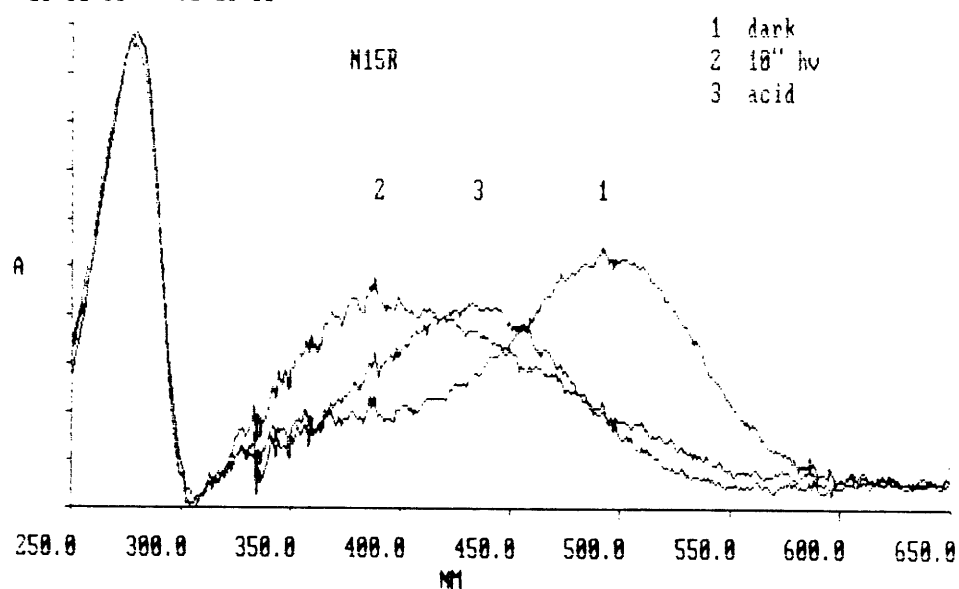
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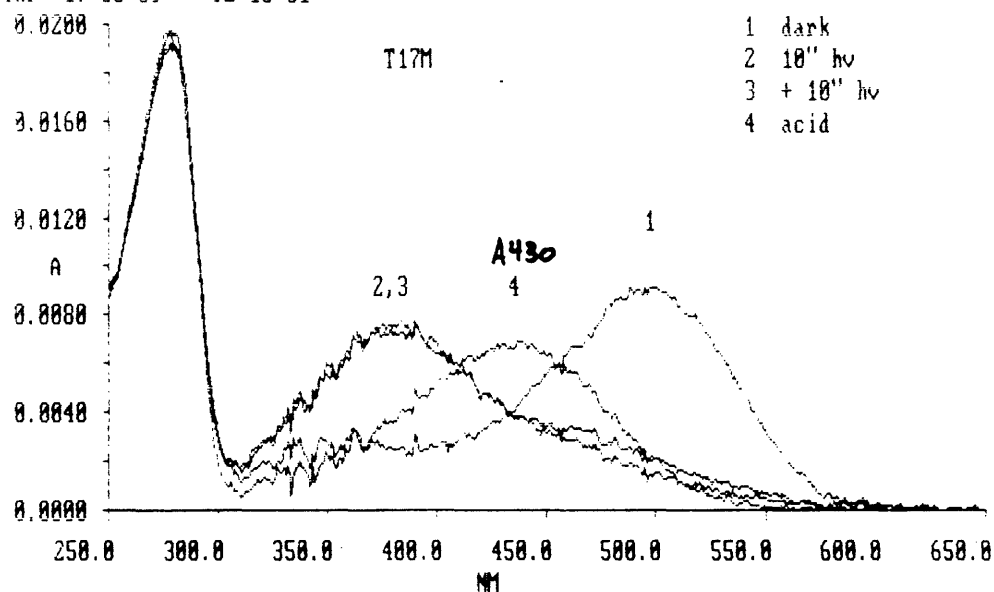
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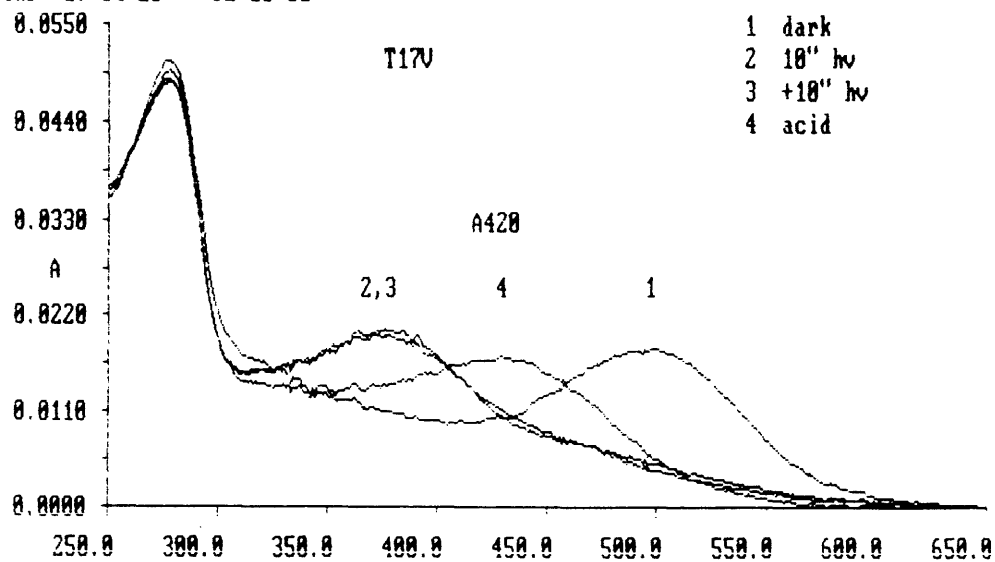
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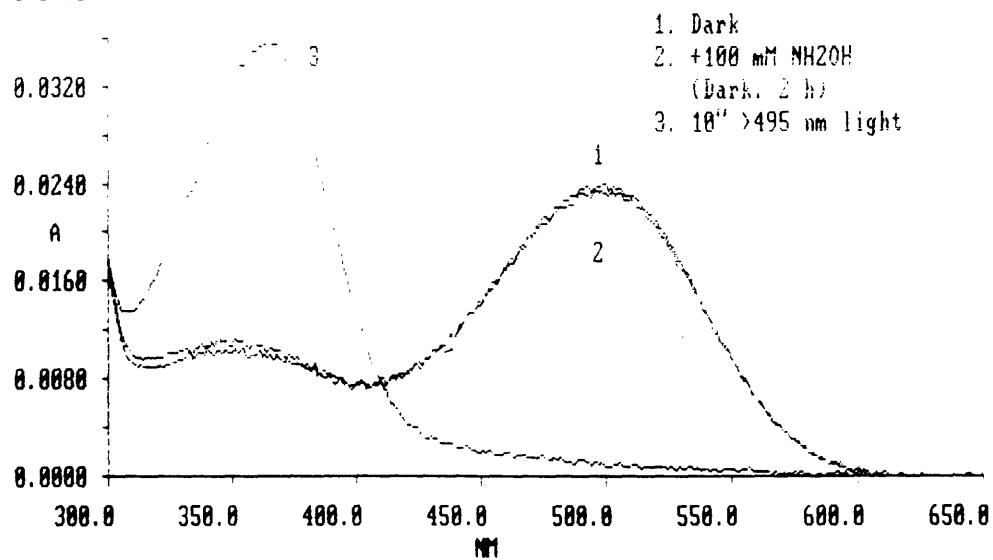
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APPENDIX 3

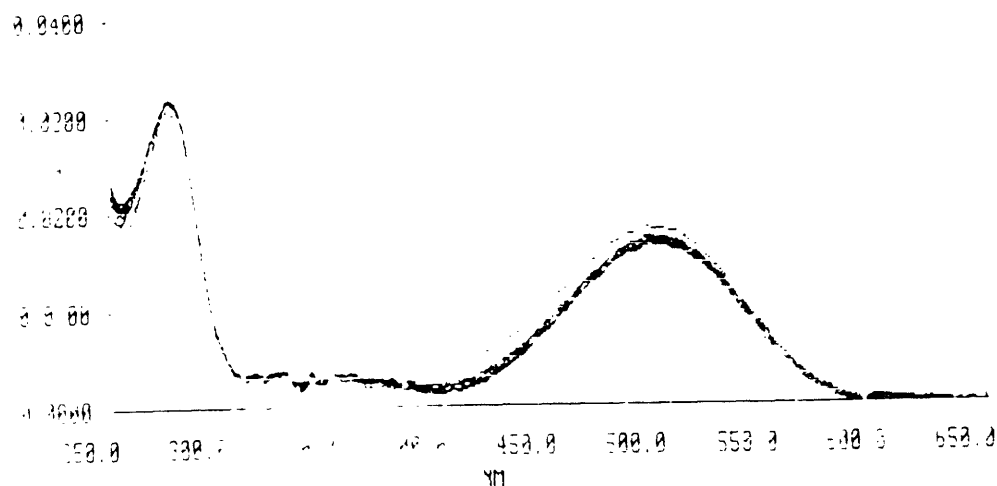
**HYDROXYLAMINE SENSITIVITY OF GLYCOSYLATION
MUTANTS**

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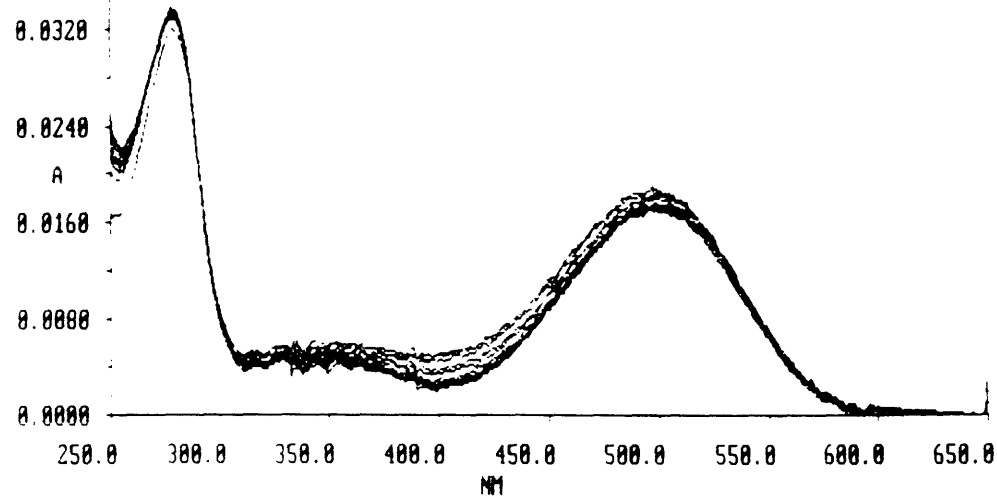
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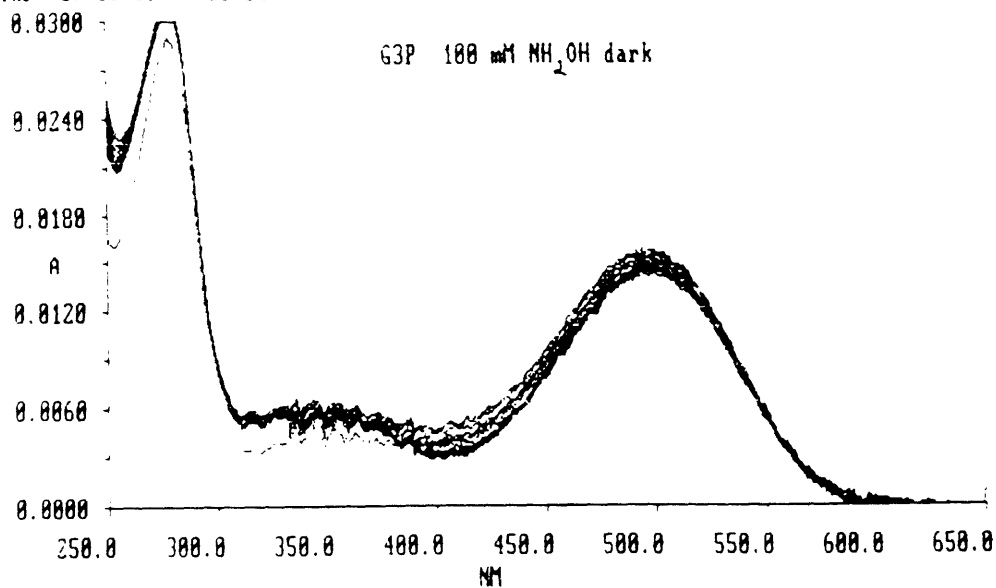


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 inf: 16:51:21 93/01/12
 0.0400 -

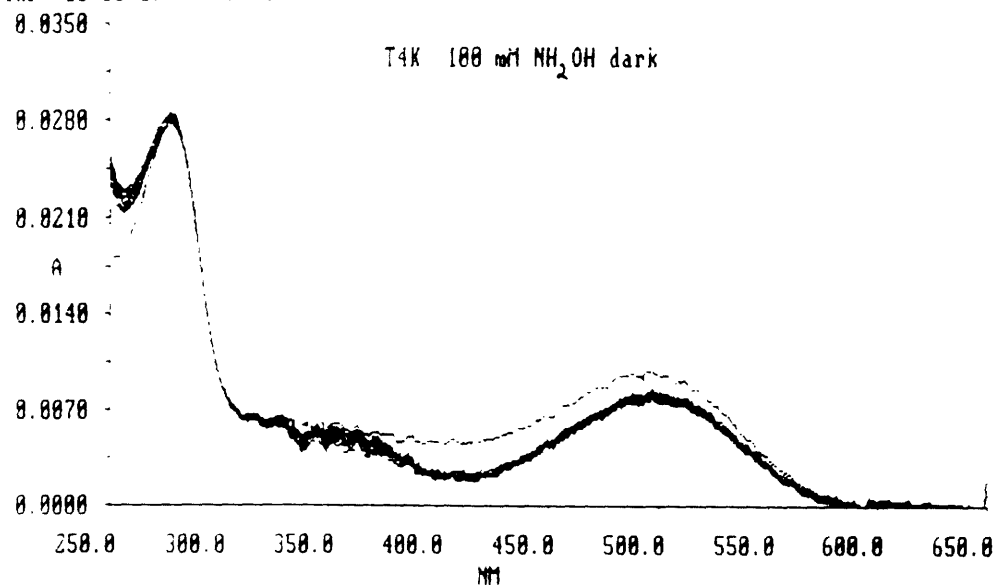
G3C 100 mM NH_2OH dark



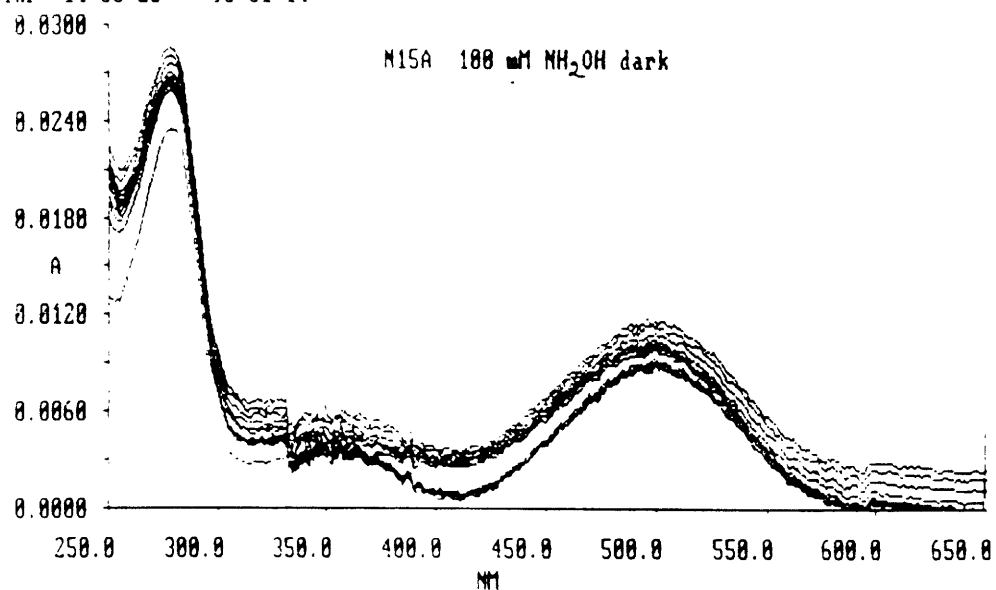
X: USER051; absc 650.0- 250.0; pts 401; int 1.00; ord -0.001-0.0314; A
inf: 17:53:09 93/01/12



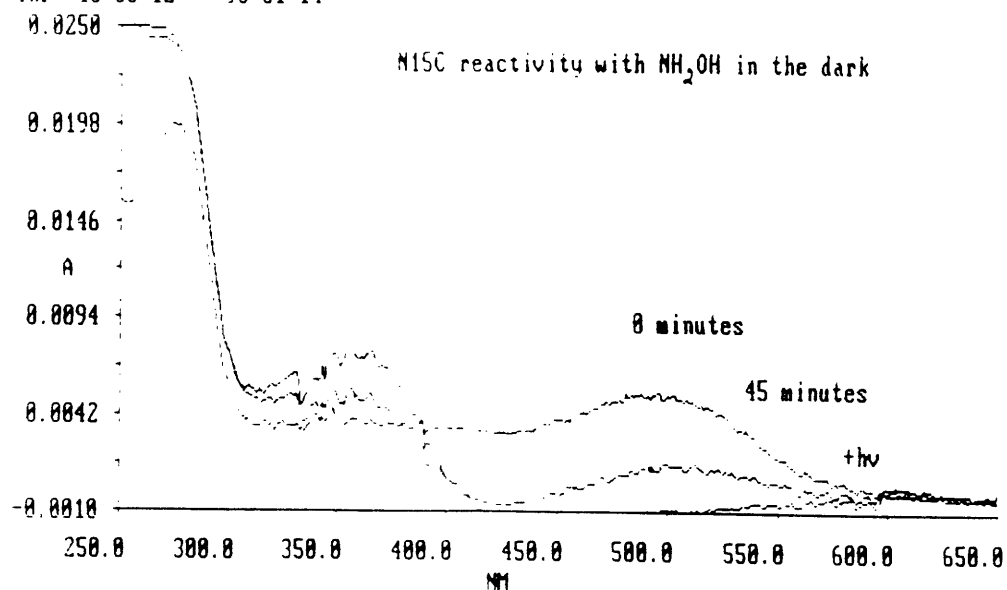
X: USER068; absc 650.0- 250.0; pts 401; int 1.00; ord -0.002-0.0286; A
inf: 18:58:17 93/01/12



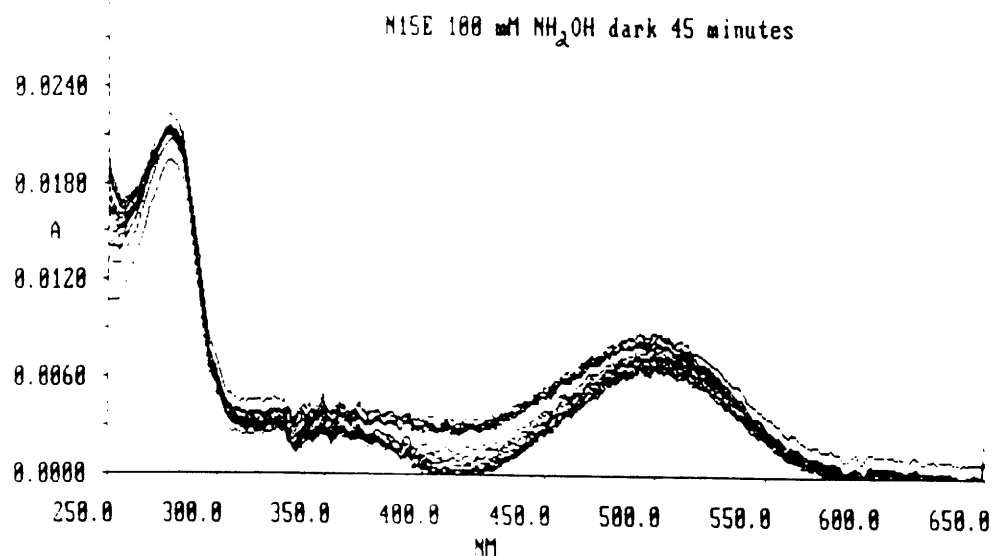
λ: USER021; absc 650.0- 250.0; pts 401; int 1.00; ord -0.000-0.0264; A
inf: 14:08:28 93/01/14



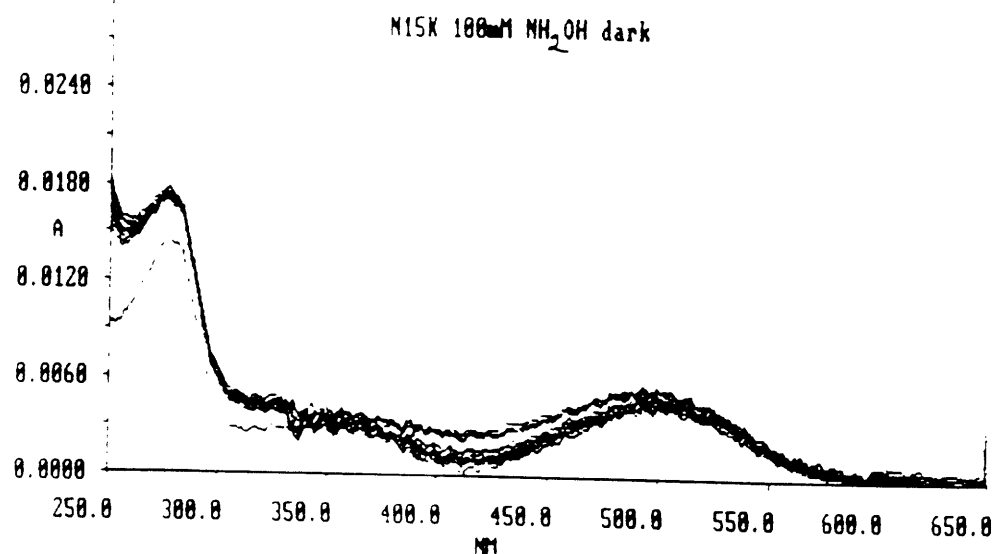
λ: USER038; absc 650.0- 250.0; pts 401; int 1.00; ord -0.002-0.0300; A
inf: 15:06:12 93/01/14



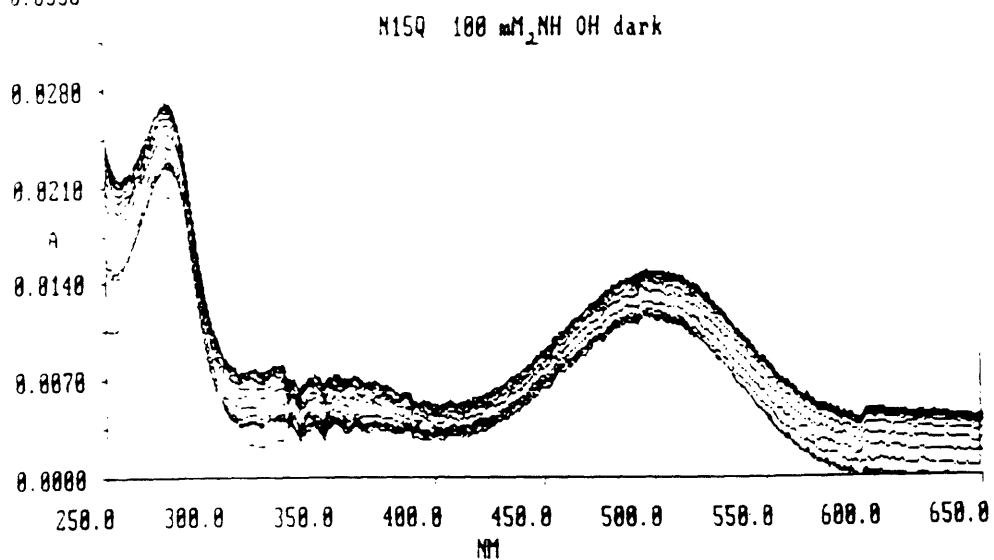
X: USER053; absc 650.0- 250.0; pts 401; int 1.00; ord -0.002-0.0215; A
inf: 16:02:46 93/01/14
0.0300



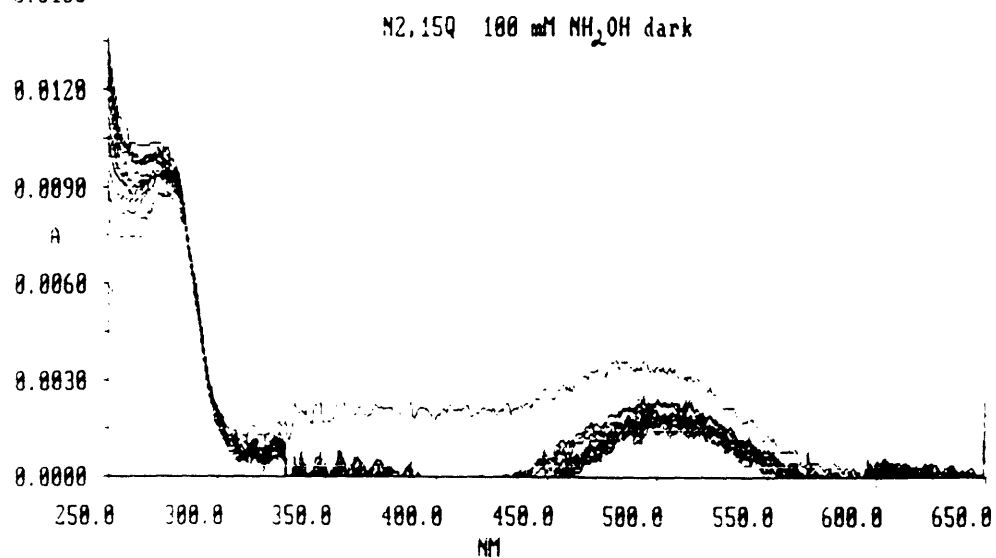
X: USER075; absc 650.0- 250.0; pts 401; int 1.00; ord 0.0000-0.0000; A
inf: 17:19:22 93/01/14
0.0300



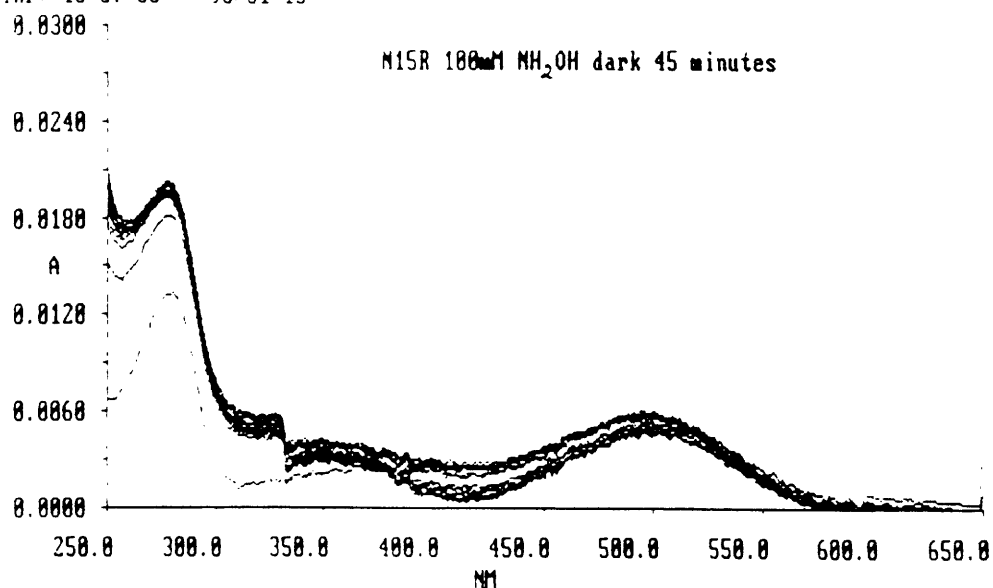
X: USER085; absc 650.0-250.0; pts 401; int 1.00; ord 0.0037-0.0274; A
inf: 20:01:55 93/01/12
0.0350



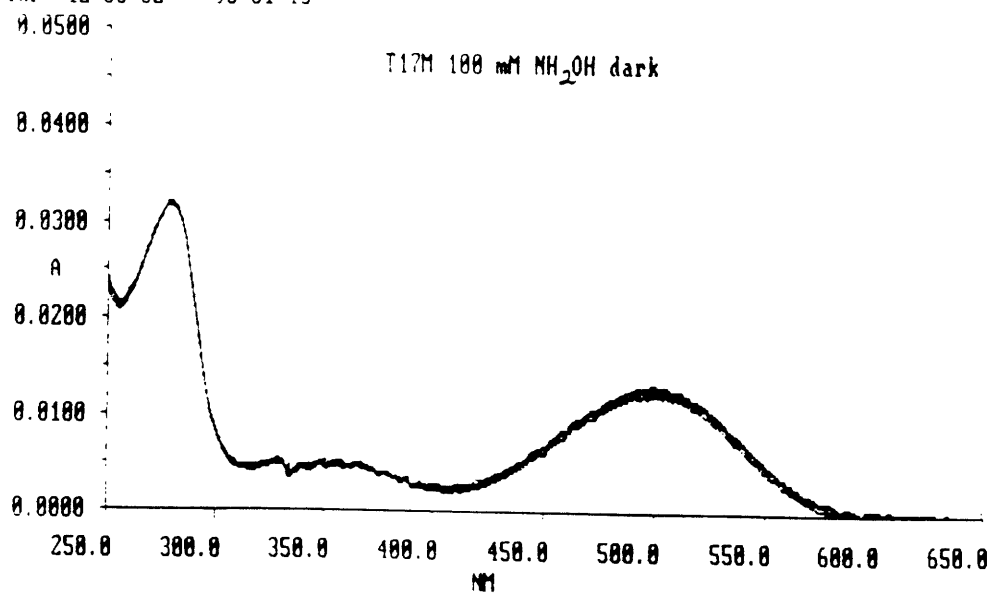
X: USER102; absc 650.0-250.0; pts 401; int 1.00; ord -0.001-0.0141; A
inf: 21:43:59 93/01/12
0.0150



X: USER015; absc 650.0- 250.0; pts 401; int 1.00; ord -0.001-0.0211; A
inf: 10:37:56 93/01/15



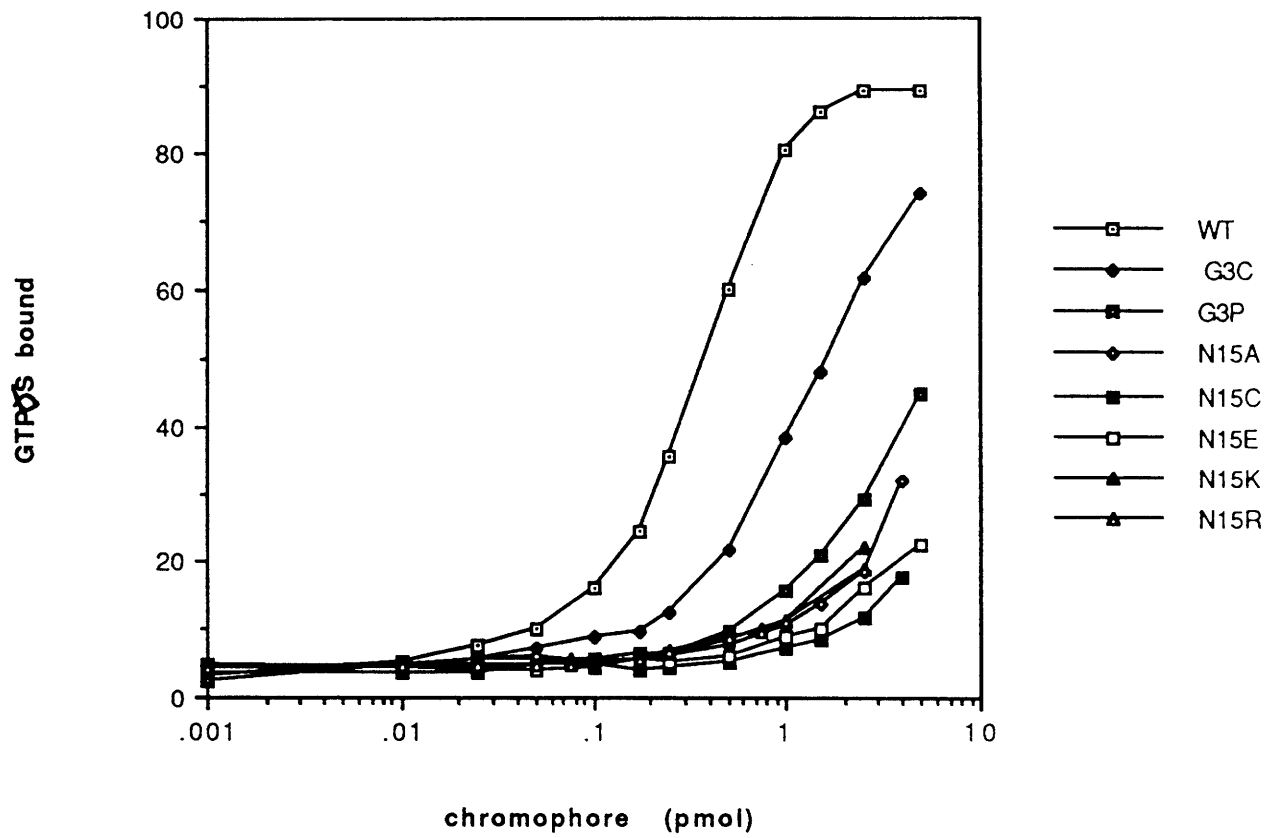
X: USER041; absc 650.0- 250.0; pts 401; int 1.00; ord -0.006-0.0318; A
inf: 12:08:32 93/01/15



APPENDIX 4

**GTP γ S EXCHANGE ASSAY OF OTHER GLYCOSYLATION
MUTANTS**

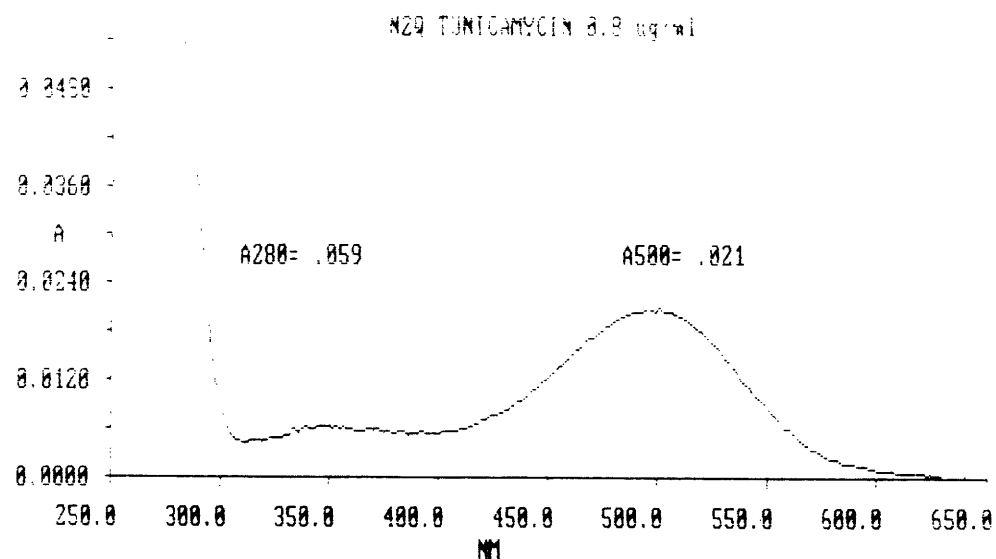
GTPγS exchange assay



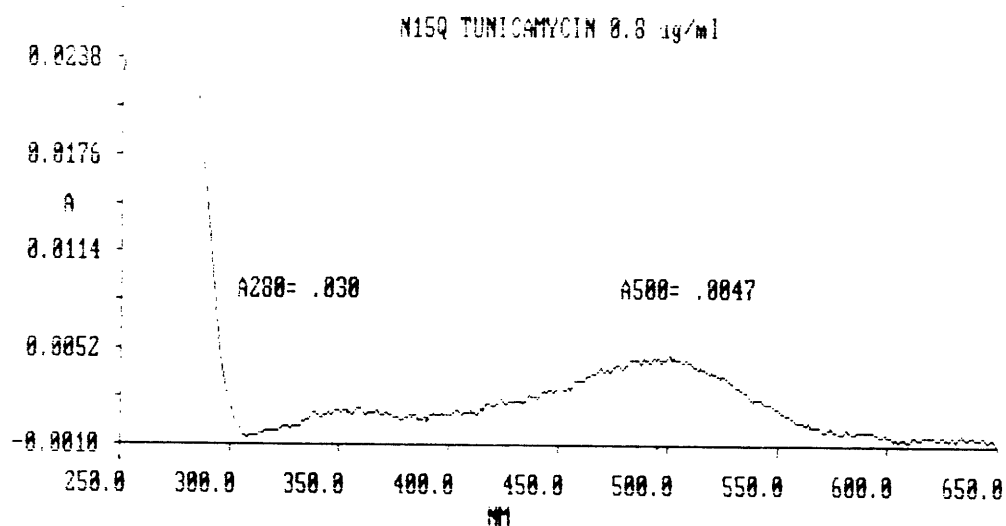
APPENDIX 5

EXPRESSION OF N2Q, N15Q AND N2,15Q IN THE PRESENCE OF TUNICAMYCIN

USER002; absc 650.0- 250.0; pts 401; int 1.00; ord -0.000-0.0592; A
 inf 20 16.36 92/03/08
 0.0600



USER003; absc 650.0- 250.0; pts 401; int 1.00; ord -0.004-0.0297; A
 inf 20 26.55 92/03/08
 0.0300



USER004. abs 550.0-250.0 pts 481. lot 1.00 and -0.001-0.0198. A

inf 20.41:16 32/03/08

0.0220

N2,15Q TUNICAMYCIN 0.2 ug/ml

0.0153

0.0116

A

0.0074

A280= .020

A500= .0026

0.0032

-0.0010

250.0

300.0

350.0

400.0

450.0

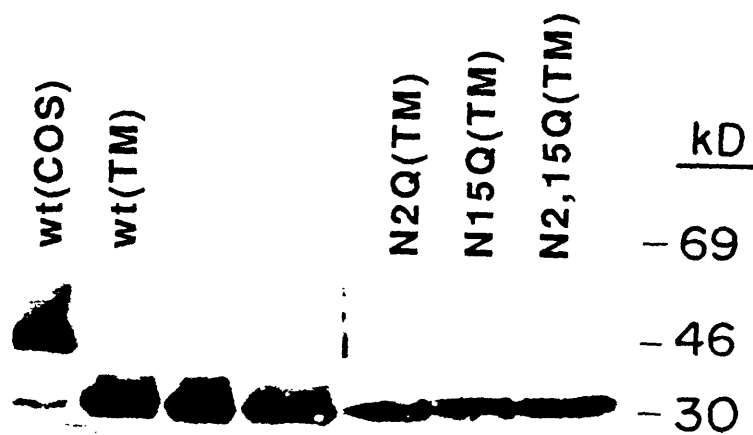
500.0

550.0

600.0

650.0

nm



Immunoblot of wild-type opsin and the glycosylation mutants expressed in the presence of tunicamycin.

APPENDIX 6

UV /VIS ABSORPTION SPECTRA OF ADRP MUTANTS

US22002; absc 650.0- 250.0; pts 401; int 1.00; ord -0.000-0.0656; 4
inf: 23:33:22 92/02/28

0.0900 -

WILD TYPE RHODOPSIN A280/A530= 1.65

0.0720 -

0.0540 -

A

0.0360 -

0.0180 -

0.0000 -

250.0 300.0 350.0 400.0 450.0 500.0 550.0 600.0 650.0
nm

X: USER005; absc 650.0- 250.0; pts 401; int 1.00; ord -0.000-0.0641; A
inf: 00:47:34 92/03/01

0.0700 -

0.0558 -

P23H

0.0416 -

A

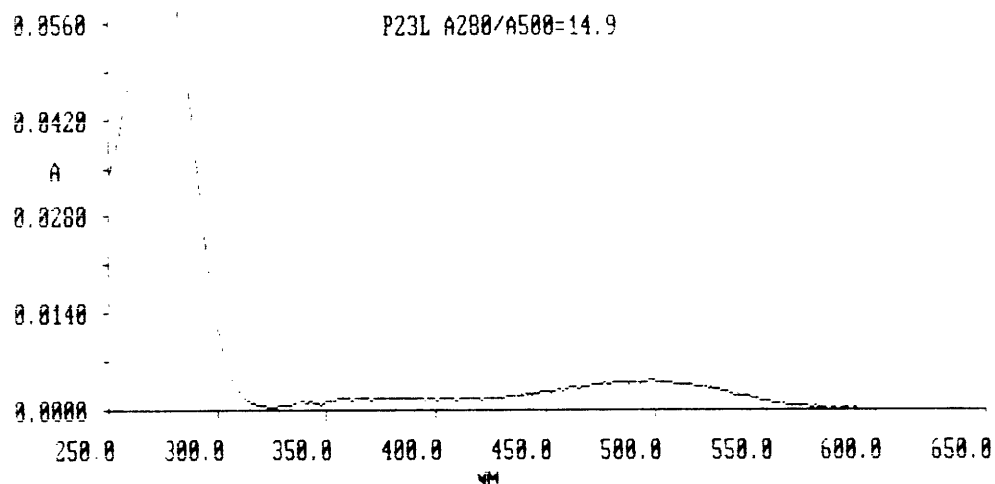
0.0274 -

0.0132 -

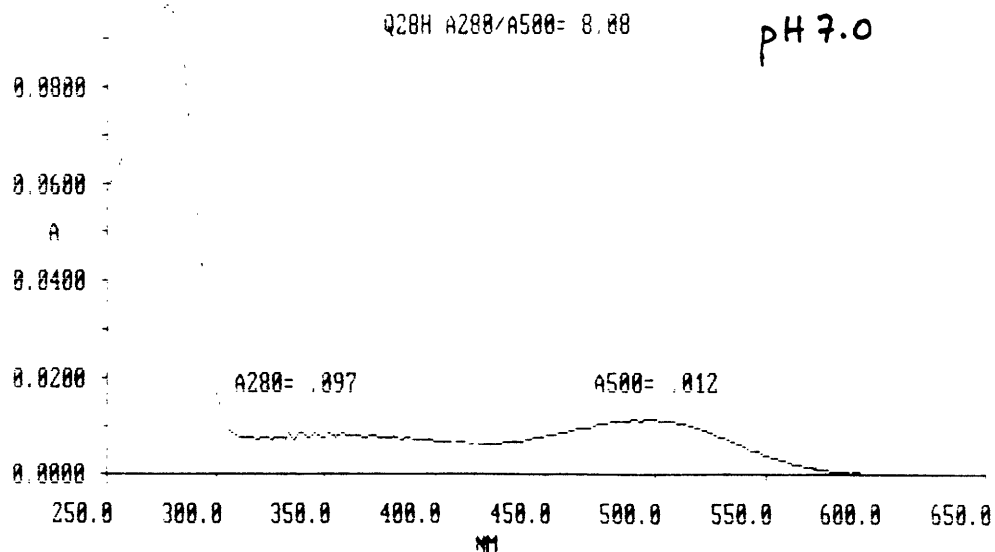
-0.0010 -

250.0 300.0 350.0 400.0 450.0 500.0 550.0 600.0 650.0
nm

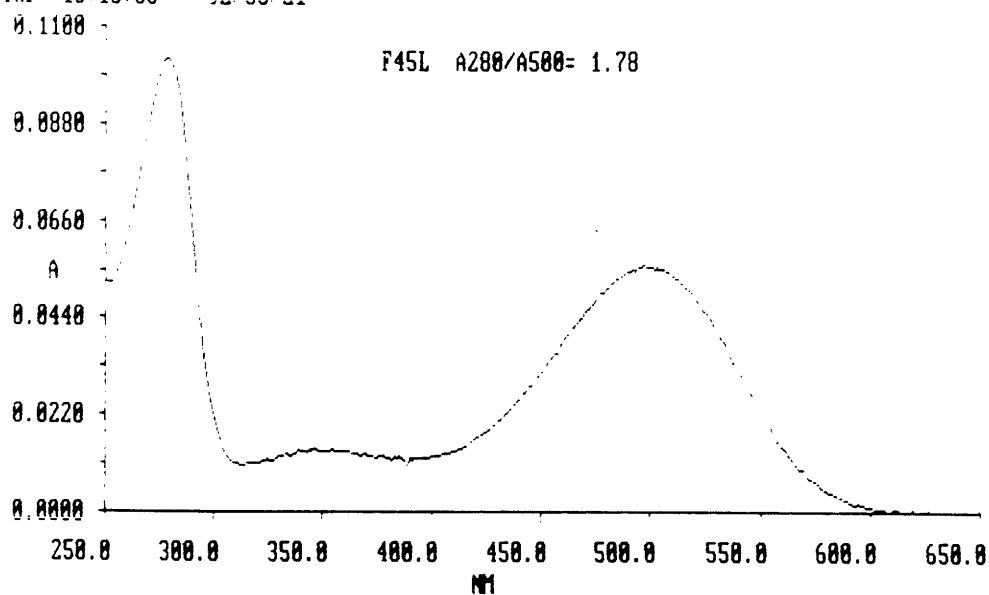
X: USER004; absc 650.0- 250.0; pts 401; int 1.00; ord -0.000-0.0628; A
 inf: 01:07:18 92/01/18
 0.0700 -



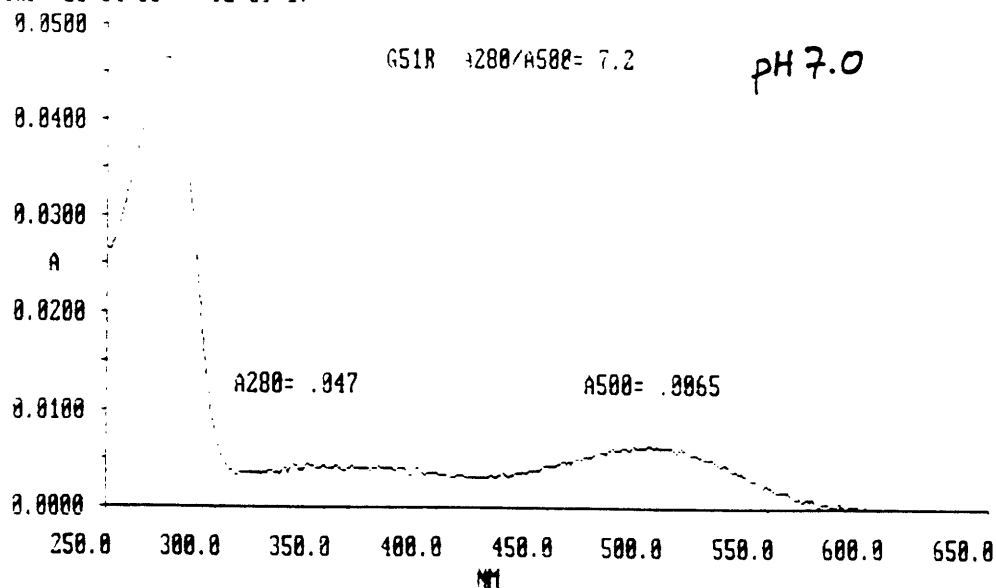
X: 0917110; absc 650.0- 250.0; pts 401; int 1.00; ord -0.001-0.0970; A
 inf: 09:10:53 92/09/17
 0.1000 -



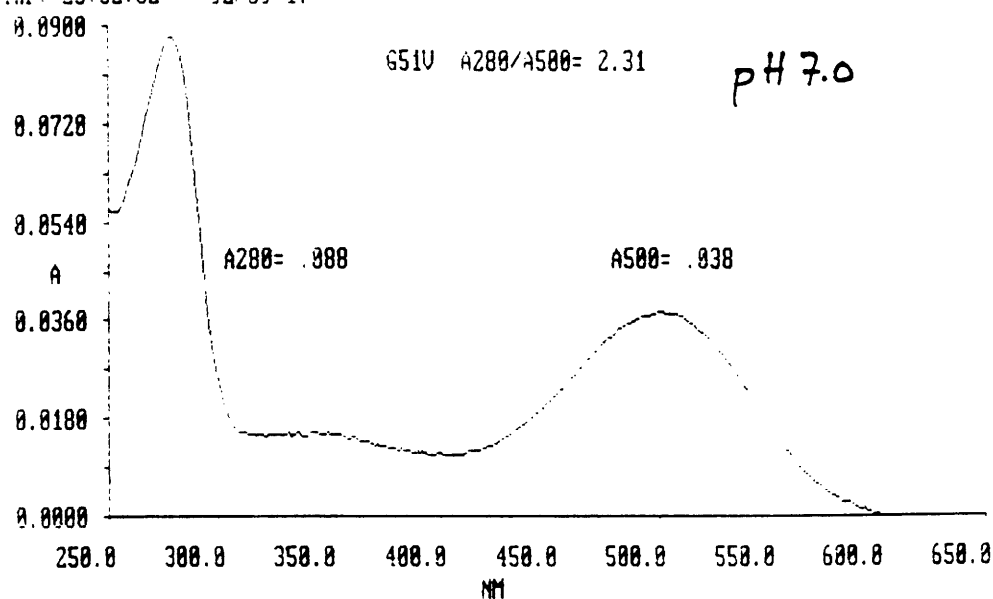
X: 852181; absc 650.0- 250.0; pts 401; int 1.00; ord 0.0000-0.1831; A
inf: 18:13:58 92/05/21



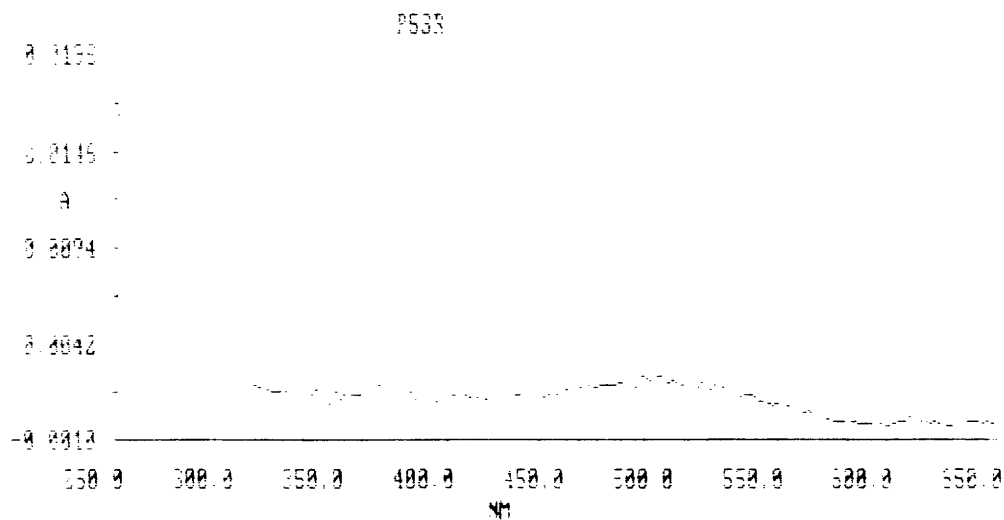
X: 8917119; absc 650.0- 250.0; pts 401; int 1.00; ord -0.000-0.0467; A
inf: 20:34:06 92/09/17



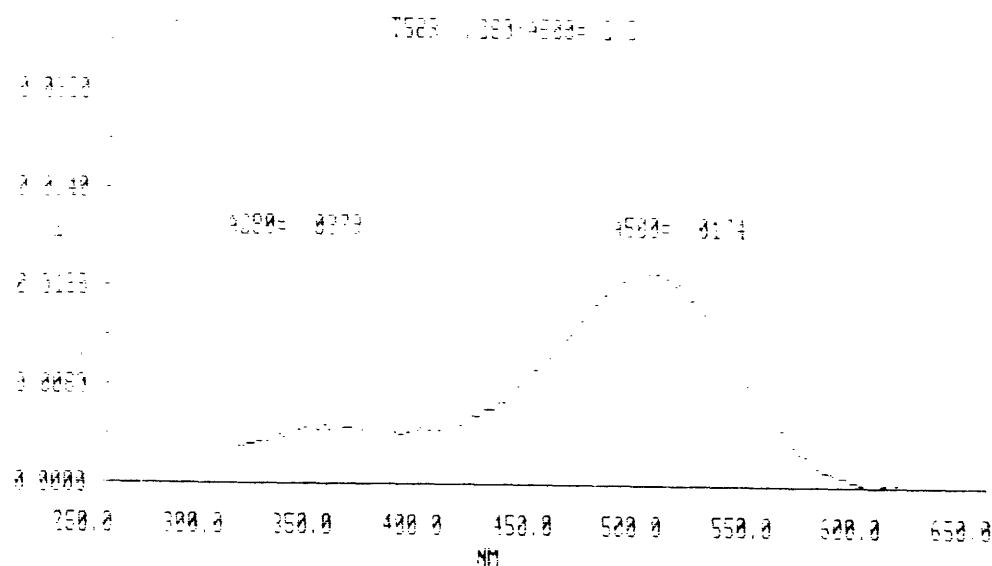
X: 8917128; absc 650.0- 250.0; pts 401; int 1.00; ord -0.006-0.0092; A
inf: 20:52:02 92/09/17



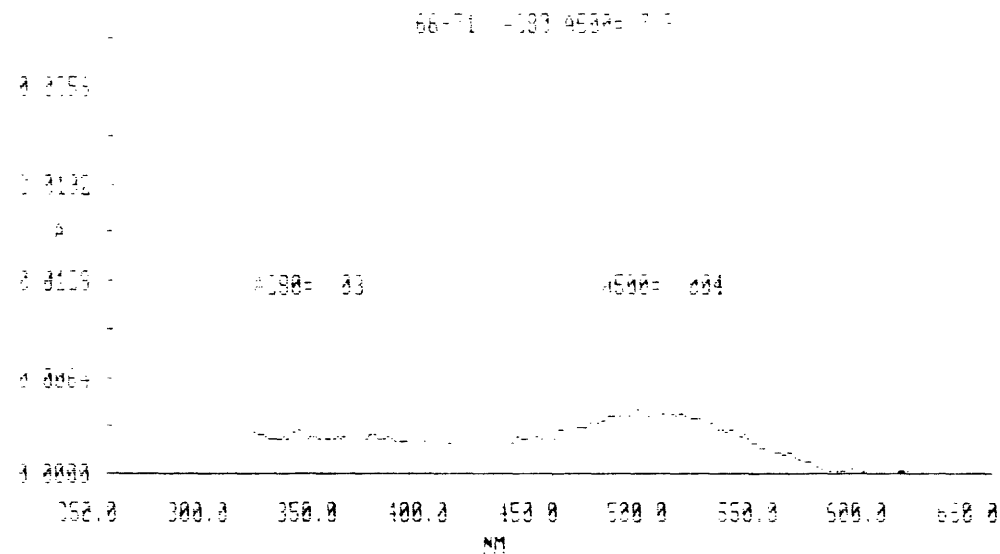
X: 888046; absc 650.0- 250.0; pts 411; int 1.00; ord -0.001-0.0117; A
inf: 00:54:19 92/02/06
0.0250



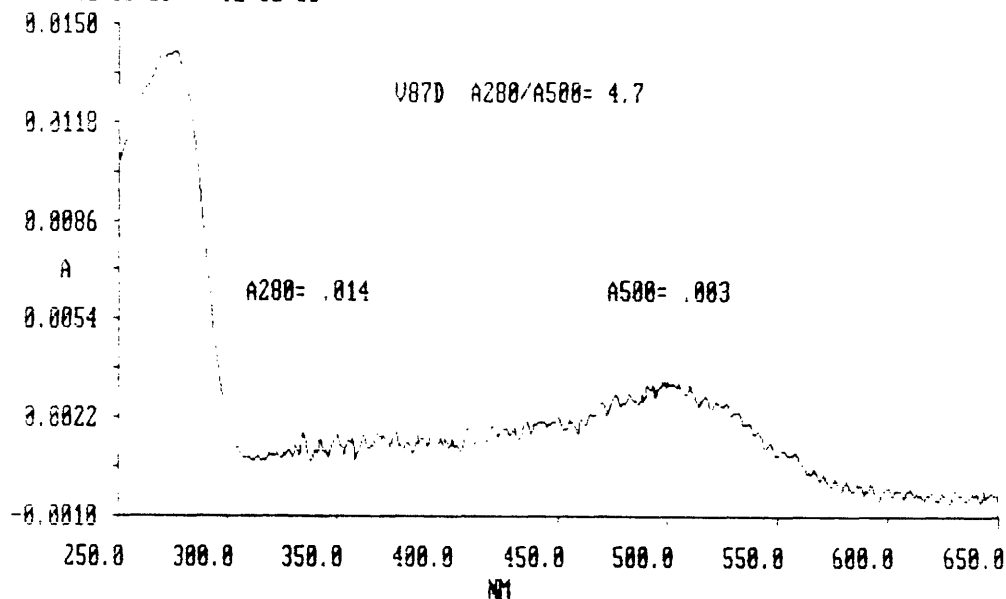
1. 0151447 Area: 148.0-158.0 gra 441 lbs 1.48 lbs to 148-1 4174
 2. 0151448 Area: 148.0-158.0 gra 441 lbs 1.48 lbs to 148-1 4174
 3. 0151449 Area: 148.0-158.0 gra 441 lbs 1.48 lbs to 148-1 4174



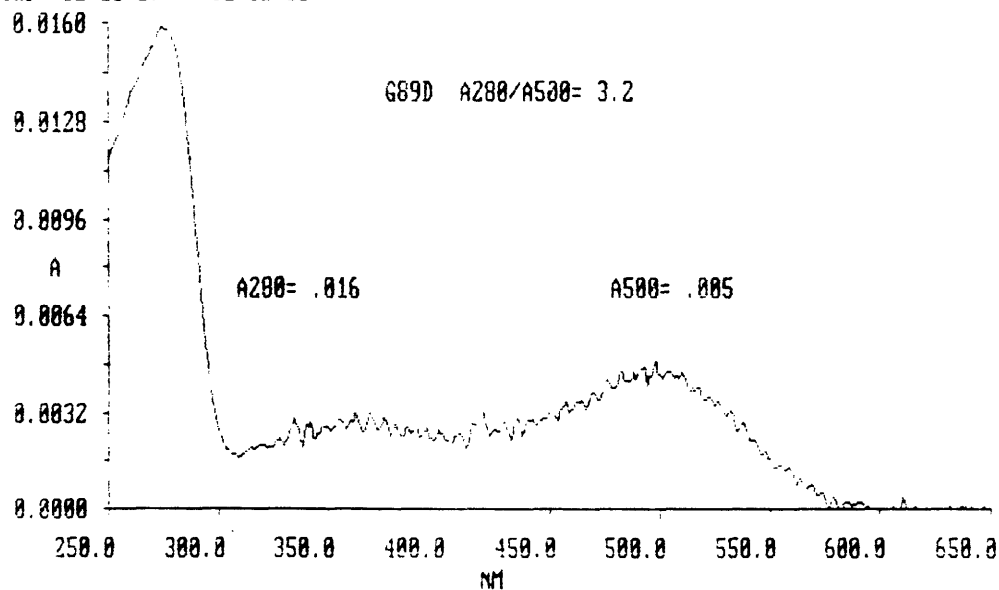
1. 0151450 Area: 148.0-158.0 gra 441 lbs 1.48 lbs to 148-1 4174
 2. 0151451 Area: 148.0-158.0 gra 441 lbs 1.48 lbs to 148-1 4174
 3. 0151452 Area: 148.0-158.0 gra 441 lbs 1.48 lbs to 148-1 4174



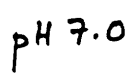
X: USER010; absc 650.0- 250.0; pts 401; int 1.00; ord -0.002-0.0141; A
inf: 01:53:20 92/02/06



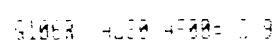
X: USER011; absc 650.0- 250.0; pts 401; int 1.00; ord -0.000-0.0159; A
inf: 02:13:27 92/02/06



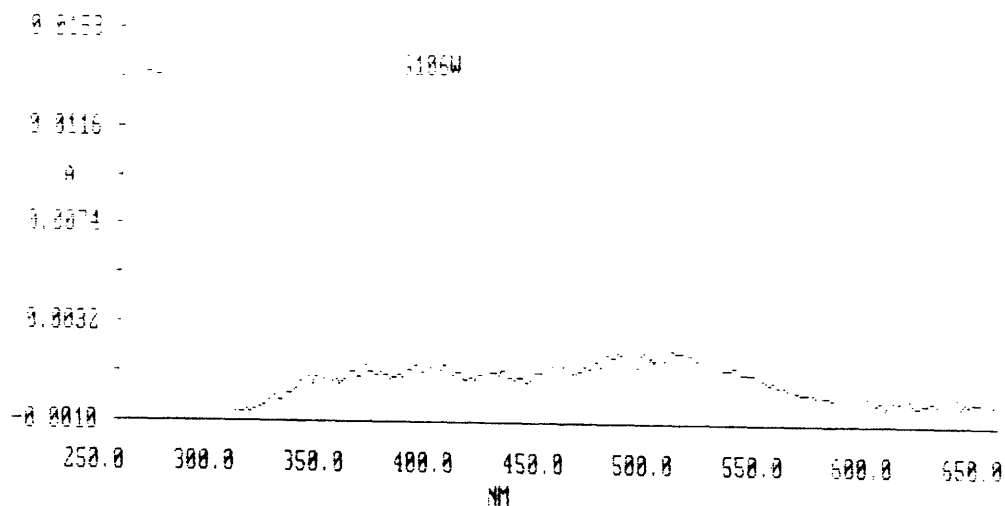
0.9550 -



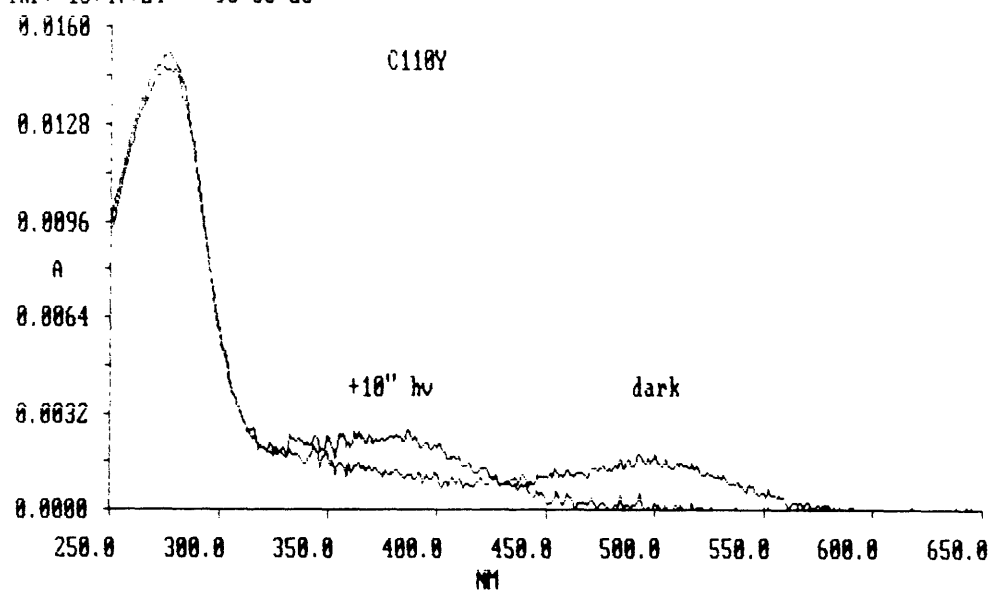
9,3453 -



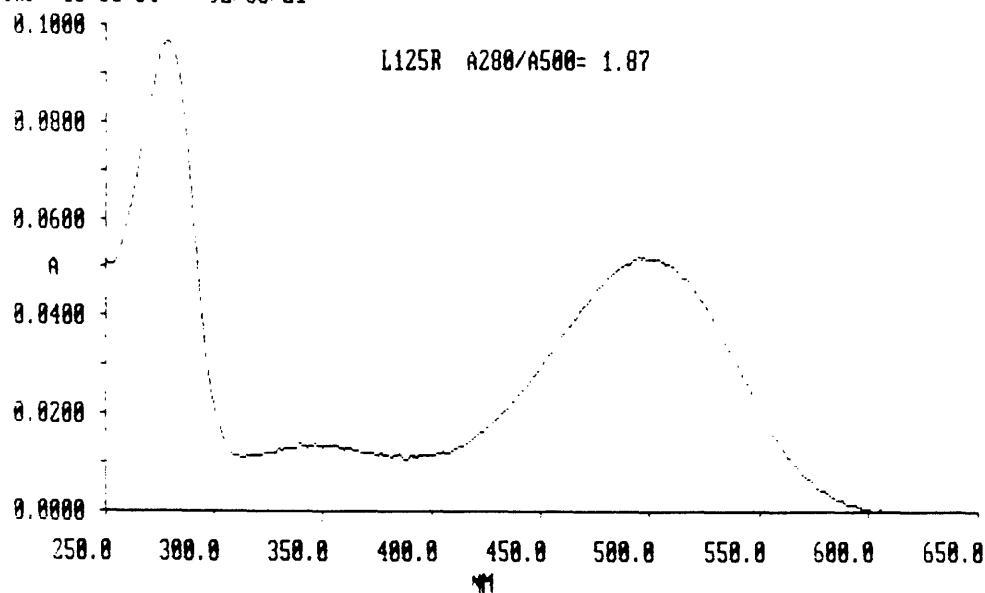
0297115; absc 650.0- 250.0; pts 401; int 1.00; ord -0.000-0.0140; A
inf: 01:03:35 93/02/04



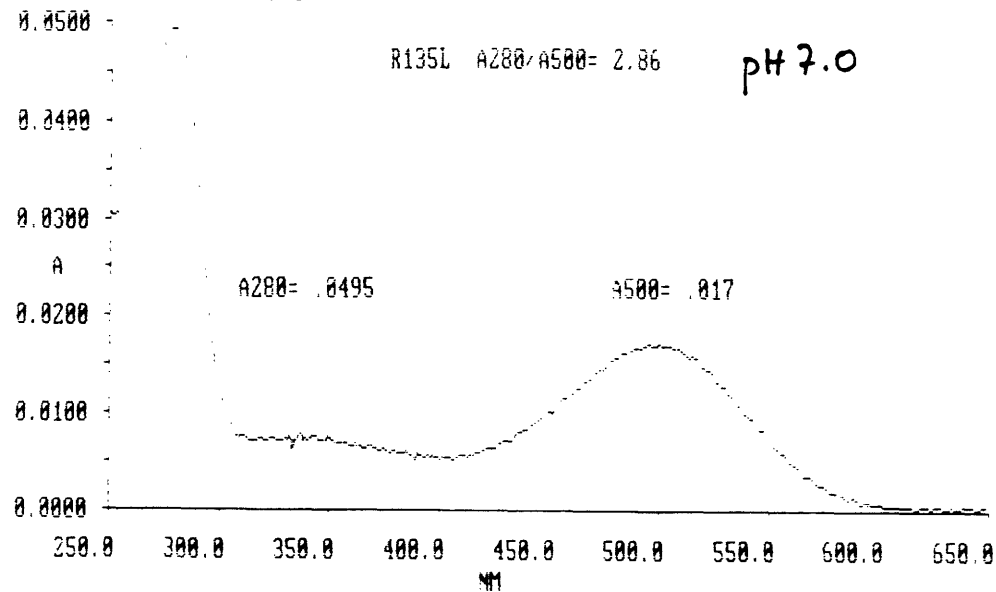
Y: USER037; absc 650.0- 250.0; pts 401; int 1.00; ord -0.001-0.0147; A
inf: 18:47:24 93/03/26



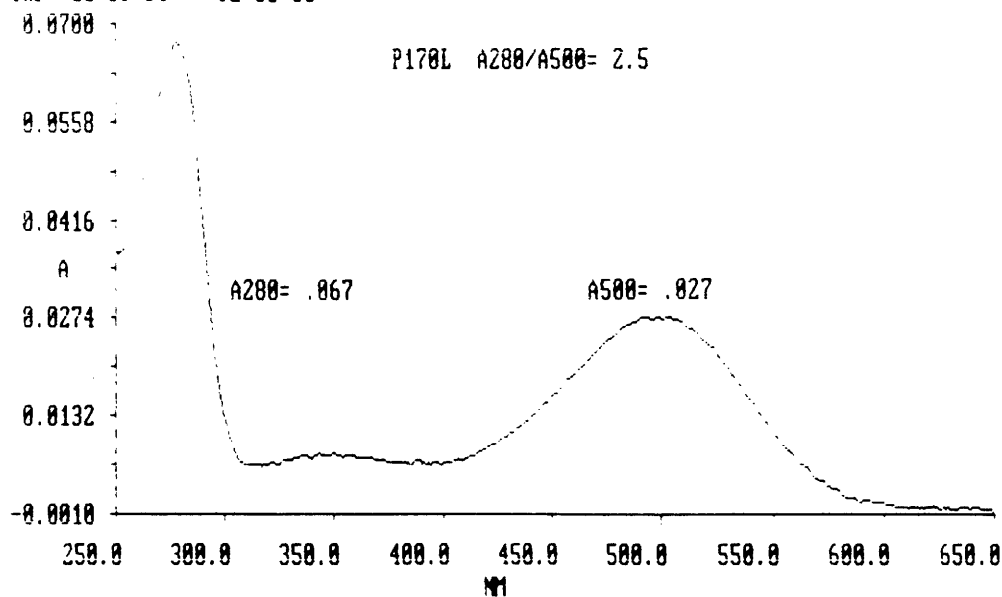
X: 052102; absc 650.0- 250.0; pts 401; int 1.00; ord -0.003-0.0969; A
 inf: 10:31:04 92/05/21



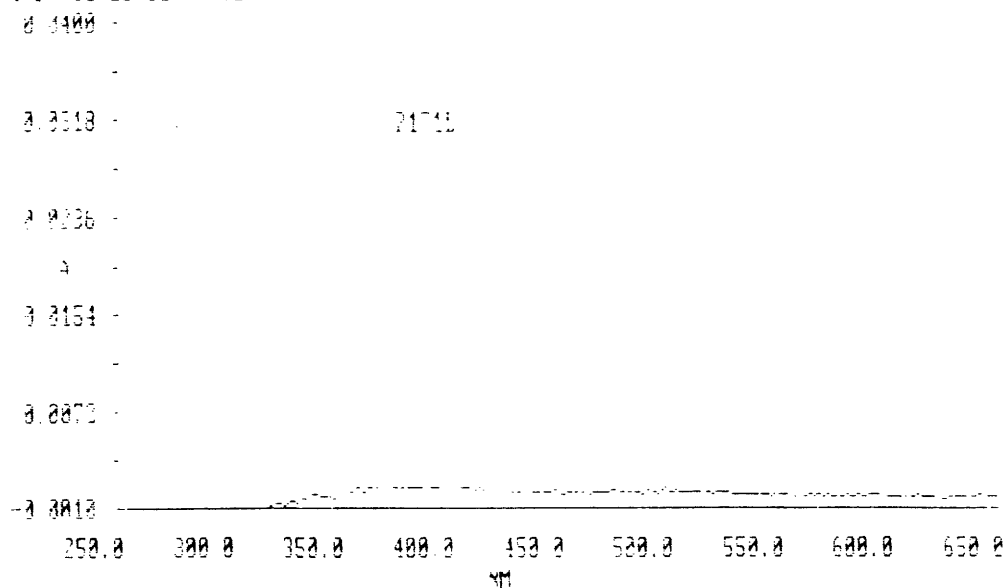
X: 0917127; absc 650.0- 250.0; pts 401; int 1.00; ord 0.0003-0.0495; A
 inf: 22:29:53 92/09/17



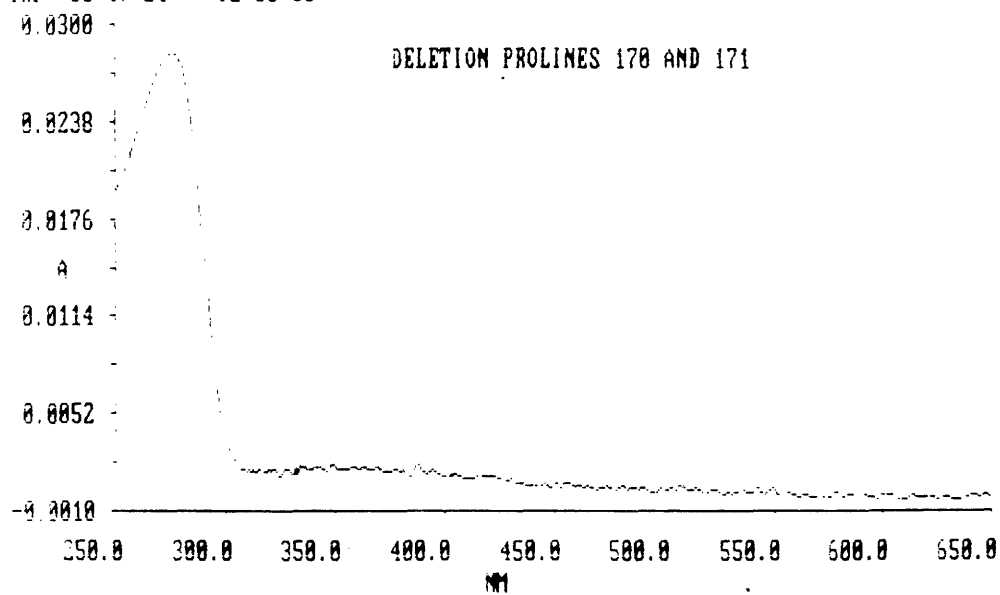
K: USER004; absc 650.0- 250.0; pts 401; int 1.00; ord -0.001-0.0675; A
inf: 11:59:54 92/06/05



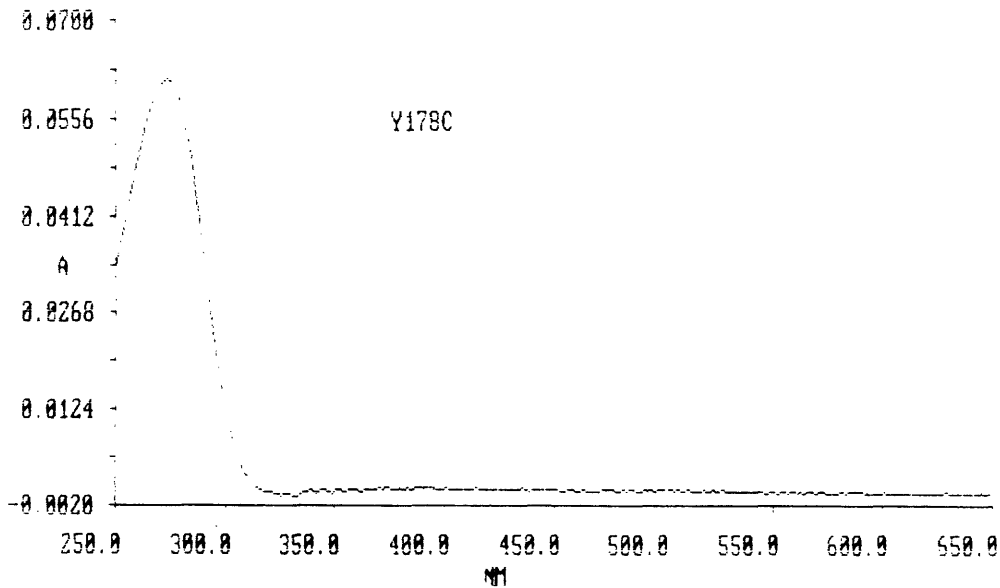
K: USER005; absc 650.0- 250.0; pts 401; int 1.00; ord -0.001-0.0313; A
inf: 01:13:01 92/01/18



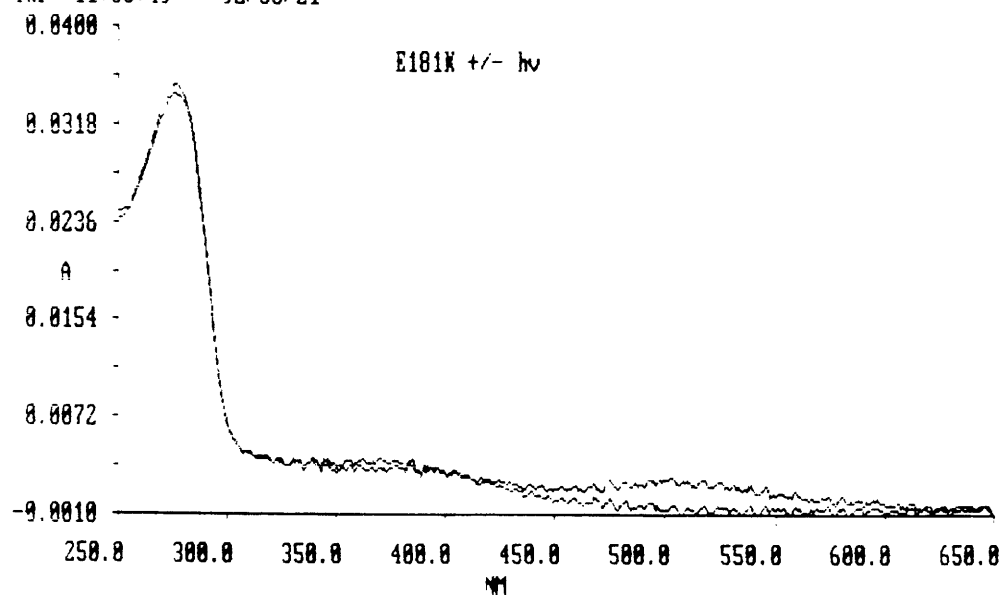
/: USER003; absc 650.0- 250.0; pts 401; int 1.00; ord -0.000-0.0283; A
 inf: 11:47:24 92/06/05



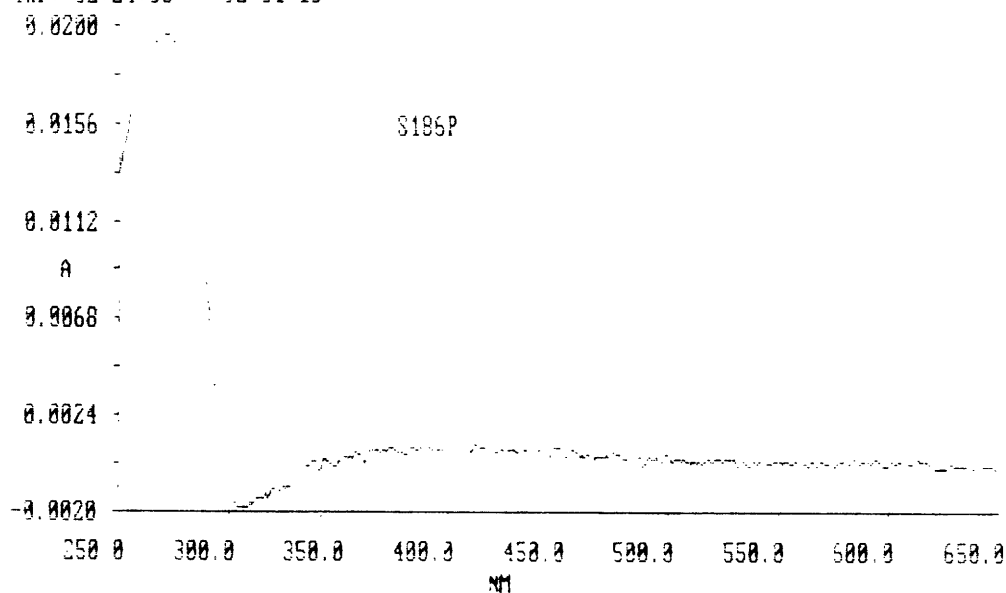
/: USER006; absc 650.0- 250.0; pts 401; int 1.00; ord -0.002-0.0615; A
 inf: 01:45:02 92/01/18



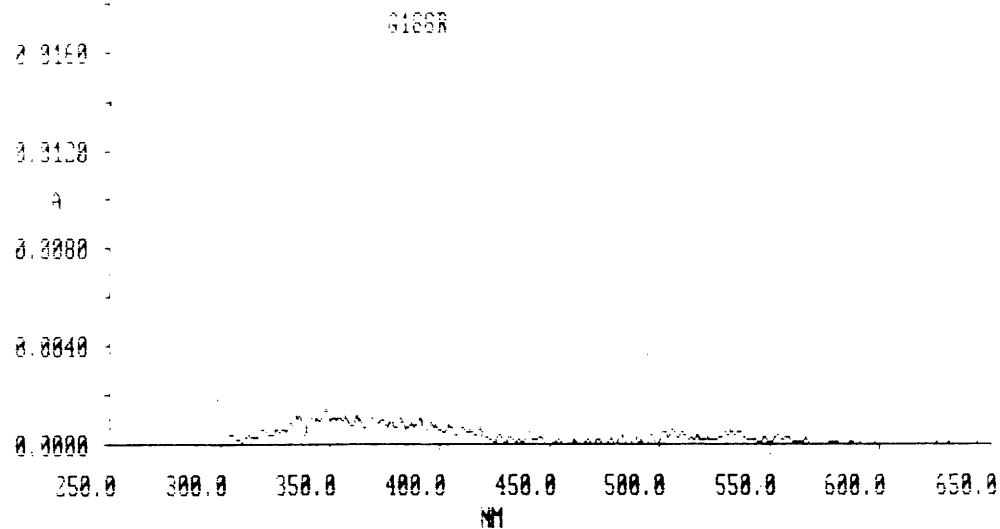
X: 052106; absc 650.0- 250.0; pts 401; int 1.00; ord -0.004-0.0345; A
inf: 11:08:49 92/05/21



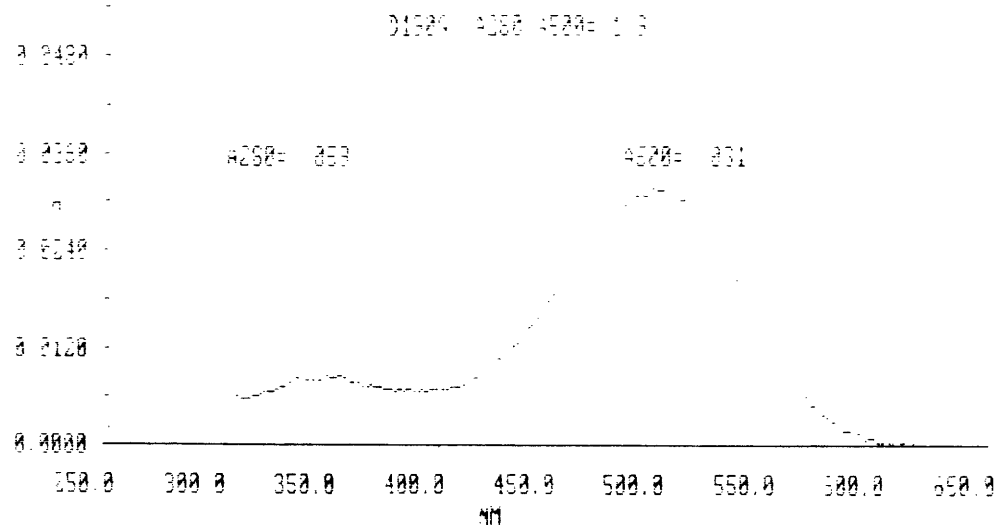
X: 052106; absc 650.0- 250.0; pts 401; int 1.00; ord -0.001-0.0197; A
inf: 02:24:06 92/01/18



0293116, abs 350.0- 250.0; pts 401; int 1.00, ord -0.002-0.0192
 inf 01 14 21 32/02/04
 0.0200 -



0293117, abs 650.0- 250.0; pts 401; int 1.00, ord -0.001-0.0553
 inf 01 10 24 32/02/04
 0.0600 -



$$f^* = f^*_{\text{max}} = f^*_{\text{min}} = f^*_{\text{avg}}$$

2 3470 -

9

3.3238 -

4. 2000 —

250.0 300.0 350.0 400.0 450.0 500.0 550.0 600.0 650.0

0.0600

8.9480

8.0368

A

0.0240

0.0120

2,0000

250.0 300.0 350.0 400.0 450.0 500.0 550.0 600.0 650.0

4298117 350.0- 650.0 pts 401.0 1.00 1.01 4.001-0.010
 32 31 34 32 31 34
 0.0110

42117

0.0031

0.0010

0.0040

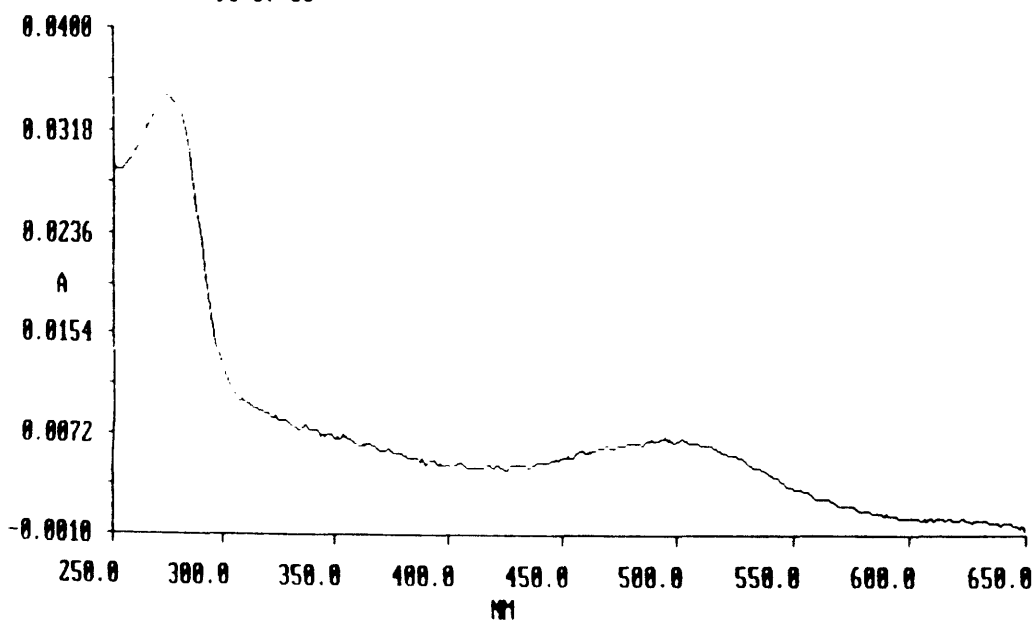
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0.0010

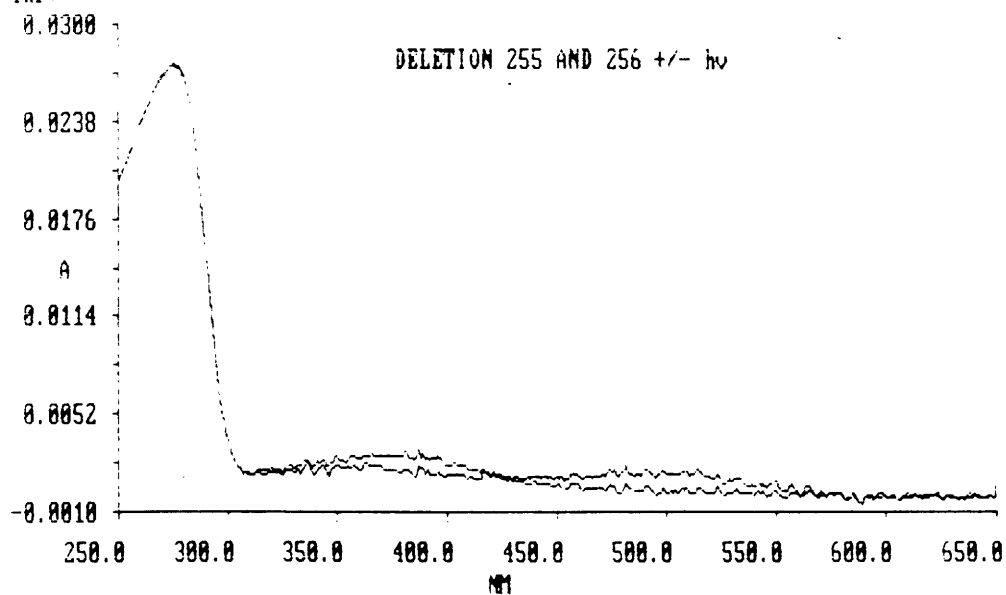
250.0 300.0 350.0 400.0 450.0 500.0 550.0 600.0 650.0
 nm

1256DEL

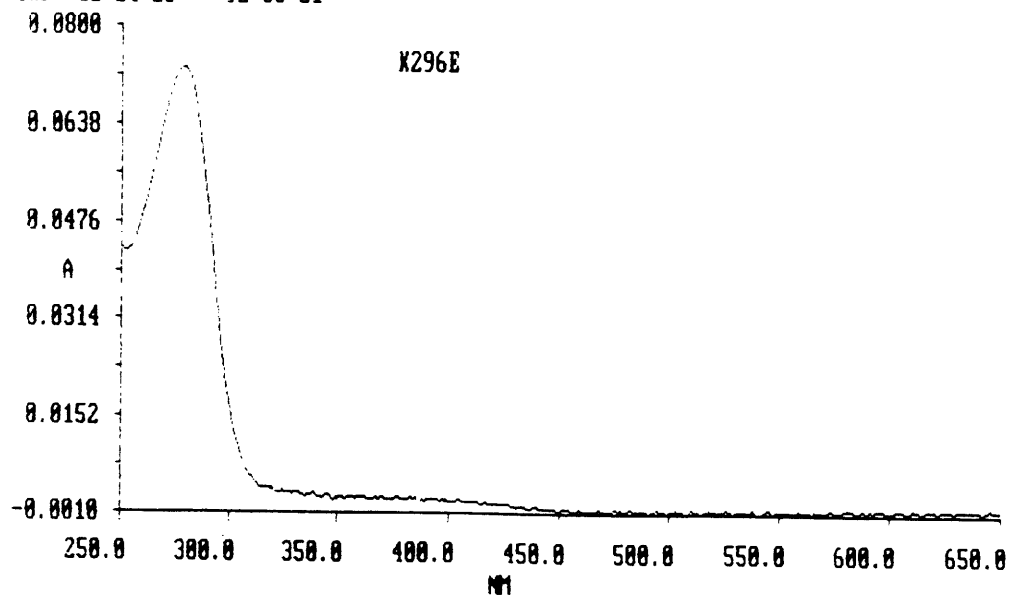
91/07/08



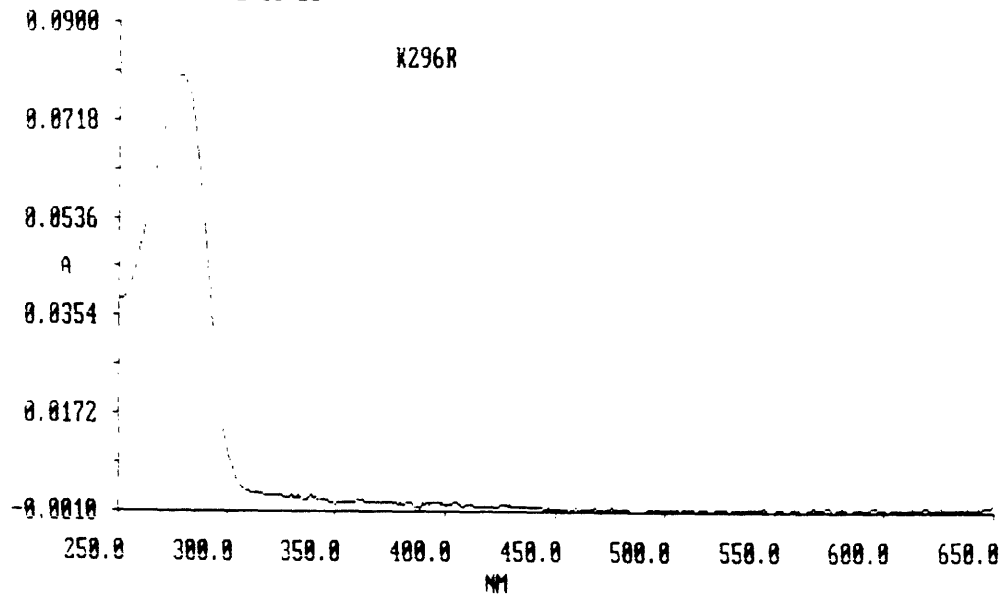
Z: 060501; absc 650.0- 250.0; pts 401; int 1.00; ord -0.000-0.0275; A
inf:



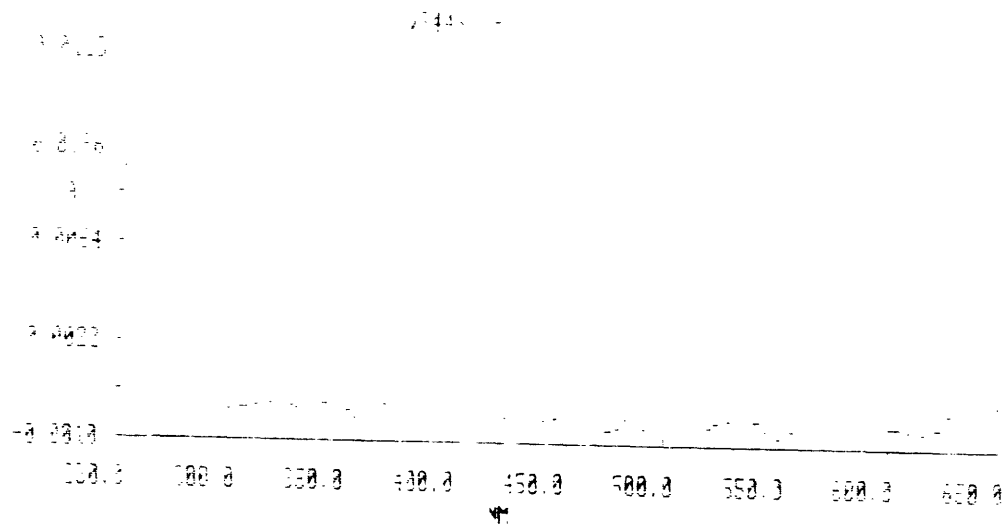
X: 052112; absc 650.0- 250.0; pts 401; int 1.00; ord -0.001-0.0734; A
inf: 12:24:26 92/05/21



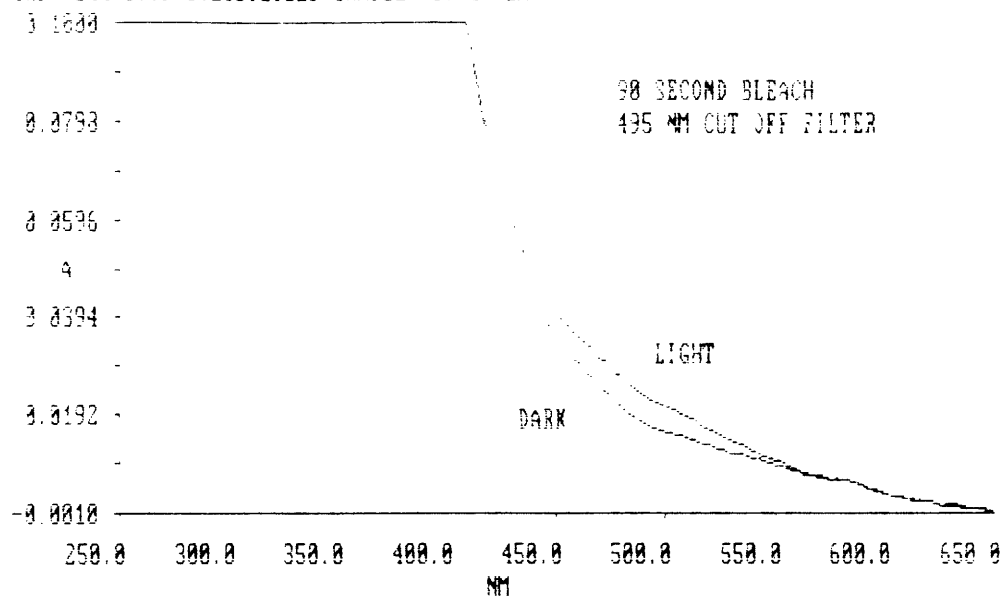
X: 052109; absc 650.0- 250.0; pts 401; int 1.00; ord -0.001-0.0002; A
inf: 11:42:11 92/05/21



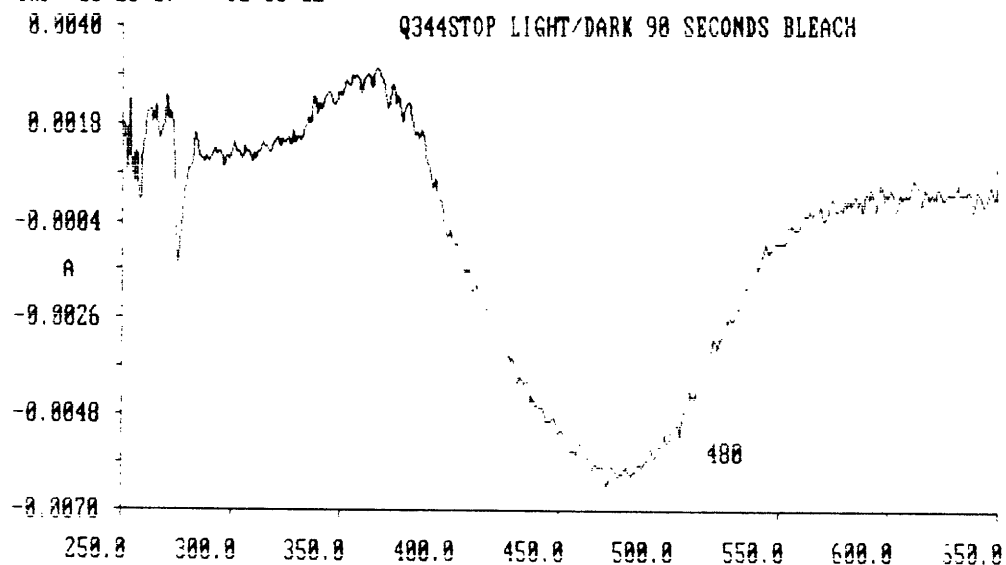
052109; absc 650.0- 250.0; pts 401; int 1.00; ord -0.001-0.0002; A
inf: 11:42:11 92/05/21



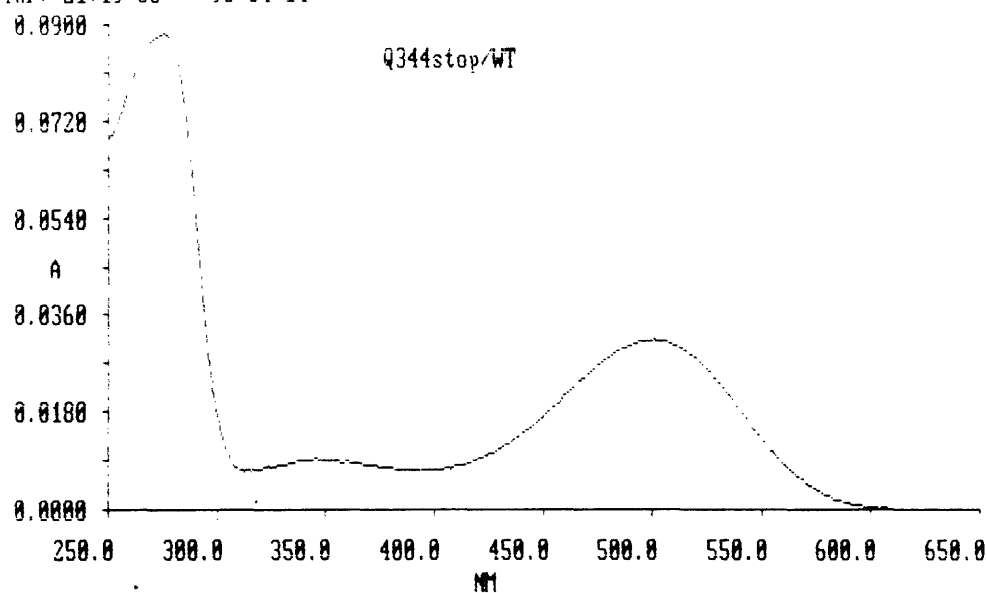
Q31112; absc 650.0- 250.0; pts 401; int 1.00; ord -0.002-1.0345; A
 inf: 344 STOP SOLUBILIZED SAMPLE VS. LM



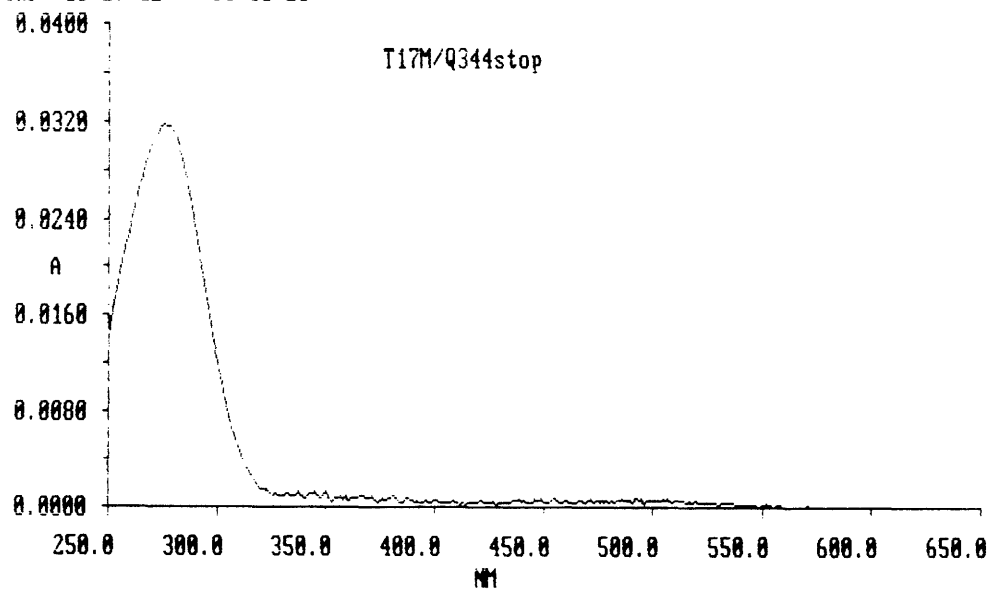
Z: USER003; absc 650.0- 250.0; pts 401; int 1.00; ord -0.006-0.0030; A
 inf: 16:25:17 92/03/12



X: USER012; absc 650.0- 250.0; pts 401; int 1.00; ord -0.001-0.0003; A
inf: 21:19:50 93/04/14



X: USER035; absc 650.0- 250.0; pts 401; int 1.00; ord -0.002-0.0318; A
inf: 18:27:12 93/03/26



1987-1988 650 0-250 01 pts 401. nat 1.000 d:1 0.0001-0.0095. 4
 12 03.00/43 02/02/11
 0.0150 -

0.0150 0.0150 0.0150 1.00

0.0150 -

0.0150 - 0.0150 0.0150 0.0150 0.0150

0.0150 -

0.0150

0.0150

250.0 300.0 350.0 400.0 450.0 500.0 550.0 600.0 650.0
 1987

1987-1988 650 0-250 01 pts 401. nat 1.000 d:1 0.0001-0.0095. 4
 12 03.00/43 02/02/11
 0.0150 -

0.0150

0.0150 -

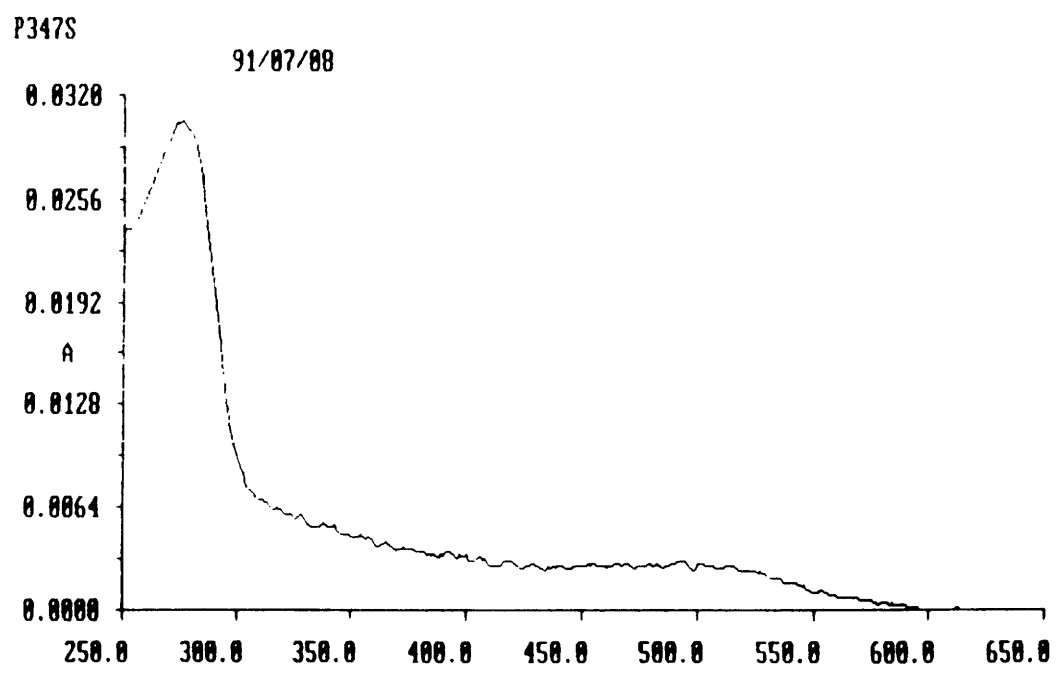
0.0150 -

0.0150 -

0.0150 -

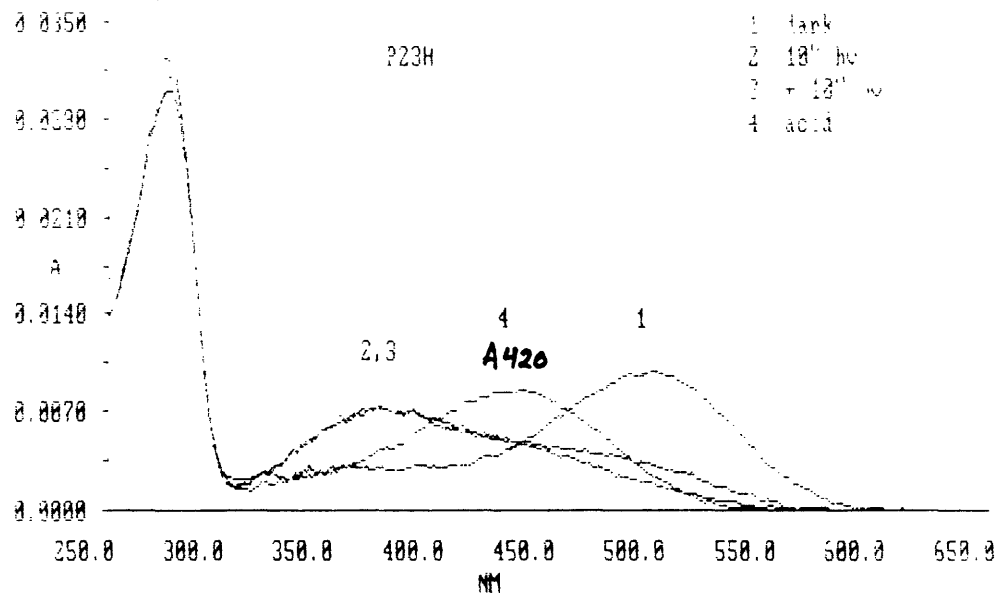
0.0150

250.0 300.0 350.0 400.0 450.0 500.0 550.0 600.0 650.0
 1987

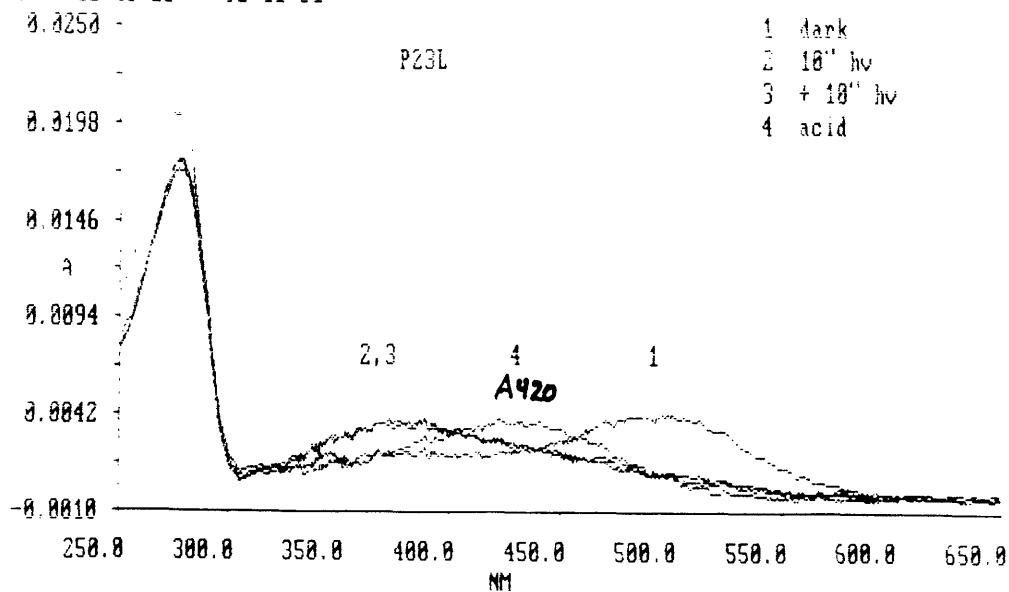


APPENDIX 7
BLEACHING SPECTRA OF ADRP MUTANTS

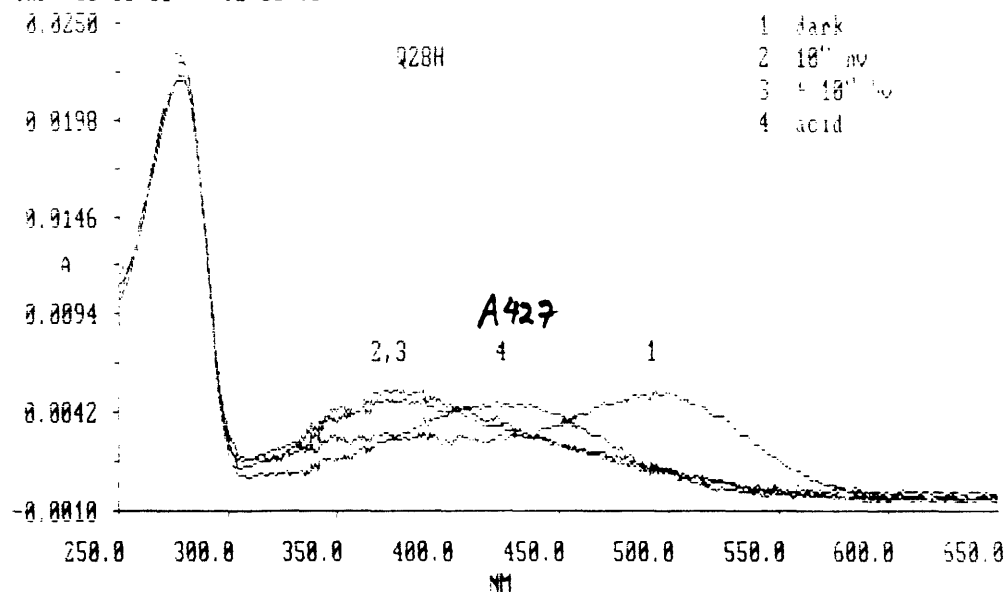
1: 1101004; absc 650.0- 250.0; pts 401; int 1.00; ord -0.000-0.0350; A
inf 11:47:13 92/11/01



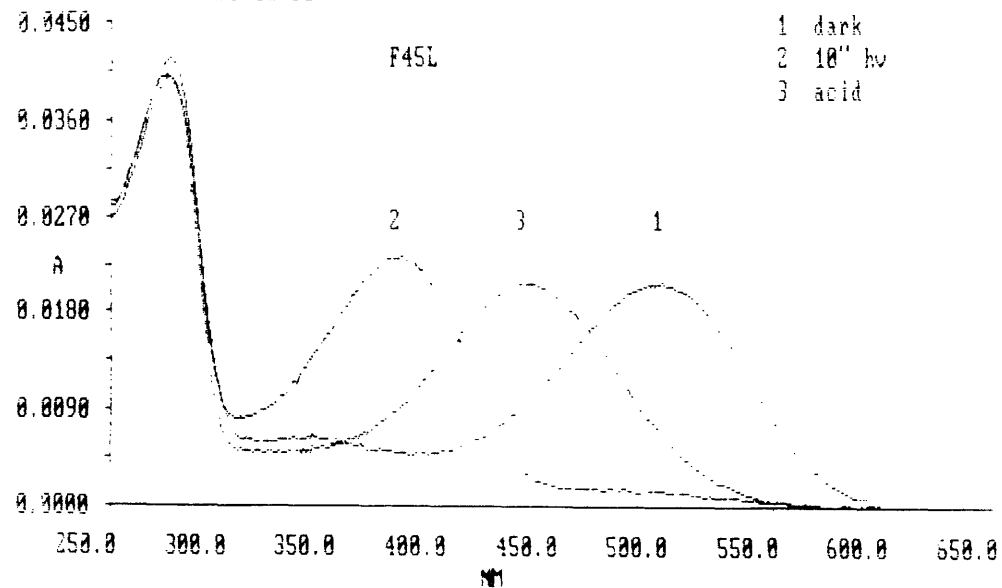
2: 1101010; absc 650.0- 250.0; pts 401; int 1.00; ord -0.001-0.0170; A
inf 12:45:28 92/11/01



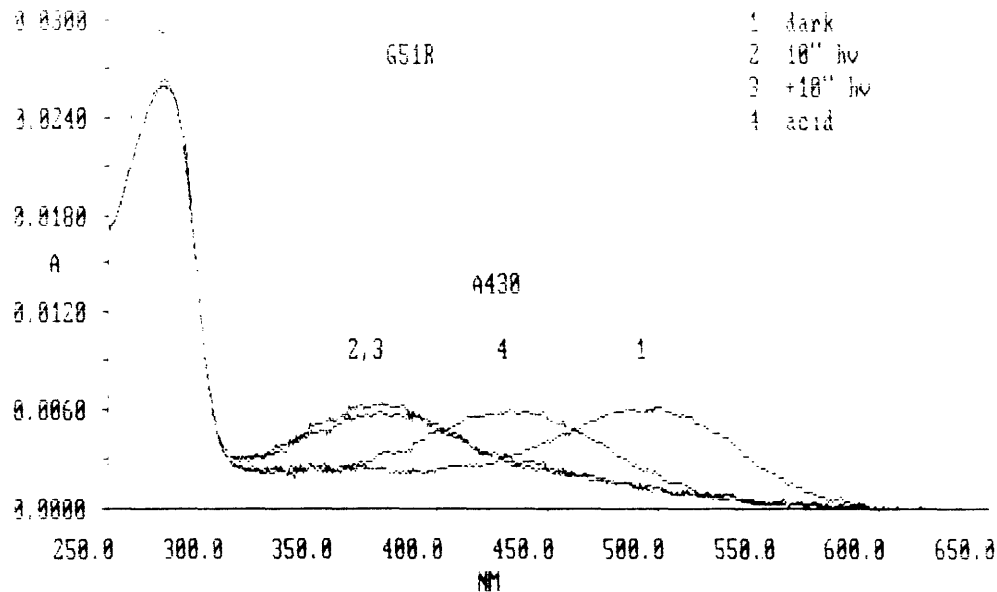
X: 1101018; absc 650.0- 250.0; pts 401; int 1.00; ord -0.000-0.0250; A
 inf 13:36:05 92/11/01



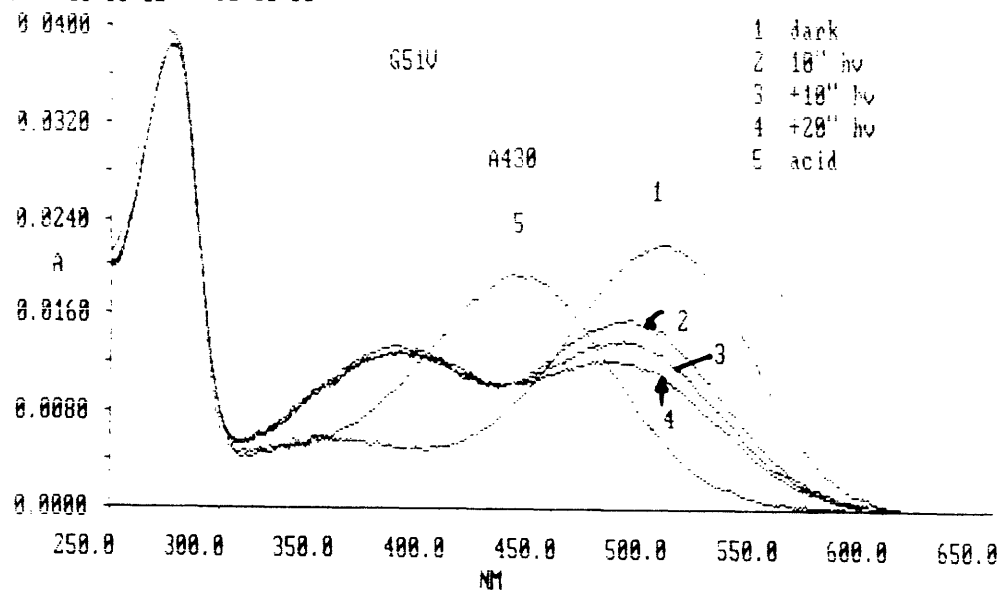
Z: 1101067; absc 650.0- 250.0; pts 401; int 1.00; ord -0.001-0.0450; A
 inf 18:14:20 92/11/01



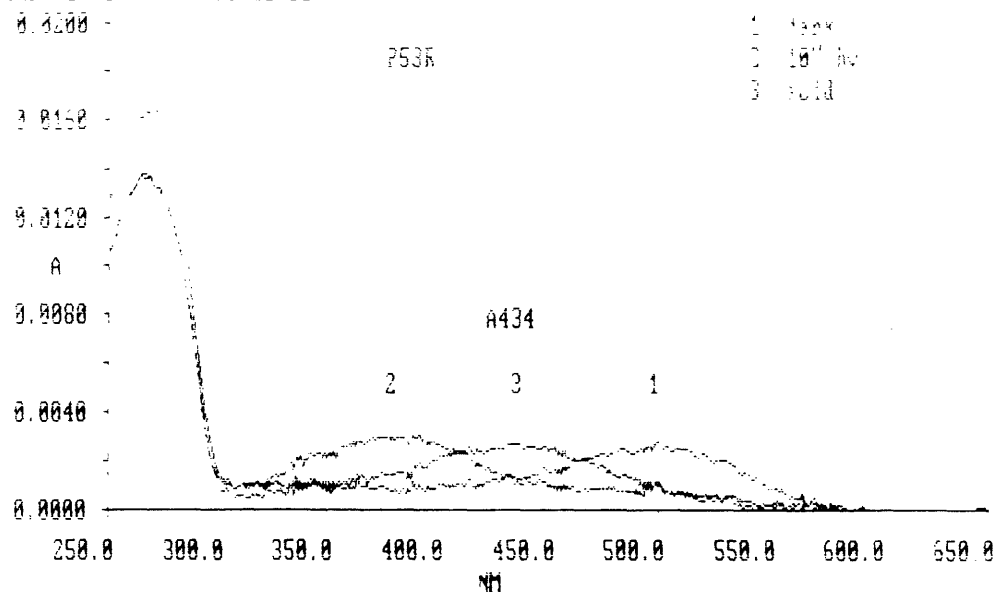
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 inf: 17:36:35 92/11/01
 0.0300 -



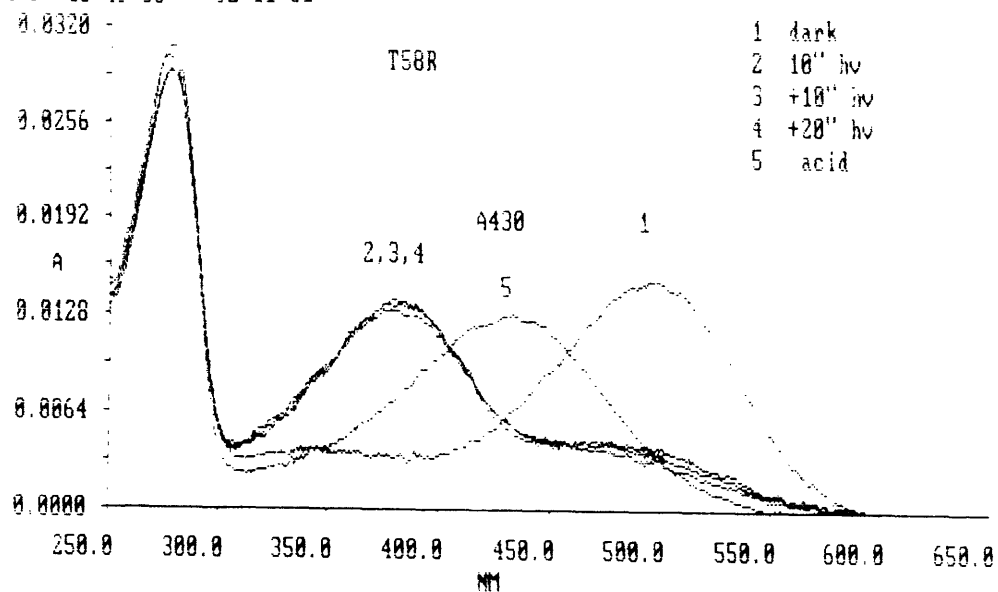
X: 1101064: absc 650.0- 250.0: pts 401: int 1.00: ord -0.000-0.0303: A
 inf: 18:00:12 92/11/01



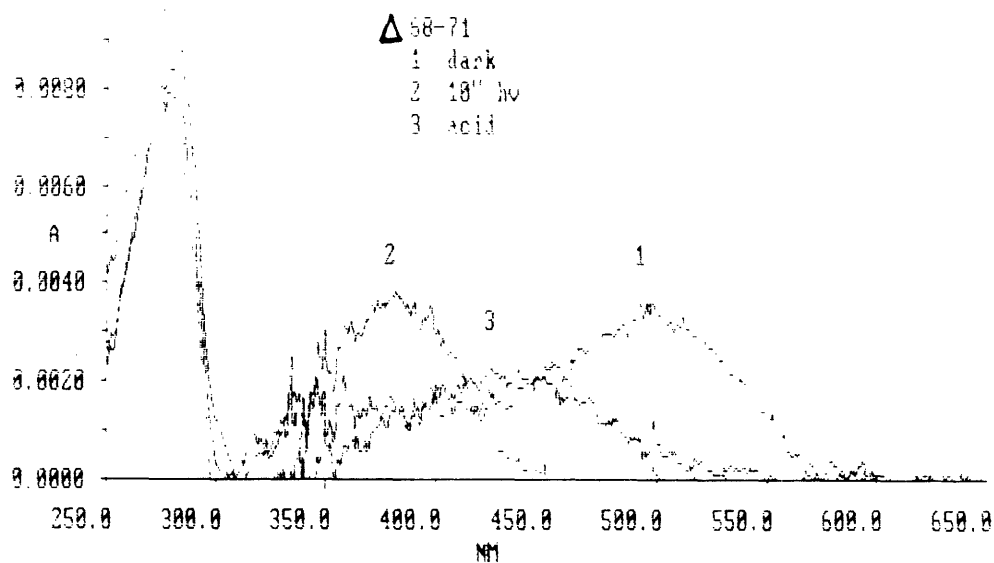
X: 1101052; absc 650.0- 250.0; pts 401; int 1.00; ord -0.000-0.0164 A
 inf: 17:15:04 92/11/01



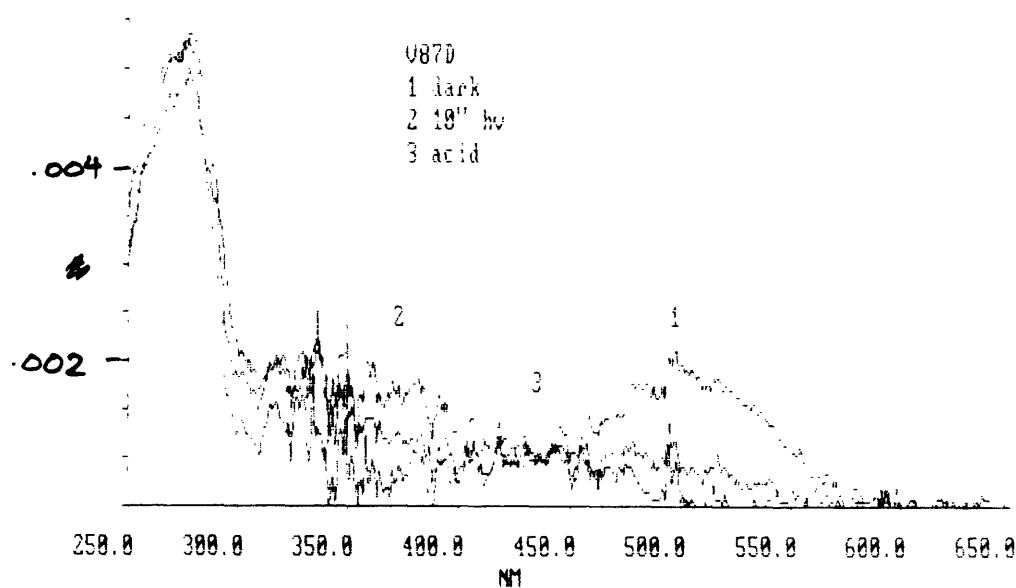
X: 1101045; absc 650.0- 250.0; pts 401; int 1.00; ord -0.000-0.0290: A
 inf: 16:49:08 92/11/01



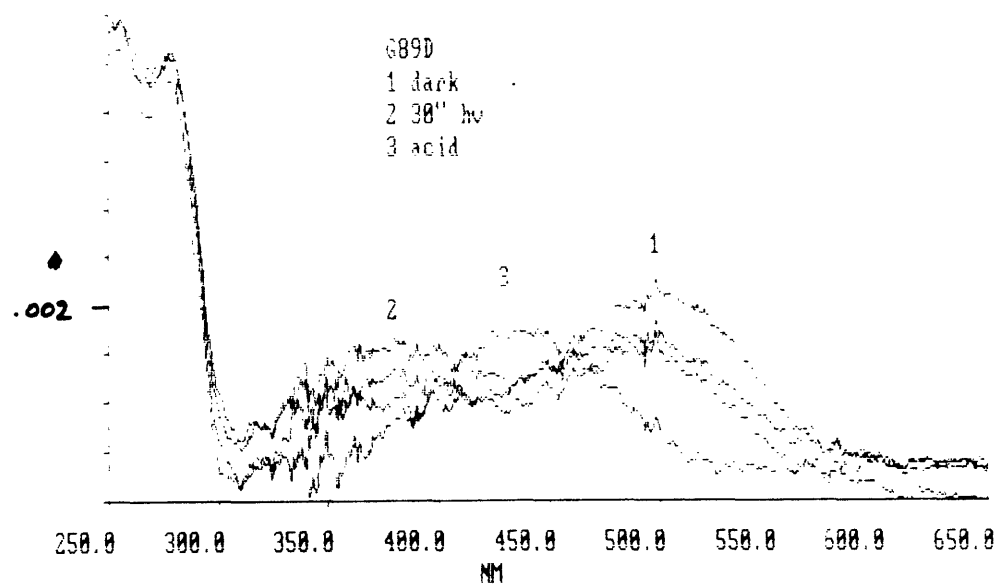
K: 1029011; absc 650.0- 250.0; pts 401; int 1.00; ord -0.001-0.0079; A
 inf: 23:00:42 92/10/29
 0.0100 -



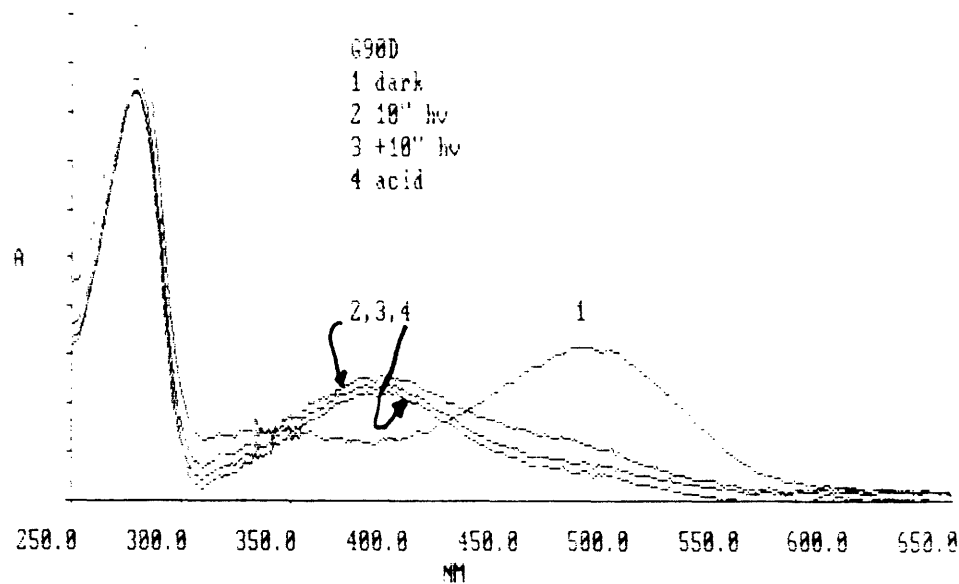
K: 1029015; absc 650.0- 250.0; pts 401; int 1.00; ord -0.001-0.0050; A
 inf: 23:24:29 92/10/29



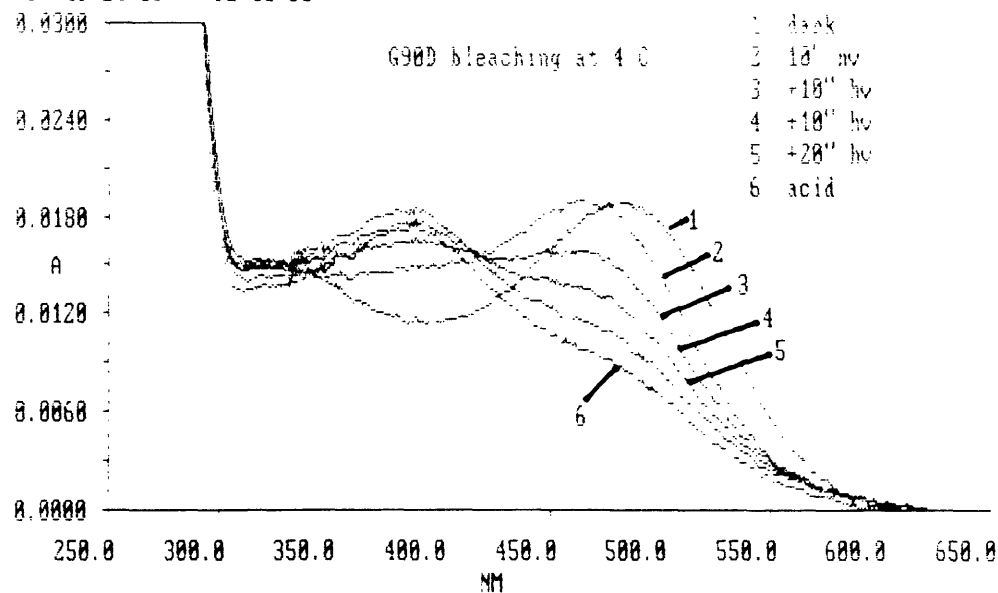
K: 1029030; absc 550.0- 250.0; pts 401; int 1.00; ord -0.001-0.0119; 4
 inf 01:05:52 92/10/30



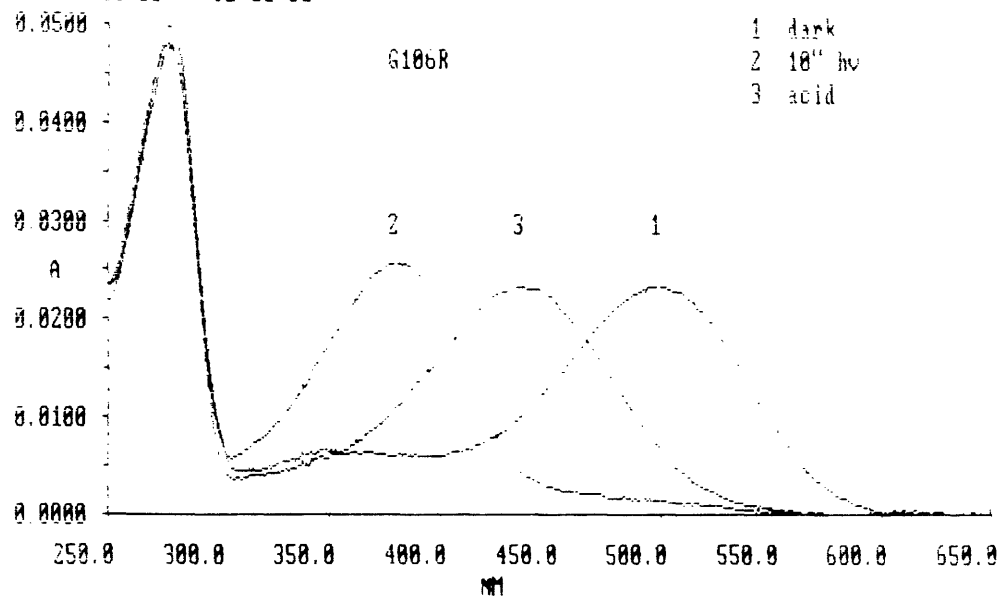
K: 1029019; absc 550.0- 250.0; pts 401; int 1.00; ord -0.001-0.0421; 4
 inf 23:52:33 92/10/29



4: 1101074; absc 550.0- 250.0; pts 401; int 1.00; ord -0.000-0.0535
inf: 19:24:10 92/11/01



5: 1101027; absc 650.0- 250.0; pts 401; int 1.00; ord -0.001-0.0470; A
inf: 14:15:38 92/11/01



1. 1101833; abs 650.0- 250.0; pts 401; int 1.00; ord -0.001-0.0120; A

inf 14 35-05 92/11/01

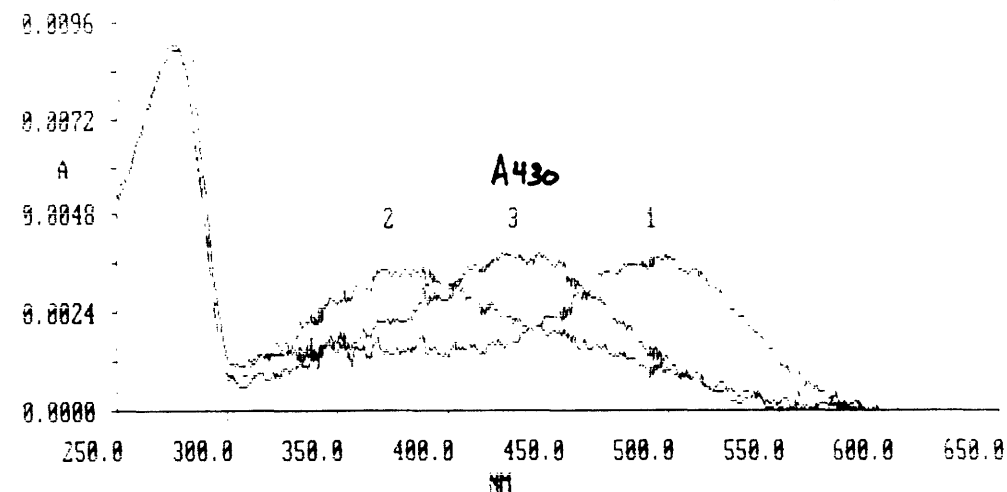
0.0120 -

G106W

1 dark

2 10" hv

3 acid



2. 1029021; abs 650.0- 250.0; pts 401; int 1.00; ord -0.001-0.0367; A

inf 00:12:20 92/10/30

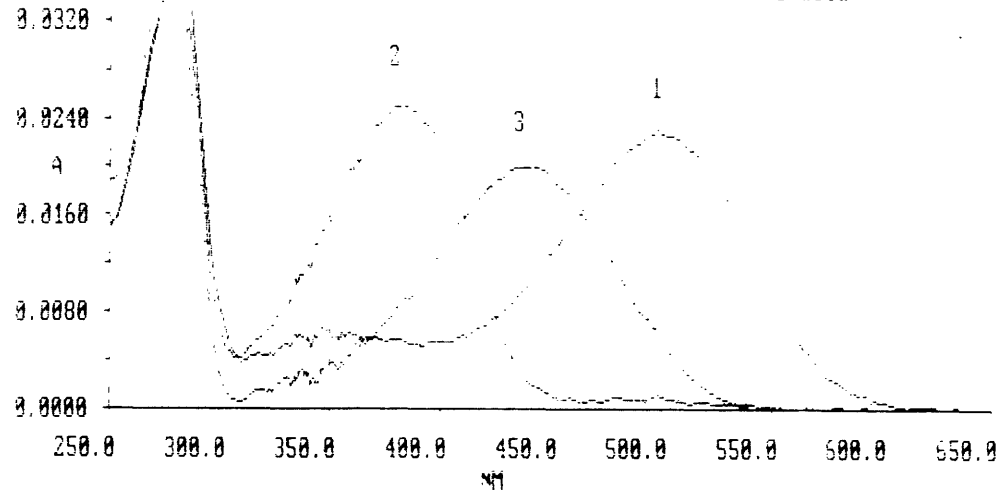
0.0400 -

R135L

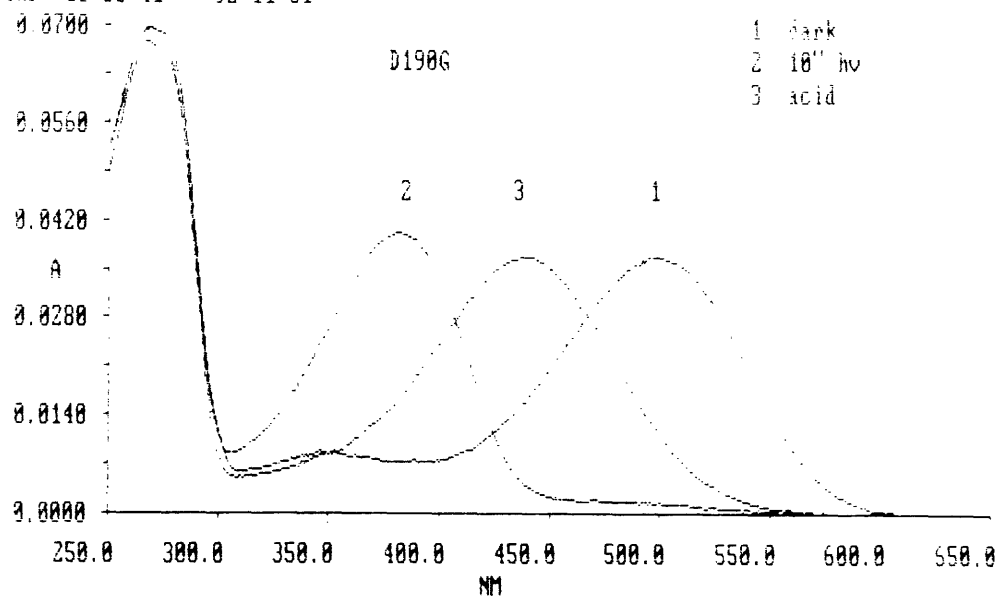
1 dark

2 10" hv

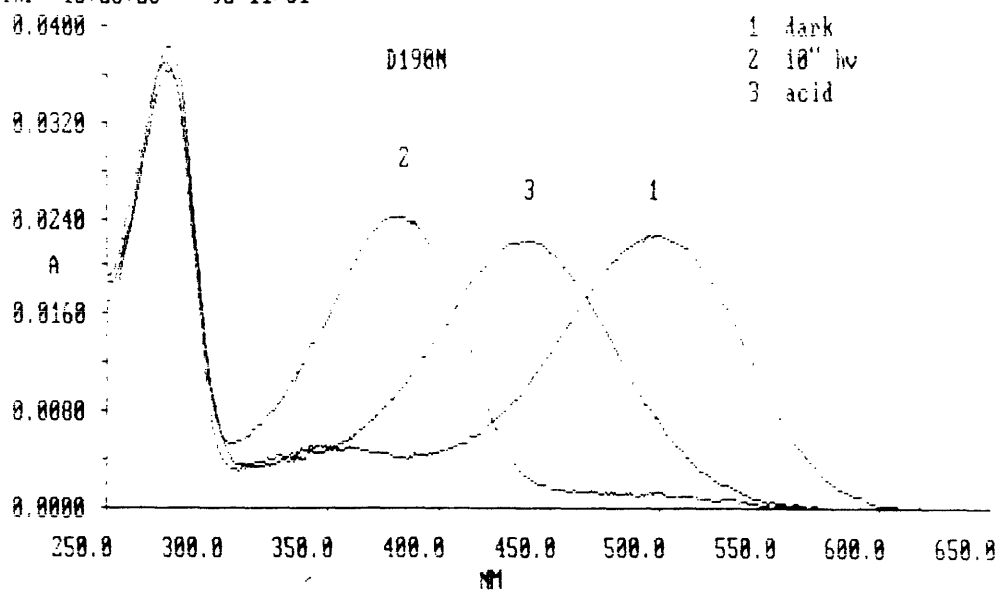
3 acid



Z: 1101022; absc 650.0- 250.0; pts 401; int 1.00; ord -0.001-0.0678; 4
inf: 13:53:41 92/11/01



Z: 1101037; absc 650.0- 250.0; pts 401; int 1.00; ord -0.001-0.0365; A
inf: 16:23:20 92/11/01



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