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# Composition, Structure And Development Of The Crystalline Cone Of The Superposition Compound Eye

Cassandra Lee Schlamp

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# COMPOSITION, STRUCTURE AND DEVELOPMENT OF THE CRYSTALLINE CONE OF THE SUPERPOSITION COMPOUND EYE

by

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## ABSTRACT

The crystalline cone, the major lens in the superposition compound eye of beetles and moths, is a transparent and solid structure consisting of a small number of highly concentrated and predominantly hydrophobic proteins.

Among the most abundant polypeptides isolated from urea-extracted cones is a 67kD protein family with members of varying pI and hydrophobicity. Members of this arthrocrystallin family are found in the cones of all 9 species examined. In addition, beetle lenses contain a 125kD polypeptide that is similar to a moth 100kD lens polypeptide in being very hydrophobic, abundant and immunologically related. The high molecular weight polypeptides may also be related to the 67kD family because (i) antibodies raised against the beetle 67kD lens polypeptide specifically cross-react with the moth 67kD and 100kD polypeptides as well as with the beetle 125kD polypeptide, (ii) enzyme digests of purified 67kD and 100kD moth polypeptides yield a small number of products of identical molecular mass, and (iii) both the purified moth 67 and 100kD polypeptides dissociate naturally into subunits of similar molecular weight. Most of the less abundant cone polypeptides appear not to be as widely distributed as the polypeptides above.

The different profiles of crystalline cone proteins in lepidopterans and beetles may result from the cone being formed in different ways. In the skipper butterfly *Calpodes*, the cone proteins are synthesized and apparently deposited in a coordinate manner in the pupa; the cone is nearly complete at adult emergence. In the beetle *Onitis*, the high

molecular weight cone polypeptides appear to be synthesized only early in lens development, members of the 67kD family only later in the pupal stage, and the cone only 70% complete by adult emergence. In both species, the crystalline cone starts to grow by deposition of granules of lens protein onto a specialized region of cone cell surface (the plasma membrane template). Subsequent growth of the cone in the skipper involves the appearance of a sponge-like scaffold which then becomes packed with lens protein. In the beetle, newly synthesized cone protein fuses uniformly around the cone axis, giving rise to cross-sectional profiles which are at first star-like, then square, and finally circular.

A template membrane connecting the four cone cells is the initial site for the ordered deposition of cone protein. The size and shape of this template defines the outer limits and volume of the mature cone. Cone proteins will not fuse with other regions of the cone cell plasma membrane, and in the template's absence form random aggregates in the cytoplasm.

A template membrane fraction can be obtained from *Calpodes* by a two-step urea solubilization of the cone proteins, leaving the template intact. The template, after solubilization in SDS, is associated with one 67kD isoform as well as the 100kD polypeptide. This 67kD polypeptide may form a link between the template membrane and the proteins that form the bulk of the crystalline cone. The 100kD polypeptide may form the scaffold to which the other cone proteins attach.

To my parents, with all my love.

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**Nomenclature:**

| | |
|---|---|
| BSA | bovine serum albumin |
| °C | degrees Celsius |
| CC | crystalline cone |
| CO | cornea |
| DGD | diethylene glycol distearate |
| DMSO | dimethyl sulfoxide |
| EDTA | ethylenediamine tetraacetic acid |
| FITC | fluorescein isothiocyanate |
| GRIN | gradient of refractive indices |
| IEF | isoelectric focussing |
| kD | kiloDalton |
| M | molar |
| mA | milliamperes |
| mM | millimolar |
| $M_r$ | relative molecular mass |
| MEM | minimal essential medium |
| $n_o$ | axial refractive index |
| PAGE | polyacrylamide gel electrophoresis |
| PBS | phoshate buffered saline |
| pI | isoelectric point |
| PMSF | phenylmethylsulfonylfluoride |
| PPO | 2,5-diphenyloxazole |
| r | radius of crystalline cone |
| RI | refractive index |
| RIA | radioimmune assay buffer |
| SBA | protein extraction buffer |
| SDS | sodium dodecyl sulfate |
| Tris | Tris(hydroxymethyl)aminomethane |

# PART 1

# INTRODUCTION

## 1.1 General Introduction

The classical studies of Exner (1891) established that insect vision is of two functional types: apposition or superposition vision. Compound eyes of both types have a radial two-dimensional array of visual units, or ommatidia, that are capable of developing and functioning independently. An ommatidium consists of a corneal facet (a lens), a crystalline cone (a second lens), and an underlying photoreceptor (the retinal unit containing a light-sensitive rhabdom) surrounded by two layers of mobile pigment cells (the iris). The two types of compound eye are distinguished anatomically from one another by the arrangement of the different cell populations. In an apposition eye, the retinal units lie directly beneath the lens elements of the ommatidia. In the superposition eye, the lenses are separated from the retina by a wide transparent space, called the 'clear zone' (Horridge, 1975). The clear zone increases the absolute sensitivity of the compound eye to light by allowing light that enters the eye through several facets to focus onto a single rhabdom. Consequently, superposition eyes are characteristic of nocturnal insects, such as moths and many beetles. In bright light, the sensitivity of superposition eyes is reduced by a

change in shape of the pigment cells. The pigment cells migrate proximally into the clear zone from between the crystalline cone cells to block the proximal tip of the cone and thus act as an iris. This lowers the amount of light entering the clear zone and striking the retina. In dark conditions, the primary pigment cells retract distally to allow the cone tip to be fully exposed to the clear zone and increase the amount of light reaching the retina (Horridge, 1975).

In a few diurnal insects with clear-zone eyes, however, the pigment remains between the cone cells to leave the tip of the cone exposed, even in bright light (Horridge *et al.*, 1972). This specialized condition is found in skipper butterflies, day-flying moths, and many scarab beetles (Horridge and Giddings, 1971; Meyer-Rochow, 1975; Caveney and McIntyre, 1981; Land, 1985). Research on these 'diurnal superposition eyes' has centered on their anatomy, optical performance and sensory neurophysiology (Horridge, 1972; Horridge, 1975; Caveney and McIntyre, 1981; McIntyre and Caveney, 1985; Warrant, 1988). While these studies suggest that the precision of the optical system is greatly dependent on the physical properties of the crystalline cone (Land, 1985; McIntyre and Caveney, 1985), the way in which this lens is made during metamorphosis, and its chemical composition, have not been examined.

The crystalline cone is an intracellular deposition of four cone (or Semper) cells that contribute equally to make a transparent, bullet-shaped lens with a precise axially-symmetrical structure (Grenacher, 1879 IN: Caveney and McIntyre, 1981). Gokan (1968) reported that the crystalline cone cells of the Velvety Chafer begin to secrete the cone forming substance at pupation. The secretions unite to form an immature cone 4 days later and as the ommatidia grow and develop, so does the crystalline

cone. However, he did not report how this was accomplished.

The optical properties of the eye depend less on the shape of the crystalline cones than on their internal structure (Caveney and McIntyre, 1981). Each cone has a graded profile of refractive index (RI) which is maximal at the center of its most distal region. From this point, the refractive index within the cone drops smoothly in every direction. The RI profile of the crystalline cone, together with its shape, position and size, optimizes the capturing, refracting, and focussing capacity of the lens system in the rhamatidium (Caveney and McIntyre, 1981).

I studied the superposition eyes of several insects to determine the protein composition of the crystalline cone and to describe its development with respect to the formation of cone shape and graded refractive index.

# PART 2

# CHARACTERIZATION OF THE CRYSTALLINE CONE PROTEINS

## 2.1 INTRODUCTION

This chapter describes the protein composition of the crystalline cone in moths and beetles and the biochemical similarities between these proteins and those of other biological lenses.

### 2.1.1 Lens Proteins of the Vertebrate Eye

Six immunologically distinct families of structural proteins have been characterized in the vertebrate lens. These families are the alpha-, beta-, gamma-, delta-, epsilon and rho-crystallins and they comprise approximately ninety percent of the soluble protein of the lens. Comparison of molecular masses, amino acid compositions, sequences and peptide maps show that all vertebrate alpha-crystallins are related (de Jong, 1981). Similar findings have been reported for beta- (Zigler, 1976; Raemakers *et al.* 1980) and gamma-crystallins (Croft, 1972), as well as the delta- and epsilon-crystallins found only in birds and reptiles (Williams and Piatigorsky, 1979; de Jong, 1981).

The alpha-crystallins have the highest native molecular masses (600kD to 4,000,000kD) and migrate with the highest mobility in free electrophoresis. The individual polypeptides have molecular masses between 20kD and 23kD (pH 5-8)(Chiou *et al.*, 1988), and are classified according to pI as alpha-acidic (alpha-A1, alpha-A2) and alpha-basic (alpha-B1, alpha-B2). The alpha-A2 and -B2 are the only primary gene products, and give rise to the alpha-A1 and -B1 polypeptides by the postsynthetic process of deamidation (Bloemendal, 1981).

The beta-crystallins are the largest of the crystallin families and have been divided into two subgroups, beta-heavy chain and beta-light chain crystallins. The beta-heavy chain crystallins have high native molecular masses between 150 and 200kD, while the light chains have low masses ranging between 52 and 80kD. All of the native beta-crystallins contain at least two monomeric subunits with masses between 23 and 28kD (pI 5.5 to 7.5) (Chiou *et al.*, 1988). As in the alpha-crystallins, some of the monomeric subunits appear to be transcribed from the same gene and then post-translationally processed (Argos and Siezen, 1983).

The gamma-crystallins, often referred to as the low molecular weight crystallins, have native masses ranging from 19kD to 23kD. These proteins generally exist as monomers of similar mass (pI 6 to 9) (Chiou *et al.*, 1988). Isolated and purified gamma-crystallins are labile, and under near- physiolgical conditions become more negatively charged. Storage of these proteins in alkaline buffers or at -20°C generates electrophoretic variants (Slingsby and Miller, 1983).

The final 3 families of structural lens proteins are the delta-, epsilon- and rho-crystallins. The delta-crystallins are predominantly found in the lens core of all birds and reptiles. They characteristically

have an aggregate mass of 200kD and contain four subunits of approximately 5\ :D (pI 9) (Narebor and Slingsby, 1985). The epsilon-crystallins are not found in all reptiles and birds. The native $M_r$ of this family is estimated to be 120,000 and they are composed of 3 identical subunits of molecular mass 38kD (pI 7.5) (Stapel *et al.*, 1985). The gene sequence for the minor epsilon-crystallin is similar to lactate dehydrogenase (Wistow *et al.*, 1987; Wistow and Piatigorsky, 1987). The rho-crystallin is the most recent addition and least studied member of the crystallin families. It is found predominantly in amphibian lenses and shows sequence homology with the bovine lung prostaglandin F synthase (Watanabe *et al.*, 1988).

The refractive index and transparency of the vertebrate lens depends on interactions between the crystallins. These interactions are determined by the sequence, conformation, and processing of the proteins. The gamma- and beta-crystallins show sequence homology and are post-transcriptionally modified at both the N- and C-terminals. These modifications are responsible for the quaternary association between various crystallins (Argos and Siezen, 1983). The alpha-crystallins show little homology in sequence to the other crystallin families, but are similar to the beta-crystallins in being composed largely of sheet secondary structures with little or no helical conformation (Schachar and Solin, 1975). Argos and Siezen (1983), have shown that hydrogen bonding occurs between the sheet configuration of the proteins and is necessary for the organized packing array found in the lens. This packing process also depends on the hydrophobicity of the proteins and the ionic environment (Benedetti *et al.*, 1981). Interestingly, the alpha-crystallins are closely related in sequence to four small heat-shock proteins from *Drosophila*.

Both of these protein groups form spontaneous aggregates after purification which is believed to be a general mechanism for protein stabilization. In the case of the alpha-crystallins, this is the result of deamidation and cleavage of amino acids from the C-terminal (Slingsby, 1985). Although the physical interactions of the crystallin proteins are not clearly understood, their structures are apparently very resistant to evolutionary change.

### 2.1.2 Lens Proteins of the Cephalopod Eye

The cephalopod eye has many structural and functional similarities to the vertebrate eye. For this reason, the lens proteins have been used to study convergent evolution of vertebrates and invertebrates at a molecular level.

The structural proteins of the cephalopod lens are unrelated to the vertebrate crystallins, but demonstrate similar characteristics (Chiou, 1984). The cephalopod lens proteins are unstable after purification, precipitate from aqueous solutions during their isolation, and consist of many basic polypeptides. The major protein of the squid lens (SIII) has a molecular mass of 60kD, and comprises 30kD and 27kD subunits. The other two major proteins (SI and SII) contain a number of acidic isoforms ranging in pI from 5 to 5.5 (Chiou, 1984). No other biochemical studies of invertebrate lens proteins have been reported in the literature.

### 2.1.3 Aims

The aim of this chapter is to determine the molecular composition of the crystalline cone in the compound eye of 2 insects, the skipper butterfly, *Calpodes ethlius,* and the dung beetle, *Onitis aygulus*. The questions specificaily asked were:

1) What are the proteins of the insect crystalline cone?
2) To what extent are the crystalline cone proteins conserved among different insect groups?

## 2.2 METHODS AND MATERIALS

### 2.2.1 Animals

Larvae of *Calpodes ethlius* Stoll (skipper butterfly; Hesperiidae) were raised on a diet of Canna leaves in a temperature controlled environment until the late fifth instar. The larvae were then collected and placed in a 22°C incubator with a 12 hour light: 12 hour dark cycle until pupation. At specified days after pupation (P+day), the animals were decapitated and the eyes removed. Adults were killed within 36 hours following emergence.

South African dung beetles (*Onitis aygulus* Fab.; Scarabaeidae) were bred and reared on cattle dung in a 28°C incubator. Pupae were collected and adult development was staged according to the degree of body tanning of the pharate adult. In the early pupal stage (<8 days), the pharate adults have cream colored bodies and tanning is not evident on the labia or the spines of the forelegs. Mid-instar pupae (8-12 days) show varying degrees of tanning on both the adult labia and spines and the body color is an iridescent green. By late pupal stage (>12 days), the entire adult body is tanned brown. The post-emergence adults used in this study were either newly emerged (<36 hours) or mature (>10 days).

Other *Onitis spp.* and the dung beetle, *Copris elphenor* Klug (Scarabaeidae) used in this study were collected in the wild from various regions in southwestern Australia during the months of March and April

1987.

Late larval stages of *Manduca sexta* (the tobacco hornworm; Sphingidae) were obtained from North Carolina Biological Supply Company. The pupal cuticle of the tobacco hornworn is soft and transparent at first, and then gradually acquires a reddish-brown color until it becomes opaque and hard. The color of the cuticle continues to deepen until it is dark brown or almost black just prior to adult emergence. For this study, pupae were classified as early or late according to the degree of cuticular tanning. All adults were killed upon emergence.

Mature adults of two species of June beetles (*Phyllophaga spp.*; Scarabaeidae) and adult stag beetles (*Pseudolucanus placidus* (Say); Lucanidae) were collected at various sites in southwestern Ontario during the months of June and July 1985, 1986 and 1987.

### 2.2.2 Preparation of Proteins

#### 2.2.2 A) Native Gel Electrophoresis

The adult eyes were removed from the insect head by an incision with a razor blade around the periphery of the cornea. The cornea and underlying tissue was peeled away from the head and placed in a physiological medium of M199 (Connaught Laboratories, Toronto, Canada) supplemented with 30mM potassium chloride (KCl), 20mM magnesium chloride (MgCl2), 50mM Trehalose, and buffered with 20mM PIPES (pH 6.75). The medium (referred to as M10) was filtered through a

Nalgene 0.2 micron filter and stored at 4°C. Dissection of crystalline cones involved removal of all connective tissue, retinular cells and the clear zone from the corneal preparation with a dissecting needle. The crystalline cones were then separated from the cornea and surrounding pigment cells with an eyelash probe and suspended in M10. Because the cones are dense, they settled out rapidly, and other tissue contaminants were washed away with several rinses of fresh medium.

For the analysis of native proteins, crystalline cones were isolated from the eyes of a minimum of six mature adult and homogenized in 50mM Tris-HCl (pH 7.2), 0.1% Triton X-100 and 1mM phenylmethyl-sulfonylfluoride (PMSF, Sigma Chemical Co., St. Louis, MO).

2.2.2 B) Denaturing Gel Electrophoresis

The eyes from 6 to 12 animals of the desired age, stage and species were used for protein analysis by denaturing polyacrylamide gel electrophoresis. Intact pupal eyes were dissected from the head and cleared of all nervous and surrounding connective tissues. An eyelash was used to remove the majority of the surrounding retinular cells and developing cornea. All pupal eye preparations contained cone cells and pigment cells. Crystalline cones were dissected from adult eyes as described above.

Samples were centrifuged for 2 minutes (Eppendorf microcentrifuge) and the supernatant was discarded. To the lens pellet was added 200μL of 9M urea, 5% beta-mercaptoethanol and 1mM phenylmethylsulphonyl- fluoride (PMSF). The mixture was then

sonicated (4 times) on ice for 3 minutes (Kontes Ultrasonic Cell Disruptor, Vineland NJ). Samples of the homogenate were used immediately or stored at -20°C until required.

### 2.2.3 Protein Determination

Protein concentration of unlabelled samples was determined by trichloroacetic acid-turbidity (Comings and Tack, 1972) or by Bradford protein assay (Bradford, 1976). In both cases, bovine serum albumin (BSA) was used as the standard. Both standards and samples were read on a Shimadzu UV-160 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). Known quantities of BSA were plotted against their absorbances at 400nm (TCA) or 595nm (Bradford) to produce an optical density curve. The amount of protein (μg/μL) in each sample was then determined by interpolation from the standard curve.

### 2.2.4 Polyacrylamide Gel Electrophoresis

#### 2.2.4 A) Native Gels

Homogenized crystalline cone samples were separated on a 10% acrylamide slab gel with a 3% stacking gel. Solutions for gels and buffers are the same as those used for one-dimensional denaturing gels (see below) except that sodium dodecyl sulfate (SDS) was excluded. Electrophoresis was performed in a cold room (4°C) at 5mA through the

stacking gel and 15mA through the separating gel. Electrophoresis was completed one hour after the tracking dye (bromophenol blue in SBA) had run completely off the gel. The proteins were visualized with Coomassie blue R-250 (Sigma Chemical Co., St. Louis, Mo) and Coomassie blue R-250/silver double stain.

2.2.4 B) One-Dimensional Gel Electrophoresis

To analyze individual polypeptides of the crystalline cone, proteins were electrophoretically separated on the basis of relative molecular weight (Mr). Denaturing gels in the presence of sodium dodecyl sulfate (SDS; BDH Chemical Co., Toronto, Ontario) polyacrylamide were prepared according to the method of Laemmli (1970). Separating and stacking gels were prepared from a stock solution of 30% (wt/vol) acrylamide and 0.8% (wt/vol) N,N-bis-methylene acrylamide (BDH Chemical Co., Toronto, Ontario). Separating gels were either 15% acrylamide slab gels or 3-15% gradient slab gels with final concentrations of 0.1% SDS and 0.375 M Tris-HCl (pH 8.9) (Atkinson, 1981). All separating gels were overlaid with a 3% acrylamide (wt/vol) stacking gel containing 0.1% SDS and 0.125 M Tris-HCl (pH 6.8). Separating and stacking gels were chemically polymerized by the addition of 0.025%-0.05% tetramethylethylenediamine (TEMED) and 0.04%-0.1% ammonium persulfate (APS).

The electrode or running buffer contained 0.025 M Tris (pH 8.3), 0.129 M glycine and 0.1% SDS. Prepared samples were mixed with an equal volume of solubilizing buffer (2% SDS, 1mM PMSF, 5% beta-

mercaptoethanol (weight/volume), 20% glycine (weight/volume) and 80mM Tris-HCl (pH 6.8)), and 0.001% bromphenol blue as the tracking dye. Generally, 50μg of protein was loaded into each well unless otherwise stated. One well of each gel was loaded with 5μL of known molecular weight standards (Sigma Chemical Co.; St. Louis MO). Electrophoresis was carried out with a current of 5mA per gel until the sample had passed out of the stacking gel. The current was then raised to 20mA per gel until 30 minutes after the bromphenol blue marker dye had completely run off the bottom of the gel. Immediately following electrophoresis, gels were stained to visualize polypeptides.

2.2.4 C) Isoelectric Focussing

Isoelectric focussing (IEF) gels were prepared with 1.33mL of Acrylamide stock (28.38g acrylamide, 1.62g bis-acrylamide made up to 100mL with distilled water and stored at 4°C in a dark bottle), 2mL of 10% Nonidet P-40 (Sigma Chemical Co., St. Louis, MO), 1.97mL of distilled water, 0.2mL of pH 3.5-10 ampholines and 0.3mL of pH 4-6 ampholines (LKB). Urea (5.5g) was dissolved in the above mixture by gently heating it with running water. Once the urea had dissolved, 11μL of 10% ammonium persulfate was added and the mixture was degassed under vacuum for 2 seconds. Following the addition of 13μL of TEMED, the solution was mixed and poured into glass tubes sealed with parafilm on one end. Each gel was poured to the height of 10cm, then overlayed with water and allowed to polymerize for a minimum of 4 hours. Once polymerized, the parafilm was replaced with dialysis membrane and the

tubes were mounted in an IEF tank. The upper buffer bath contained 2g of sodium hydroxide (NaOH) per liter and the lower buffer bath contained 8mL of 85% phosphoric acid per liter. Gels were prerun at 200 volts for 15 minutes, 300 volts for 30 minutes and then 400 volts for 30 minutes. Following the prerun, the upper buffer bath was replaced with fresh buffer and the samples were loaded onto the top of the gels. The loaded samples were run at 400 volts for 12-16 hours and then at 800 volts for 2 hours. Gels were removed from the tubes by exerting pressure with a 10mL syringe connected with Tygon tubing, equilibrated in Laemmli Sample Buffer (SBA) for 10 minutes, and used for two dimensional analysis.

To determine the pH gradient of IEF gels, a companion gel, on which no sample was loaded, was sliced into 0.5cm pieces. Each slice was placed into a test tube containing 2mL of cold, previously boiled, distilled water and kept at 5°C overnight to remove the ampholines. The pH (Radiometer PHM61, Copenhagen) of the solubilized extract from each slice was determined and used as a standard for comparison to the other gels of the same run (Saleem and Atkinson, 1976).

2.2.4 D) Two-Dimensional Gel Electrophoresis

Two-dimensional analysis (IEF-SDS-PAGE), the separation of proteins on the basis of their isoelectric points in the first dimension and Mr in the second dimension, was essentially the method of O'Farrell (1975). Samples, labelled with L-[35S]-methionine were loaded according to the number of counts per minute per microliter. For samples that were

unlabelled, 200μg of total protein were loaded onto each IEF gel. Extruded gels were equilibrated in 10mL of Laemmli Sample Buffer (SBA) for 15 minutes (SBA; 2% SDS, 5% beta-mercaptoethanol, 20% glycerol in 80mM Tris-HCl (pH 6.8) and 0.01% bromphenol blue. To load the equilibrated gel on to the second dimension, a 1% solution of melted agarose (prepared in 1% SDS at 80°C) was first layered onto the 3-15% gradient SDS gel. The IEF gel was quickly placed upon the agarose and let stand until the agarose solidified. The second dimension was then run at 5mA until the tracking dye completely entered the gradient gel; the current was then increased to 15mA until the dye front ran off the gel (O'Farrell. 1975).

2.2.5 Staining

2.2.5 A) Coomassie Blue R-250

For general protein staining, gels were placed in 0.2% (wt/vol) Coomassie blue R-250 (Bio-Rad Laboratories, Richmond, California) in 45% methanol and 10% glacial acetic acid. Gels were stained overnight with continual mechanical agitation (Gyrotory shaker, model G2). Primary destaining occurred in 50% methanol and 10% acetic acid for 2 hours. Secondary destaining took place in 5% methanol and 10% glacial acetic acid with agitation until complete (approximately 12 hours). Gels were stored in 7% glacial acetic acid for photography.

2.2.5 B) Coomassie Blue/Silver Double Stain

The procedure for double staining with Coomassie blue R-250 and silver followed that of Dzandu *et al.* (1984). Following electrophoresis, each gel was fixed in 500mL of 40% methanol and 10% glacial acetic acid for 1 hour at room temperature (22 °C). Residual SDS was removed by two 30 minute washes of 10% ethanol and 5% glacial acetic acid. Each gel was oxidized in 200mL of 3.4mM potassium dichromate and 3.2mM nitric acid for 10 minutes and then rinsed with several changes of distilled water. Once clear, the gels were placed in 200mL of 20mM silver nitrate for 30 minutes and again rinsed with water, this time to remove any unbound silver. The absence of residual silver was verified by adding one drop of 1M HCl to 1mL of the final water rinse. If the solution turned cloudy, indicating the presence of silver, another rinse was necessary.

Band development was initiated by the addition of 200mL of warm (40°C) 0.28M sodium carbonate and 0.008% formalin which was replaced as soon as it turned light brown in color. To stop development, the developer was decanted and the gels were rinsed with distilled water as previously described. To enhance the yellow banding pattern, each gel was placed into 200mL of 10% glacial acetic acid until it was photographed or counterstained with Coomassie blue R-250. To counter-stain, silver stained gels were placed in 200mL of 0.1% (wt/vol) Coomassie blue in 25% methanol and 7.5% glacial acetic acid for 1 hour. They were destained with 25% methanol and 7.5% glacial acetic acid and then photographed (Dzandu *et al.*, 1984.

### 2.2.6 Photography

Gels were photographed on a light box with Technical Pan Film #2415 (Kodak Canada Inc., Toronto, Canada) through a #25 red filter with an exposure of 1/4 second at f 5.6/8. Films were developed for 10 minutes with Kodak 110 developer and printed on Ilford Multigrade paper (Ilford Inc.).

### 2.2.7 Electroelution and Concentration of Proteins

Isolation and concentration of the native proteins of the crystalline cone was achieved according to the method recommended by Bhown *et al.* (1980). Numerous native gels with a single wide well were loaded with approximately 300-500µg of protein. The gels were run as above and a narrow side strip was removed, stained and destained. This strip was used as a marker to locate the desired bands of proteins. A strip of gel containing the protein of interest was removed from the gel with a razor blade, then cut into small pieces and loaded onto the electroelutor (ISCO Model 1750 Concentrator).

The samples were loaded in a cup buffer containing 0.05M Tris (pH 8.5) and 0.001% SDS. Proteins were electroeluted overnight at a constant current of 4-7mA into a sucrose buffer (2% sucrose, 0.05MTris (pH 8.5), 0.001% SDS). Outer chamber buffer contained 0.1M Tris (pH 8.5) and 0.001% SDS while the inner chamber buffer was 0.057M Tris (pH 8.5) and 0.001% SDS (Bhown *et al.*, 1980). Concentrated proteins were either stored at -20°C or prepared for analysis by PAGE.

### 2.2.8 Hydrophobic Interaction Chromatography

After isolation of the crystalline cones from *Calpodes ethlius* and *Onitis aygulus*, the tissue was solubilized in 100µL of 50mM Tris-HCl (pH 7.5) and 1mM beta-mercaptoethanol. The samples were sonicated on ice for 3 minutes and the concentration of protein in each was determined by Bradford (1976) protein assay, using BSA as a standard. Samples were frozen at -20°C until required.

Equal volumes (70µL) of each sample were loaded onto a Phenol Sepharose CL-4B column (Pharmacia Fine Chemicals, Piscataway, N.J.) for hydrophobic interaction chromatography. The column was equilibrated with 50mM Tris-HCl (pH 7.5), 1mM beta-mercaptoethanol and 0.1mM calcium chloride (column buffer), and the slurry was poured into a cotton plugged 1cc syringe. The column volume was approximately 100µL.

The samples were then layered onto the top of the column and collected as follows. The first 5 fractions were eluted with 200µL of column buffer to remove any of the hydrophilic polypeptides in the samples that were not bound to the column. Fractions 6-10 were eluted with 200µL of column buffer and 0.5M sodium chloride to remove ionically bound peptides and peptides with weak hydrophobic groups. Fractions 11-15 were eluted with 200µL of distilled water and the last 5 fractions were washed with 200µL of 8M urea. Both of these elutants were necessary to remove hydrophobically bound polypeptides.

Each fraction was then solubilized in SDS so that the final sample

was made to 2% SDS, 80mM Tris-HCl (pH 6.8), 1mM PMSF, 1mM beta-mercaptoethanol, and approximately 10% sucrose. A known volume of each sample was then loaded onto a one-dimensional polyacrylamide gel (3-15% gradient).

2.2.9 Triton X-114

According to Bordier (1981), extraction with Triton X-114 not only separates proteins that are hydrophilic and hydrophobic but can further subdivide hydrophobic proteins into three categories. Each category is based on the degree of solubility of the proteins during temperature phase changes of the Triton X-114. Each phase can easily be identified and isolated according to its position and appearance in the extraction tube (Bordier, 1981).

Following the above procedure, 40-200µg of total crystalline cone proteins were solubilized in 200µL of 10mM Tris-HCl (pH 7.4), 150mM NaCl, and 1% Triton X-114 (Sigma Chemical Company, St. Louis, Missouri). The solution was vortexed and incubated at 0°C for 15 minutes. Each sample was overlaid onto a 300µL cushion of 6% sucrose (wt/vol), 10mM Tris-HCl (pH 7.4), 150mM NaCl, and 0.06% Triton X-114 in a 1.5mL Eppendorf tube and incubated at 40°C for 15min. After the solutions turned cloudy they were spun at 300 x g in a clinical centrifuge for 3 minutes.

The upper aqueous phase was removed and mixed with fresh, cold (0°C) Triton X-114 to a final concentration of 0.5%. This solution was then layered on top of the same sucrose cushion, incubated at 30°C for 15

minutes and centrifuged as before. Sample could be divided into four phases: the upper, clear phase was the most hydrophilic; the cloudy phase below the aqueous fraction was the soluble hydrophobic phase; the sucrose cushion containing protein of intermediate hydrophobicity, and the bottom, most hydrophobic phase. Each of the four phases were removed and stored separately at -20°C, or were prepared for SDS-PAGE analysis.

### 2.2.10 Polysaccharide Stains

Isolated crystalline cones from mature adults of *Calpodes ethlius* and *Onitis aygulus* were stained with periodic acid Schiff's reagent (PAS), for up to 30min to detect the presence of polysaccharides, glycogen in particular. Some of the samples were treated with alpha-amylase for 30min prior to staining with PAS (Lillie and Fullmer, 1976). Alpha-amylase extracts glycogen and in conjunction with PAS staining will indicate its presence.

The lectin, Concanavalin A, which preferentially binds to mannose and glucose residues, was used to determine if any of the crystalline cone proteins were glycosylated. The binding of the lectin was visualized by a color development on Western transblots (Hawkes, 1982, see transblot section 2.2.11).

### 2.2.11 Immunology

Six rabbits (New Zealand Whites) were acclimatized for 6 weeks and

then bled from the marginal ear vein for preimmune sera. Blood samples were collected in glass tubes and placed in a 37°C water bath for 1 hour to allow blood to clot. Samples were stored at 4°C overnight to enable the clot to contract. Serum was pipetted from the clot and placed into a 15mL conical bottom tube and spun at 1,000 x g for 8 min to remove contaminating red blood cells. Serum was heated at 56°C in a hot water bath for 30 minutes to inactivate complement proteins and then frozen in 2mL aliquots at -70°C.

2.2.11 A) Concentration of Antigen

Polyclonal antibodies were raised against two SDS-denatured polypeptides (125kD and 67kD) of *Onitis aygulus* crystalline cones. Isolated crystalline cones were solubilized in 9M urea and loaded as a single well onto a 3-15% polyacrylamide gel. After electrophoresis, the outer edges of the well were cut from the gel and stained in Coomassie Brilliant blue-R. The remainder of the gel was wrapped in plastic wrap (still on the electrophoretic plate) and stored at 4°C. When the strips were destained, they were re-aligned with the gel and the 125 and 67kD bands were excised. Each band was then cut into small pieces for electroelution and concentration (ISCO 1750 Concentrator; see section 2.2.7).

2.2.11 B) Immunization

The antigen (100µL) was emulsified in Complete Freunds'

Adjuvant (Gibco Chemical Co.) reaching a total volume of approximately 500μL per rabbit. Emulsion was injected intramuscularly into the rabbits' hindlimb. Two rabbits each were used to produce polyclonal antibodies to the 125kD and 67kD polypeptides of the crystalline cone of *Onitis aygulus*. Rabbits received "booster" shots of 100μg of antigen emulsified in Incomplete Freund's Adjuvant (Gibco) every 4 weeks. One week after each of these injections, 15mL of blood was collected by ear bleeding and tested for the presence of circulating antibody. The final boost was 100μg of antigen in an aqueous solution injected intramuscularly. Five days later, rabbits were killed and bled by cardiac puncture. All samples collected were then treated as described previously.

2.2.11 C) DEAE Sephadex A-50

Reactive cationic functional groups on DEAE Sephadex bind the negatively charged serum proteins. The isoelectric point of IgG is sufficiently basic so that IgG carries a predominantly positive charge at pH 6.6 and thus passes through the column.

In preparation of the column, 5g of Sephadex A-50 was swollen in 2 liters of 0.02M phosphate buffer (pH 6.6) overnight. The buffer was decanted and another 2 liters added. After mixing, the gel was allowed to sediment and the buffer was again decanted. This procedure was repeated twice. The gel was transferred to a 25 x 500mm glass column (25 x 250mm was packed) and 200mL of 0.02M phosphate buffer was run through the column.

Equal volumes of serum and phosphate buffer (20mL each) were

mixed and filtered. Next the filtrate was added to the column and eluted with buffer. Twenty fractions of 10mL each were collected for each sample. The concentration of protein in each was then determined by measuring absorbance at 280nm.

2.2.11 D) Ammonium Sulfate Precipitation

To isolate immunoglobulin from serum, 0.5mL of saturated ammonium sulfate was added dropwise to 1.0mL of serum. Solution was mixed gently overnight and then centrifuged for 15min at 2,500 x g. The supernatant was decanted, the pellet dissolved in 0.1M sodium bicarbonate, and dialyzed against 2 liters of 0.1M sodium bicarbonate for 24 hours. Protein concentration was again determined by measuring the absorbance at 280nm.

2.2.11 E) Immunoblots

Western transblot analysis (as outlined by Bio-Rad Laboratories, Richmond, CA) was used to determine the reactivity of the crystalline cone proteins to antibodies and the lectin, Concanavalin A. Proteins were separated by 1D-SDS-PAGE and transferred onto nitrocellulose paper for 5-12 hours at 30V, 40mA. The transfer or blotting buffer was 25mM Tris, 192mM glycine, and 20% v/v methanol (pH 8.3).

Subsequent to the transfer, nitrocellulose membranes were blocked in Tris-buffered saline (TBS; 20mM Tris (pH7.5), 500mM NaCl) with 3%

gelatin for a minimum of 1 hour at room temperature. Excess blocking solution was removed with two 5 minute washes with TTBS (TBS, 0.05% Tween 20).

The Western blots were then challenged with one of several primary antibodies in a 1:200 dilution in TTBS and 1% gelatin for a minimum of 4 hours at room temperature. The antibodies used in this study were anti-125kD, anti-67kD, (generated against the 125 and 67kD polypeptides of the crystalline cone of *Onitis aygulus*), and commercially purchased anti-tubulin, and anti-actin. The transblots were washed 4 times (10min. each) in TTBS prior to exposure to the secondary antibody.

Goat anti-rabbit IgG, heavy and light chains conjugated to N-hydroxysuccinimidobiotin (Jackson ImmunoResearch Laboratories) was the secondary antibody. The membranes were challenged for a minimum of 4 hours at room temperature in a 1:3000 dilution of secondary antibody in TTBS with 1% gelatin and then thoroughly washed in TTBS (4 times, 10min each). Following this, blots were bathed in 1:3000 dilution of avidin-egg white peroxidase (Jackson ImmunoResearch Laboratories) in TTBS and 1% gelatin for 2-6 hours and rinsed in TBS (4 times, 10 min. each). Sixty mg of 4-chloro-1-napthol dissolved in 20mL of ice cold methanol was added to 100mL of TBS containing 60μL of 30% hydrogen peroxide and used immediately to develop bound secondary antibody containing the enzyme tag. The maximum development time was 15 minutes.

2.2.11 F) Immunoprecipitation

Antibodies made to the 125kD and 67kD lens proteins of *Onitis*

*aygulus* were used for immunoprecipitation. The immunoprecipitated crystalline cone proteins from *Calpodes ethlius* were analysed by SDS-PAGE.

Cones were solubilized in 200μL of 9M urea with 1mM PMSF and sonicated (4 x 15 seconds) with a Kontes micro-ultrasonic cell disruptor. The samples were centrifuged for 2 min and the supernatant was removed and stored at -20°C (fraction #1). To the remaining pellet was added 200μL of radioimmune precipitation buffer, RIA [0.05M Tris-HCl (pH 7.2), 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 0.15M NaCl and 1mM PMSF (Atkinson *et al.*, 1983)]. The sample was sonicated for 2 min, placed in boiling water for 3 min and centrifuged for 5 min. The supernatant containing the RIA-soluble proteins was removed and stored at -20°C (fraction #2).

A fresh sample of cones was solubilized in 200μL of RIA, sonicated, boiled and centrifuged as above. The resulting supernatant was stored as fraction #3. To the pellet from the above sample was added 200μL of SBA. The sample was extensively vortexed and then sonicated and boiled as before. After centrifugation (3 min), the supernatant was removed and stored as #4.

Volumes equivalent to 50μg of protein for each sample were aliquotted and diluted four-fold with RIA. All samples were mixed with either the anti-125kD or anti-67kD lens antibody in amounts equivalent to 1:100 dilution. To each mixture was added 10μL of a suspension of Staphylococcus aureus Cowan I strain (Calbiochem-Behring, a division of American Hoescht Corp., La Jolla, CA; standardized to bind 2+/-1mg IgG/mL). Samples were vortexed gently and then placed on ice for 30min. Following the incubation on ice, the samples were microfuged and the

pellets washed thoroughly 3 times with RIA. The precipitates were solubilized in 50μL of 60mM Tris-HCl (ph 6.8), 2% SDS, 1mM PMSF, 5% 2-mercaptoethanol, boiled for 3min and then microfuged for 5min (Atkinson et al, 1983). Samples were either used immediately or stored at -20°C.

2.2.12 Protein Digests

Purified samples of the 100kD and 67kD polypeptides of the crystalline cone of *Calpodes ethlius* were enzymatically digested and exposed to freezing and thawing conditions to determine the similarity between the polypeptides. Purified samples of each protein were repeatedly frozen and thawed for a minimum of 25 times, 30 min in duration. Two percent sucrose was added to the samples for comparative purposes with the enzyme digest experiments (below). The degradation products, before and after boiling, were separated by SDS-PAGE.

Samples of the same proteins (50μg each), which were not denatured, were mixed separately with 5μL of four different enzymes. Two percent sucrose was added and the mixture was vortexed and placed on ice for 15 min. Two of the enzymes, trypsin and chymotrypsin, have specific sites at which they hydrolyze peptide bonds. Trypsin catalyzes the hydrolysis of polypeptides whose carbonyl function is donated by either a lysine or an arginine residue. Chymotrypsin cleaves the polypeptide at phenylalanine, tyrosine and tryptophan residues. The other two enzymes used, protease X and protease XXV do not have specific cleavage sites (Lehninger, 1977). The products from enzymatic digests were separated by SDS-PAGE.

## 2.3 RESULTS

### 2.3.1 Physical Properties of the Crystalline Cone

There are approximately 3,000 crystalline cones in the superposition compound eye of *Calpodes ethlius* and slightly fewer in that of *Onitis aygulus*. (Fig. 1). Each crystalline cone functions by refracting light that has passed through the corneal facet above it with an internal gradient of refractive index. The light is then projected across the clear zone of the eye. Collectively, the cones focus an image on the underlying retina, or rhabdom. The crystalline lens is formed as an intracellular deposition with each cell contributing an identical quadrant. The quadrants are held together by two perpendicular planes of plasma membrane that are tightly apposed, which facilitates the separation of the intact lens free of the cone cell cytoplasm.

The crystalline cone of *Calpodes ethlius* has a cylindrical body with a pliable and tapered proximal tip. An isolated lens can easily be crushed or be split into its four quadrants with slight pressure. The crystalline cone of *Onitis aygulus*, on the other hand, is a harder and more brittle lens.

### 2.3.2 Native Crystalline Cone Proteins of *Calpodes ethlius*

The crystalline cone of *Calpodes ethlius* contains a small number of native proteins. The protein detected as most abundant with Coomassie

Figure 1:

External morphology of the compound eye of *Calpodes ethlius* (top panel, mag. 55x) and *Onitis aygulus* (bottom panel, mag. 55x).


C. ethi
O. aygulus

blue R-250 staining (Fig. 2, lane a) has the highest relative mobility. When a similar preparation was co-stained with silver and Coomassie blue, more proteins were observed (Fig. 2, lane b). These proteins fall into three groups based on their relative mobility. The first group consists of 4 or 5 proteins with high molecular mass that barely enter the separating gel (Fig. 2, #1). The second group consists of a smear of poorly resolved bands of various mass (Fig. 2, #2). The major band with the highest mobility in the native gels is only one protein. This protein stains most intensely with Coomassie blue (Fig. 2, #3).

The first group of native proteins comprises six distinct polypeptides with molecular masses of 145, 100, 80, 67, 21 and 19kD. The second group, although it appears as numerous proteins in native gels, resolves into a cluster of bands around 67kD. The third group contains only one polypeptide with a molecular mass of 67kD.

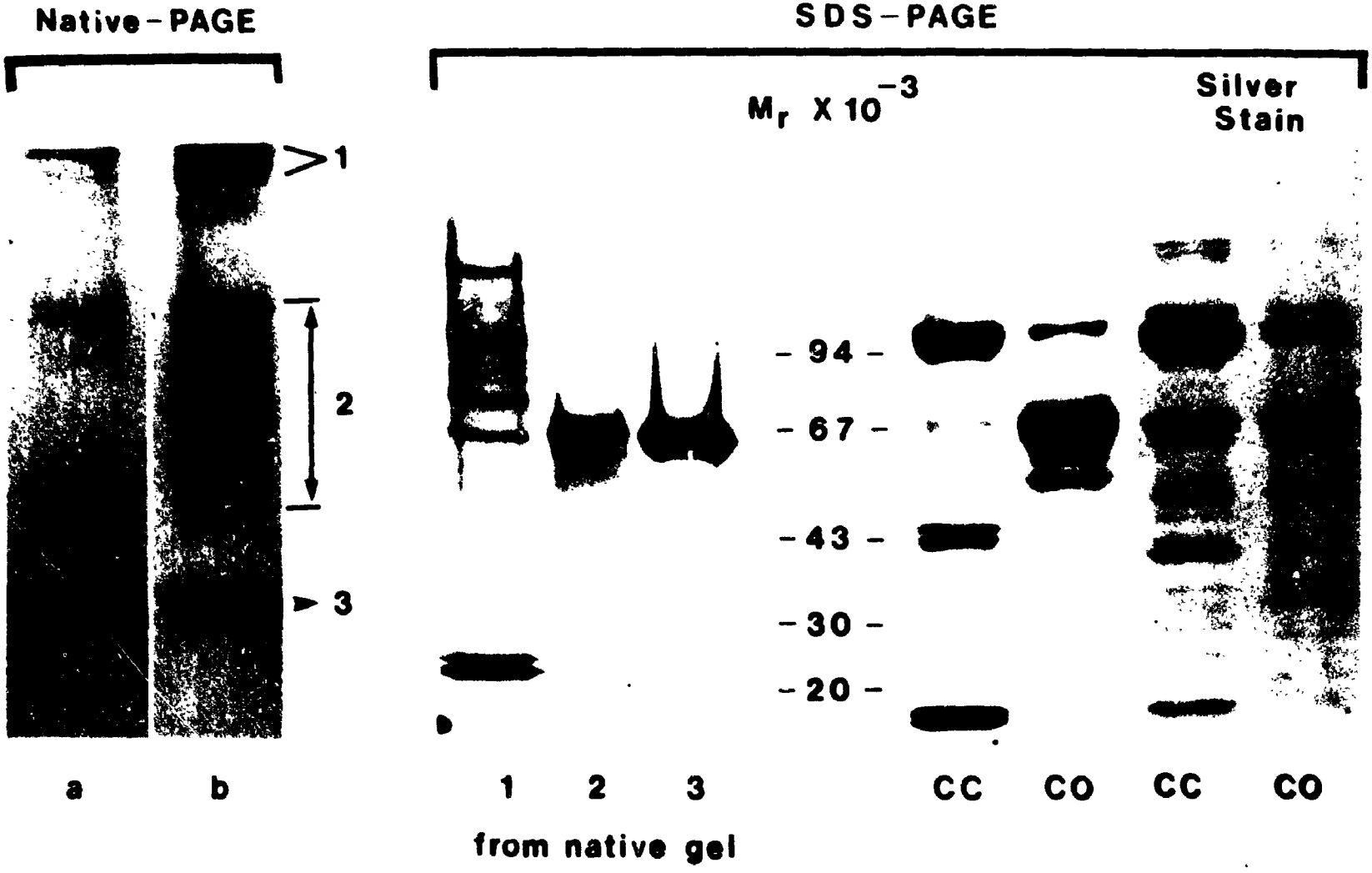
### 2.3.3 Polypeptides of the Adult Crystalline Cone and Cornea of *Calpodes ethlius*

A similar polypeptide pattern to that described above was obtained after solubilization of crystalline cones and corneas in urea. The major cone polypeptides have molecular masses of 145, 108, 100kD, a group of between 39-45kD, and at 21 and 19kD (Fig. 2). The 100kD polypeptide is most abundant. Corneal polypeptides were low in yield and hence faint on Coomassie blue stained gels, but appeared to have the same banding pattern as that seen in the cone. Both purified cones and cornea have several minor polypeptide bands not easily resolved by this staining

Figure 2:

Native proteins and urea-soluble polypeptides of the crystalline cone and cornea from the adult eye of *Calpodes ethlius*.

The native proteins, separated by non-denaturing PAGE and visualized with Coomassie blue alone (a) or double stained with silver nitrate (b), fall into 3 groups. The polypeptides in each group were solubilized by urea and separated by 1D-SDS-PAGE (center panel). For comparison, the urea-soluble polypeptides of the entire crystalline cone (CC) and cornea (CO) are shown separated by SDS-PAGE and stained with Coomassie blue stain (right panel, left) or Coomassie blue/silver stain (right panel, right). The crystalline cone and corneal polypeptides stain more strongly with silver double stain than with Coomassie blue alone.


Native-PAGE
SDS-PAGE
$M_r$ X $10^{-3}$
Silver Stain
1
2
3
94
67
43
30
20
a
b
1
2
3
CC
CO
CC
CO
from native gel

procedure.

Silver double-staining of 1D gels of adult crystalline cones and cornea identified extra polypeptides. The most prominent bands seen, however, were those which had previously been visualized with Coomassie blue alone, with the notable exception of the silver-stain specific 67kD polypeptide. Two minor crystalline cone polypeptides of approximately 70kD and 57kD also became apparent along with numerous polypeptides of low molecular weight. The protein profile of the cornea was greatly enhanced with silver stain revealing the presence of polypeptides of low molecular mass. The most notable difference between the two banding patterns was abundance of the 100kD polypeptide in the crystalline cone in comparison to the cornea.

### 2.3.4 Native Proteins of the Crystalline Cone of *Onitis aygulus*

The crystalline cone of immature or teneral adults (<5 days old) and mature adults (>10 days old) of *Onitis aygulus* also contain only a few native proteins. The protein patterns were the same in the teneral adult (TCC) as in the mature adult (CC) (Fig. 3, top left) and no additional polypeptides were observed after double staining with silver nitrate (Fig. 3, top right), compared to staining with Coomassie blue alone.

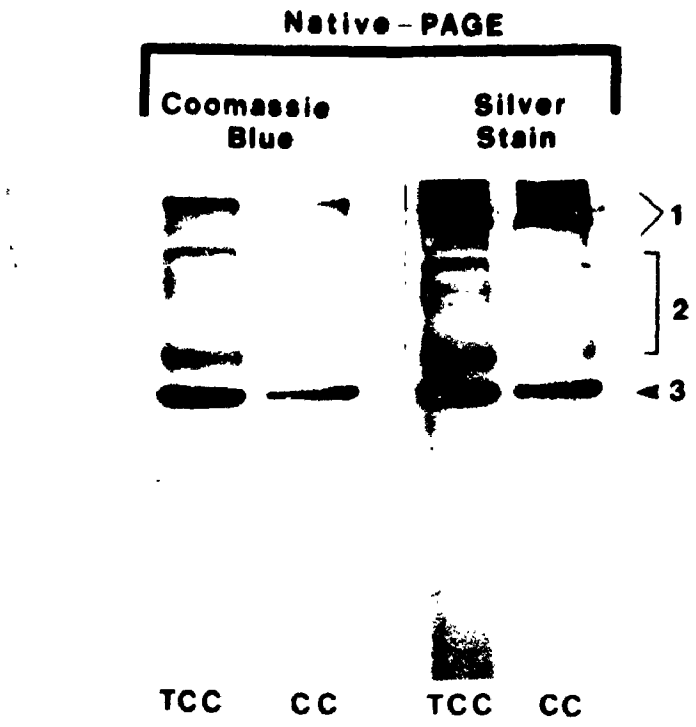
The native protein gels were divided into 3 groups as described earlier for *Calpodes ethlius* (Fig. 3, top panel). The first group of native proteins, which barely enter the separating gel (Fig 3, #1), contains the majority of the crystalline cone polypeptides. The second group contains three or four poorly resolved native bands which have relatively low

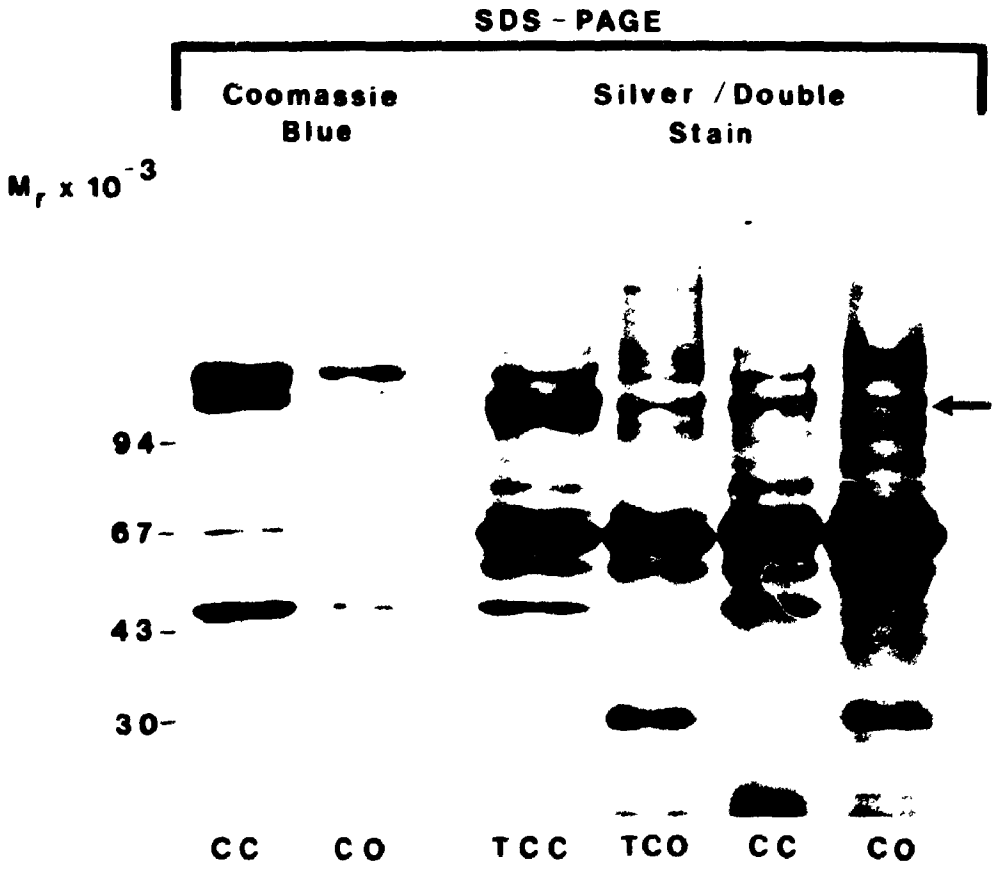
Figure 3:

Comparison of crystalline cone and corneal proteins from teneral and mature adults of *Onitis aygulus.*

Native proteins (top panel): The crystalline cone proteins from the teneral adult (TCC) and mature adult (CC) were separated by non-denaturing PAGE and visualized with Coomassie blue stain (left) or Coomassie blue/silver double stain (right). The protein profiles from both stages are identical and fall into three groups (1, 2 and 3).

Urea-soluble polypeptides (bottom panel): Total lens and corneal polypeptides separated by SDS-PAGE were visualized with Coomassie blue (left) and Coomassie blue / silver stain (right). A 125'-D polypeptide (arrow) is more abundant in the immature (TCC) than in the mature (CC) crystalline cone. No major differences exist between the total corneal lysates of a teneral (TCO) and mature (CO) adult.


Native-PAGE
Coomassie Blue
Silver Stain
1
2
3
TCC
CC
TCC
CC


SDS-PAGE
Coomassie Blue
Silver / Double Stain
$M_r \times 10^{-3}$
94-
67-
43-
30-
CC
CO
TCC
TCO
CC
CO

mobility within the gel, and a more distinct band which is most abundant and has the highest mobility of this group (Fig. 3, #2). These proteins resolved into a high concentration of polypeptides around 67kD. The third group contains only one native protein which is the most abundant of all the native proteins and has the greatest molecular mobility (Fig. 3, #3). Following denaturation in SDS, this protein resolves into only one polypeptide with a molecular mass of 67kD. Since these findings are the same as those for Calpodes in Fig. 2, they are not shown in a figure.

### 2.3.5 Polypeptides of the Adult Crystalline Cone and Cornea of *Onitis aygulus*

The predominant polypeptides of the crystalline cone of *Onitis aygulus,* when visualized with Coomassie blue alone, have molecular masses of 135, 125 and 45kD, and more faintly staining bands at 145, 80 and 67kD (Fig. 3, bottom panel). Corneal proteins produced a polypeptide pattern similar to that of the cones, although resolution was very poor with this staining technique.

After Coomassie blue and silver double staining, the three most prominent polypeptide bands from crystalline cones of teneral adults have molecular masses of 135, 125, and 67kD. The staining of the 67kD polypeptide is greatly enhanced by silver. The remaining polypeptides have molecular masses of 80, 70, 57 and 45kD. The major corneal polypeptides of a newly emerged adult had molecular masses of 125, a group between 80 and 90, 70, 67, 57, and 30kD. Of these, the 67kD polypeptide is most prominent. The corneal protein profiles differ from

that of the cone by having a reduced amount of the 45kD polypeptide and an increased amount of the 30kD polypeptide.

As the cone matures in the adult, the most noticeable changes in the protein profiles are a reduction in the relative abundance of the 125kD polypeptide and an apparent increase in the amount of the 80, 67, and 45kD proteins. The amount of the 67 and 57kD polypeptides increases in the cornea in comparison to that of the newly emerged adult. The cornea of the mature adult also showed an apparent increase.

### 2.3.6 Determination of the Isoelectric Points of the Polypeptides of the Crystalline Cone

Several difficulties were encountered in the analysis of the crystalline cone proteins by two-dimensional SDS-PAGE. One problem was finding the appropriate conditions under which the hydrophobic lens polypeptides would enter the isoelectric focussing gel. A related problem was the inability to focus the proteins clearly. This caused extensive streaking in the gels. These difficulties were not due to the pH of the gels as nonequilibrating pH gels (NEpHGE) were also employed but were less successful. The results shown in Fig. 4 were consistently obtained, although the relative abundance of each polypeptide varied in different preparations.

### 2.3.6 A) Two Dimensional Analysis of the Crystalline Cone Polypeptides of *Calpodes ethlius*

Although the 100kD band is the most abundant polypeptide in the crystalline cone of *Calpodes ethlius.*, it barely enters the IEF gel. Consequently, it appears in only trace amounts as a streak between pH of 6.5 and 7.5 (Fig. 4, top panel, arrows).

The 67kD polypeptide of the cone focusses at a minimum of three different isoelectric regions. The first region extends between pI 8.0 and 8.2, the second between pI 7.2 and 7.7 and the third between pI of 5.5 and 5.8 (top panel, arrowheads). Due to the abundance and overlapping nature of many of the isoforms in 2D gels, it is difficult to resolve them all and thus determine the exact number. At best, it can be concluded that there is a large group of polypeptides with apparent molecular mass of 67kD, and they are considered here to be a family of proteins.

To compound the difficulties in characterizing the 67kD family, the cone contains several other polypeptides of similar pI and apparent molecular weights, namely all of the polypeptides between 57kD and 72kD. These polypeptides also focus as large masses which could only be identified as pI ranges. One group appeared at pI 6.9, a second group between pI 6.6 and 6.8, a third group between pI 6.3 and 6.5 and a final group between pI 5.5 and 5.8. These isoforms would have been included with the 67kD proteins when the 67kD proteins were cut from 1D gels during purification (Fig. 4, brackets). To avoid confusion, any further reference to the 67kD polypeptides includes these closely associated polypeptides as members of the 67kD family.

Other notable cone polypeptides have masses of 80, 21 and 19kD. The

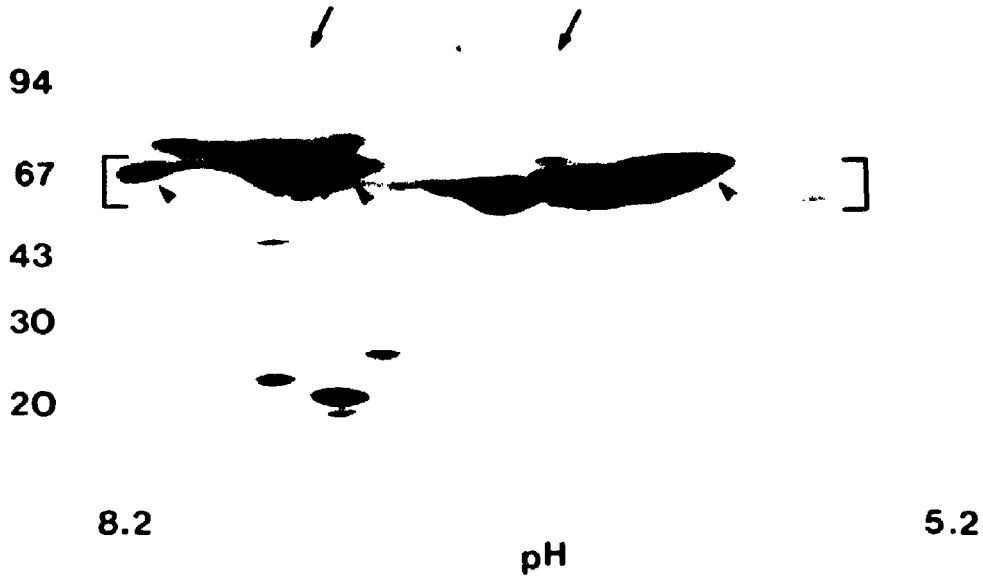
Figure 4:

Two-dimensional separation of the crystalline cone proteins from the mature eye showing the diversity of the 67kD arthrocrystallin family.

*Calpodes ethlius* (top panel): The crystalline cone contains at least 3 isoforms of the 67kD polypeptide (arrowheads) which are in close proximity to other isoforms of polypeptides of similar molecular weights. The brackets denote the polypeptides included in the 67kD family of proteins. Only trace amounts of the 100kD polypeptide are detectable (arrows).

*Onitis aygulus* (bottom panel): The crystalline cone also contains three main isoforms of the 67kD polypeptide (arrowheads), but their pI differ from those of Calpodes ethlius. Brackets denote the polypeptides included in the 67kD family of proteins.

Calpodes ethlius




Onitis aygulus

80kD polypeptide consists of three isoforms with pI of 7.3, 7.6, and 7.9. Both the 19 and 21kD polypeptides appear to have only one isoform of pI 7.4.

### 2.3.6 B) Two Dimensional Analysis of the Crystalline Cone Polypeptides of *Onitis aygulus*

Two major crystalline cone polypeptides of *Onitis aygulus* have molecular masses of 135 and 125kD. As in *Calpodes ethlius*, these high molecular weight polypeptides fail to enter isoelectric focussing gels and therefore their pI's could not be determined.

The 67kD major cone polypeptide appears in large quantities focussing at three pH ranges (bottom panel, arrowheads). The first group barely enters the IEF gel and has a pI of approximately 8.2. The second group ranges in pI from 6.9 to 7.4. The final group appears as a large mass between pI 5.7 and 6.2. Clearly, the 67kD band seen on a 1D gel includes numerous isoforms and polypeptides of molecular weight close to 67kD. For simplicity, all of these polypeptides are here also considered a 67kD family of polypeptides.

The beetle 57kD polypeptide appears as three isoforms, the first at pI 7.0 to 7.2, the second at 6.4 to 6.5, and the third ranges in pI from 6.1 to 6.2. A 47kD polypeptide has a single pI ranging between 6.5 and 6.7.

Numerous cone polypeptides did not enter the isoelectric focussing gels, and those that did failed to focus as discrete spots but tended to streak or smear. The majority of the polypeptides detected, however, range in pI between 5.8 and 7.6. The poor separation of cone polypeptides is probably due to their hydrophobic nature, which is now explored.

## 2.3.7 Hydrophobicity of the Crystalline Cone Polypeptides

### 2.3.7 A) Determination of Protein Hydrophobicity by Column Chromatography

Crystalline cones isolated from the eyes of mature adult *Calpodes ethlius* and *Onitis aygulus* were separated according to their hydrophobicity on a phenyl-sepharose column and the polypeptides in different eluates were analysed by one-dimensional SDS-PAGE.

The first eluant used was a Tris-based column buffer which removed hydrophilic polypeptides not bound to the matrix of the column. Virtually no polypeptides were present in this eluate from the lens sample of *Calpodes*, with the exception of trace amounts of a 100kD polypeptide (Fig. 5, top left, lane 1). No *Onitis* lens polypeptides were eluted from the column with the Tris buffer (top right, lane 1).

The second eluant contained a higher concentration of salt which removed polypeptides ionically bound to the column. Four of the major cone polypeptides of *Calpodes* were present in the eluate. These polypeptides had masses of 100, 67, 21 and 19kD (Fig 5, top left, lane 2). No polypeptides were evident in the corresponding fraction for *Onitis* (top right, lane 2).

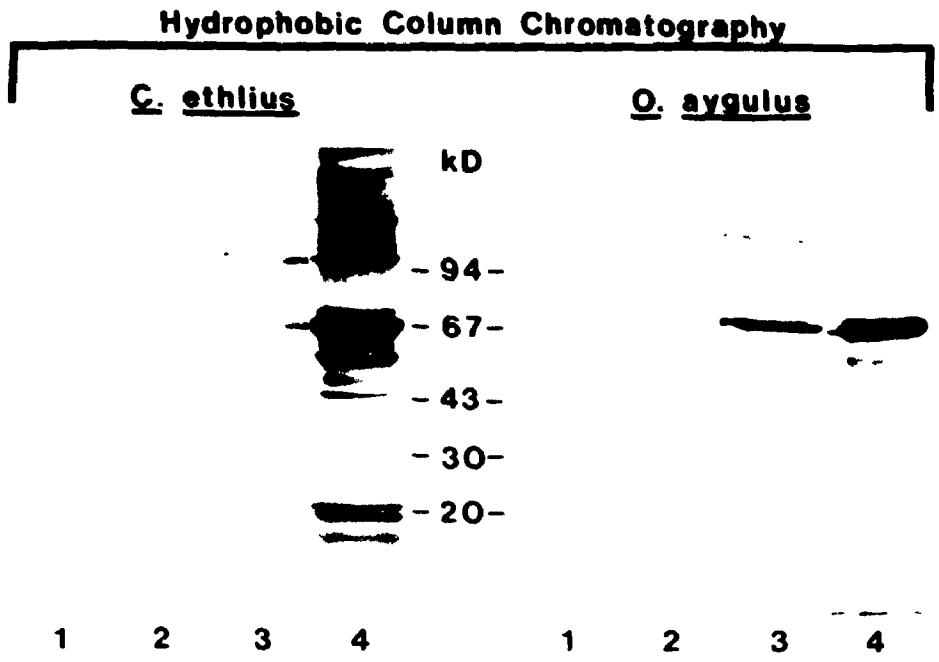
The third eluant was distilled water, which removes bound polypeptides of moderate hydrophobicity. For *Calpodes*, the protein profile was very similar to that obtained with the previous wash. Polypeptides with masses of 100, 67, 21, and 19kD were present in approximately the same quantity. Trace amounts of some other polypeptides, such as a 43kD

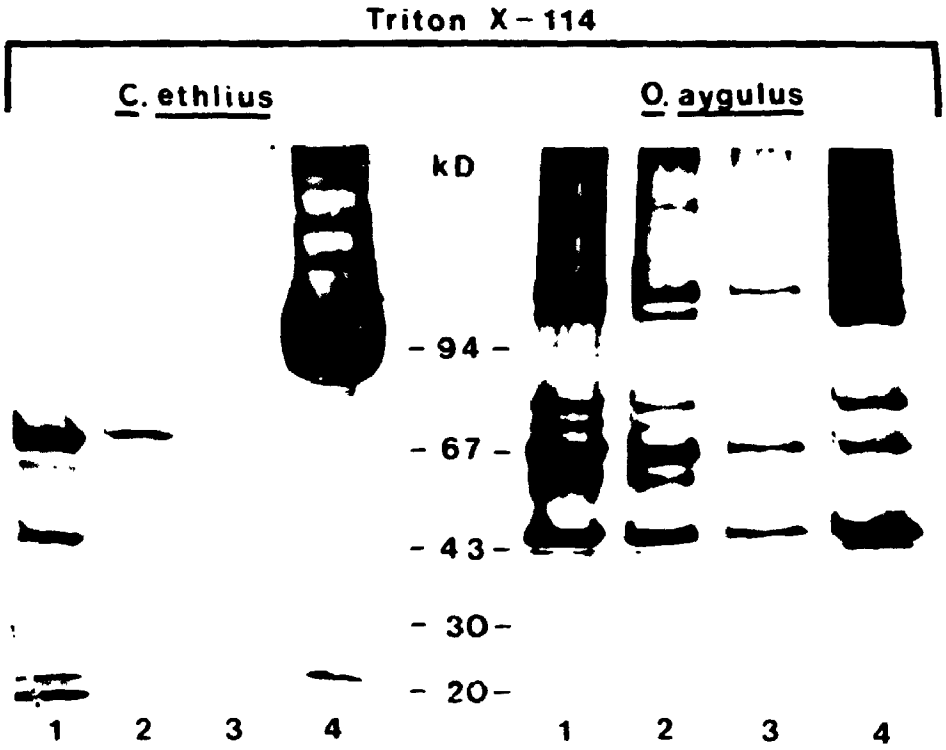
Figure 5:

Crystalline cone proteins of *Calpodes ethlius* and *Onitis aygulus* are predominantly hydrophobic.

Phenol sepharose hydrophobic column chromatography (top panel): Polypeptides were eluted from the column by a series of buffer solutions: (1) low salt buffer to remove hydrophilic polypeptides, (2) high salt buffer to remove polypeptides with weak hydrophobic groups, (3) distilled water to remove hydrophobic polypeptides and (4) 8M urea to remove the most hydrophobic polypeptides. Each fraction (40uL) was analyzed by 1D-SDS-PAGE and double stained with Coomassie blue and silver nitrate.

Phase-partitioning in Triton X-114 (bottom panel): Proteins varying in hydrophobicity can be isolated over a sucrose cushion according to their solubility in temperature-induced phases of Triton X-114. (1) Upper clear phase containing hydrophilic polypeptides. (2) Upper cloudy phase containing soluble hydrophobic polypeptides. (3) Sucrose cushion containing polypeptides of intermediate hydrophobicity. (4) Bottom phase containing the most hydrophobic polypeptides. The fractions (40uL) were analyzed by 1D-SDS-PAGE and stained with Coomassie blue and silver nitrate.


Hydrophobic Column Chromatography
C. ethlius
O. aygulus
kD
94
67
43
30
20
1
2
3
4
1
2
3
4


Triton X-114
C. ethlius
O. aygulus
kD
94
67
43
30
20
1
2
3
4
1
2
3
4

band, were also evident (Fig. 5, top left, lane 3). For *Onitis*, the most abundant polypeptide found in this eluate had a molecular mass of 67kD. Trace amounts of a 125kD, 47kD, and some of the very high molecular mass polypeptides were also detected (top right, lane 3).

The final wash of 8M urea removes the most hydrophobic polypeptides bound to the column. All the polypeptides of the crystalline cone of *Calpodes ethlius* were present in this eluate. The most prevalent bands had molecular masses of 145, 100, 74, 67, 60, 21 and 19kD (Fig. 5, top left, lane 4). The results, however, were not as clear for *Onitis aygulus.*

The most notable band had a molecular weight of 67kD. Small quantities of a 135, 125, 80, and a group between 57 and 70kD polypeptides were also apparent (Fig. 5, top right, lane 4). Although trace amounts of numerous polypeptides were present in the final wash, it did not appear that all polypeptides of the crystalline cone were recovered. Some of the proteins may have remained bound to the column.

### 2.3.7 B) Determination of Protein Hydrophobicity by Temperature-Induced Phase Separations of Triton X-114

Based on the above analysis, crystalline cone proteins are largely hydrophobic. To determine the degree of hydrophobicity, the proteins were fractionated by temperature-induced phase changes of Triton X-114. According to Pryde (1986), the size of a micelle formed by the detergent is proportional to the number of hydrophilic moieties of the proteins. The solubility of different sized micellar aggregates is temperature dependent. Each phase can easily be identified and isolated according to its position

and appearance in the extraction tube (Bordier, 1981).

Following the procedure outlined by Bordier (1981), the uppermost fraction in the extraction tube is the aqueous phase and contains proteins readily solubilized in water. From the lens of *Calpodes ethlius*, this fraction (Fig. 5, bottom left, lane 1) contains detectable amounts of all the major cone polypeptides. The most prevalent bands had molecular masses of 67, 45, 21 and 19kD. Trace amounts of the 145, 100, 80 and 57kD polypeptides were also present. The corresponding fraction from the crystalline cone of *Onitis aygulus* contained considerable amounts of 135, 125, 80, 67, and 47kD polypeptides (Fig. 5, bottom right, lane 1).

Lying just beneath the clear aqueous phase in the extraction tube is a cloudy phase that contains the soluble hydrophobic proteins. The polypeptide profile for this phase from the skipper butterfly was similar to that seen from the aqueous fraction (Fig. 5, bottom left, lane 2). Although the same polypeptides were present, the relative amount of each was reduced. Similarly, the cone polypeptides of *Onitis aygulus* recovered in this phase are the same as in the aqueous phase (Fig. 5, bottom right, lane 2).

The third phase was the sucrose cushion which contains polypeptides of moderate hydrophobicity. Few cone polypeptides were recovered in this phase from either the skipper butterfly or the dung beetle lens samples (Fig. 5, bottom, lane 3). In *Calpodes ethlius,* trace amounts of the 100kD and 67kD bands were present. In *Onitis aygulus*, the 135, 67 and 47kD polypeptides were barely detectable.

The detergent rich phase, which also appears cloudy, lies beneath the sucrose cushion and contains the most hydrophobic polypeptides. Many polypeptides were present in this fraction from the cones of both

species (Fig. 5, bottom, lane 4). In the skipper butterfly, all the high molecular mass polypeptides, especially the 100kD, were present in significant amounts. Polypeptides with masses less than 100kD were also present but in smaller quantities. In the dung beetle, the majority of the cone proteins were present in this fraction. The relative abundance of the cone polypeptides was highest in the detergent-rich fraction with the exception of the 67kD polypeptide which was more prominent in the less hydrophobic fractions.

### 2.3.8 Detection of Carbohydrates in the Crystalline Cone

Intact crystalline cones of adult *Calpodes ethlius* and *Onitis aygulus* were stained with periodic-acid Schiff's reagent to detect the presence of carbohydrate. A PAS-positive reaction was observed along the margin of the crystalline cone in both species (Fig. 6, top). The peripheral region of the crystalline cone of *Calpodes* also stained PAS-positive (top left). No positive reaction was observed in the body of the crystalline cone of *Onitis aygulus* (top right). Following treatment with alpha-amylase, which digests glycogen, the positive staining with Schiff's reagent was significantly reduced. This suggests that the crystalline cone of *Onitis* is wrapped in a thin, well defined sleeve of cytoplasmic glycogen. The crystalline cone of *Calpodes* is also encased in a glycogen sheath and may contain some glycogen in the cone body. This may account for the more pliable (more hydrated) cone in the skipper butterfly.

To determine further the carbohydrate content of the crystalline cone, the lectin Concanavalin A was used to assay for the presence of

Figure 6:

Carbohydrates associated with the crystalline cone.

Glycogen stain (top panel): The margins of isolated crystalline cones of *Calpodes* (left) and *Onitis* (right) stain positively with periodic-acid Schiff's reagent (PAS). A negative PAS-reaction was observed after the cones were treated with alpha-amylase, which extracts glycogen (not shown). This suggests the crystalline cone is surrounded by a glycogen sheath.

Concanavalin-A-binding (bottom panel): The cone polypeptides of *Calpodes* (lanes b and c) and *Onitis* (lanes d and e) were transferred to nitrocellulose and challenged with Concanavalin A. This lectin did not bind to any of the crystalline cone polypeptides, suggesting that they are not glycosylated. Polypeptides in lanes b and d were extracted in 4.5M urea and polypeptides in lanes c and e were extracted in 9M urea. Pure samples of ovalbumin (43kD, lanes a, f) and bovine serum albumin (67kD, lane g) serve as positive and negative controls for polypeptide glycosylation, respectively.

Periodic Acid-Schiff (PAS)

Calpodes ethlius Onitis aygulus

Concanavalin A

$M_r \times 10^{-3}$

94 -
67 -
43 -
30 -
20 -
14 -

a b c d e f g

glycosylated lens proteins (Fig. 6, bottom). None of the crystalline cone polypeptides extracted with 4.5M and 9M urea appears to be glycosylated in either species.

### 2.3.9 Specificity of the Antibodies Against Polypeptides of the Crystalline Cone of *Onitis aygulus*

Antibodies were raised against the 125 and 67kD polypeptides of the crystalline cone of *Onitis aygulus*. Purity of the lens antigens was determined by 1D and 2D SDS-PAGE (Fig. 7, panels a, b). The 125kD polypeptide did not focus as a discrete spot, but appeared as a streak which ranged in pI from approximately 6.6 to 8.0 (Fig. 7, panel a). A significant amount of the sample did not enter the IEF gel. This polypeptide is thought to have degraded upon storage because traces of lower molecular weight polypeptides were evident in the one- and two-dimensional profiles.

The purified 67kD lens antigen focussed into three major pI regions, but also showed evidence of degradation or possibly contamination from the isolation procedure (Fig. 7, panel b). Polypeptides with isoelectric points between 6.9 to 7.4 and 5.8 to 6.1 had molecular masses of 67kD. The third major spot, with a pI range between 6.3 and 6.6, had a molecular mass slightly below 67kD.

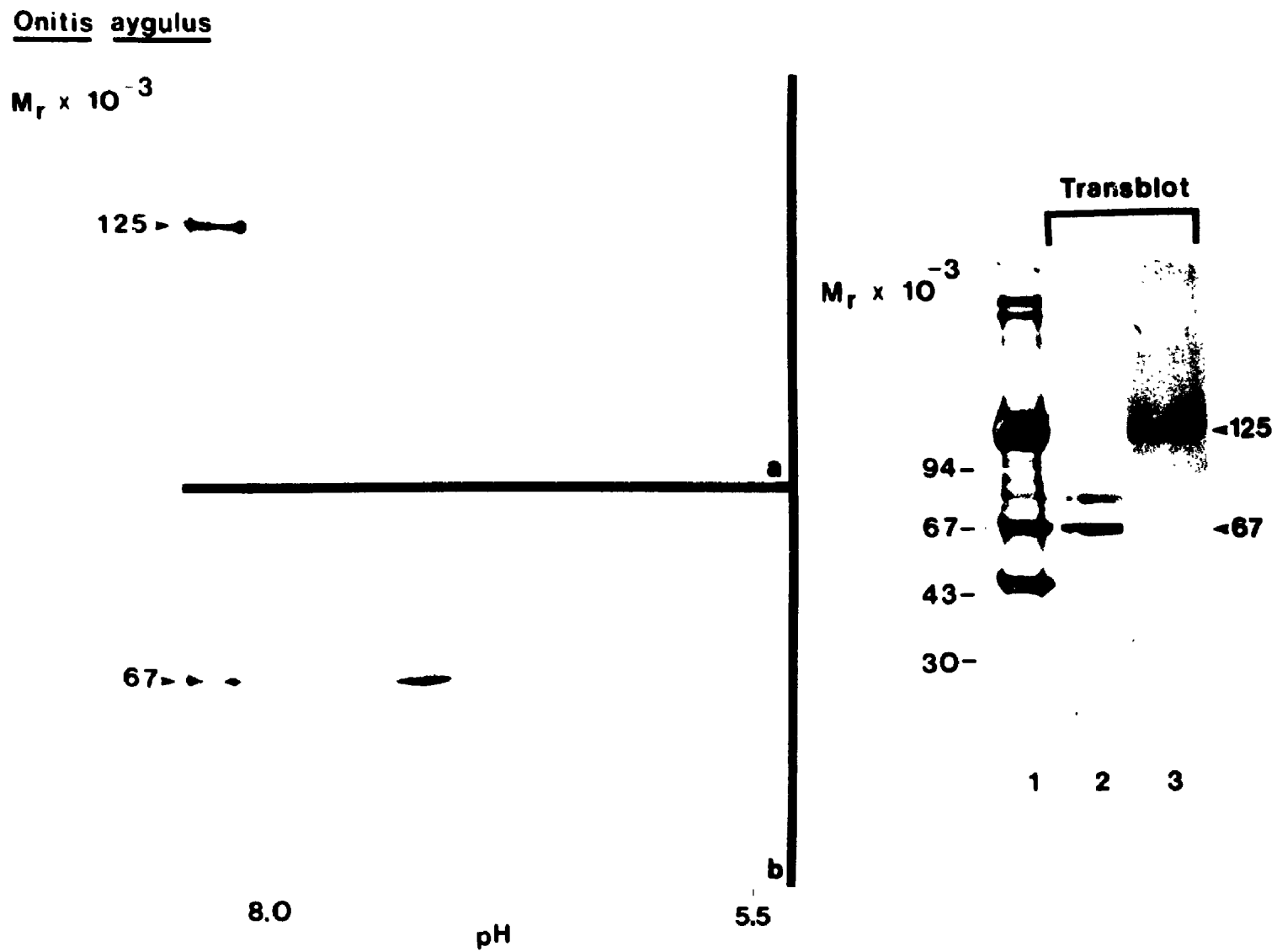
The 125kD and 67kD antigens were injected into two rabbits each. Rabbits #1 and #2 were used to raise polyclonal antibodies to the 125kD polypeptide, and rabbits #3 and #4 were used for the production of anti-67kD polyclonal antibodies. Antibodies raised to both antigens were challenged against the preimmune serum and against the total protein

Figure 7:

Specificity of antibodies raised against two major lens polypeptides.

Left: One- and two-dimensional SDS-PAGE separation of the 125kD (panel a) and 67kD (panel b) *Onitis* lens antigens cut from gels and used in the production of polyclonal antibodies (double stained with Coomassie blue and silver nitrate, see methods).

Right: The specificity of the antibodies raised against these two antigens was tested on the transblotted polypeptide profiles of the total proteins of the crystalline cone of *Onitis* (double stained with Coomassie blue and silver nitrate, lane 1). The anti-67kD antibody recognizes its antigen strongly but also cross-reacts with polypeptides of 80 and 125kD (lane 2). The 125kD antibody only recognizes the antigen against which it was raised.


Onitis aygulus
$M_r \times 10^{-3}$
125
67
a
b
8.0
pH
5.5
Transblot
$M_r \times 10^{-3}$
94-
67-
43-
30-
125
67
1
2
3

profile of the crystalline cone of *Onitis aygulus* (Fig. 7, lane 1). The preimmune serum from all 4 rabbits tested negative for the antibodies which they were used to produce. The anti-125kD antibody from both rabbit #1 and #2 showed high specificity to its antigen and did not cross-react with other lens polypeptides. The anti-67kD antibodies from rabbits #3 and #4 showed strong specificity to its antigen. The polyclonal antibodies produced in rabbit #4 also reacted to a lesser degree with the 125 and 80kD polypeptides of the cone (lane 2). In either case, positive reaction to the 125kD polypeptide by the anti-125kD antibody was not as strong as the positive reaction to the 67kD polypeptide by the anti-67kD antibody (see Table 1 for summary).

### 2.3.10 Cross-Reactivity of the Cone Antibodies with the Crystalline Cone of *Calpodes ethlius*

The 1D-SDS-PAGE separated polypeptides from the adult crystalline cone of *Calpodes* were transferred onto nitrocellulose and exposed to polyclonal antibodies produced by the 4 rabbits against *Onitis* lens antigens. Antibodies from each of the rabbits resulted in different patterns of positive reaction (Table 1). The anti-125kD antibody produced by rabbit #1 cross-reacted with the 100 and 80kD crystalline cone polypeptides of the skipper butterfly. The anti-125kD antibody raised in rabbit #2 cross-reacted with the 100kD polypeptide of *Calpodes*. The anti-67kD antibody produced by rabbit #3 only reacted with the 67kD polypeptide of *Calpodes* . The polyclonal antibodies raised against the 67kD polypeptide in rabbit #4 showed a positive reaction to the 100, 80 and 67kD polypeptides of the

**Table 1.**

**Transblotted protein profiles of the crystalline cone of *Onitis aygulus* and *Calpodes ethlius* were exposed to the four lens antibodies and scored according to the degree of reactivity. Strong positive reaction (+++), positive reaction (++), weak positive (+) and no reaction (-).**

| | | Proteins Recognized | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | *Onitis aygulus* | | | | *Calpodes ethlius* | | | |
| Antibody | Ab # | 125 | 80 | 67 | Other | 100 | 80 | 67 | Other |
| 125 | 1 | ++ | - | - | - | ++ | + | - | - |
| | 2 | ++ | - | - | - | ++ | - | - | - |
| 67 | 3 | - | - | +++ | - | - | - | ++ | - |
| | 4 | + | ++ | +++ | - | ++ | + | ++ | - |

skipper butterfly.

Since the polyclonal antibodies raised against the lens proteins of *Onitis* showed variable reaction patterns with the lens polypeptides of *Calpodes ethlius*, only the least cross-reactive, namely the anti-125kD polyclonal antibodies produced in rabbit #2 and the anti-67kD polyclonal antibodies produced in rabbit #3 were used for further analysis.

### 2.3.11 Immunoprecipitation of the 100kD and 67kD Polypeptides of *Calpodes ethlius*

The anti-125kD and 67kD lens antibodies of *Onitis aygulus*, which specifically recognized the 100kD and 67kD polypeptides respectively of the crystalline cone of *Calpodes ethlius* (Fig. 8, transblots), were used for immunoprecipitation.

The anti-125kD antibody precipitated the 100kD polypeptide from fractions #2 and #4, and to a lesser extent, fraction #1 of the extraction procedure (Fig. 8, top right). Fraction #1 contained urea soluble proteins. Fraction #2 resulted from the solubilization of the urea pellet in the RIA buffer and fraction #4 was obtained by the solubilization of the RIA pellet in SBA. Apparently, best recovery of the 100kD polypeptide occurred in the presence of SDS.

The anti-67kD antibody exclusively precipitated the 67kD polypeptide of the crystalline cone of *Calpodes ethlius* from fraction #3 (Fig. 8, bottom right). This fraction was obtained by solubilization in the RIA buffer which contains Triton X-100. The solubility properties of the 100kD and 67kD polypeptides are quite different.

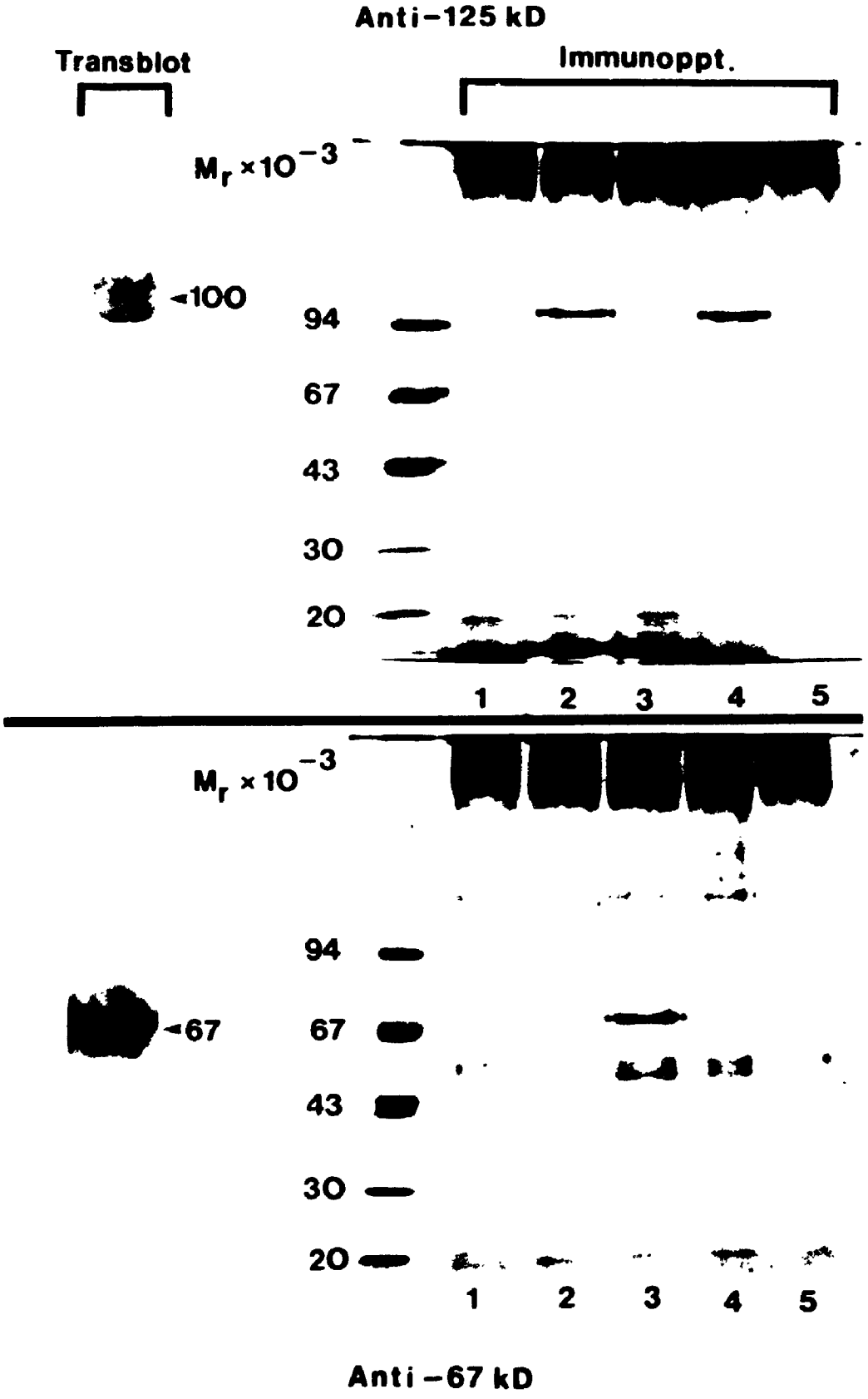
Figure 8:

Lens proteins in insect orders are immunologically related.

Cross-reactivity by transblot analysis (left): The anti-125kD (top) and anti-67kD (bottom) antibodies raised against the lens proteins of the beetle, specifically recognize the 100kD and 67kD lens polypeptides, respectively, of the skipper butterfly.

Cross-reactivity by immunoprecipitation (right): The same antibodies as above were used to immunoprecipitate lens polypeptides following cone extraction with: (1) 9M urea (2) RIA buffer of the insoluble urea pellet (3) RIA buffer (4) SBA buffer of the insoluble RIA pellet (5) Control lane containing no cone polypeptides (see text for details). The 100kD polypeptide is precipitated predominantly in extraction solutions containing SDS (lanes 2 and 4) while the 67kD protein is recovered exclusively from the fraction containing Triton X-100 (lane 3).

**Calpodes ethlius**



### 2.3.12 The 100kD and 67kD Lens Polypeptides of *Calpodes ethlius* May Be Related

The 100kD and 67kD polypeptides of the crystalline cone have similar denaturation products. This was first evident after repeated use of the purified samples that had been stored at -20°C (Fig. 9, center panel). Continual freezing and thawing of the samples resulted in similar breakdown products of both polypeptides. The 100kD polypeptide denatured into trace amounts of an 80, 67, 38 and 21kD bands. The 67kD polypeptides denatured into bands with molecular masses of 80, 38 and 21kD. Boiling the samples increased the quantity of the breakdown products.

Enzymatic digests were performed to determine further the degree of similarity between the 100 and 67kD polypeptides (Fig. 9, right panel). Treatment with trypsin resulted in identical banding patterns for both polypeptides. Digest patterns following treatment with chymotrypsin, protease X and protease XXV were also very similar for both polypeptides. The most noticeable difference was the intensity of the products: only minor differences occurred in the polepeptide profiles. For example, a 38kD band was not present in the protease X digest pattern for the 67kD but was evident in the 100kD digest profile. Similarly, two bands around 67kD were not present in the protease XXV pattern of the 100kD polypeptide but were present in the 67kD digest pattern.

### 2.3.13 Comparison of Lens Polypeptides in Different Species

To determine the similarity between the lens proteins of different

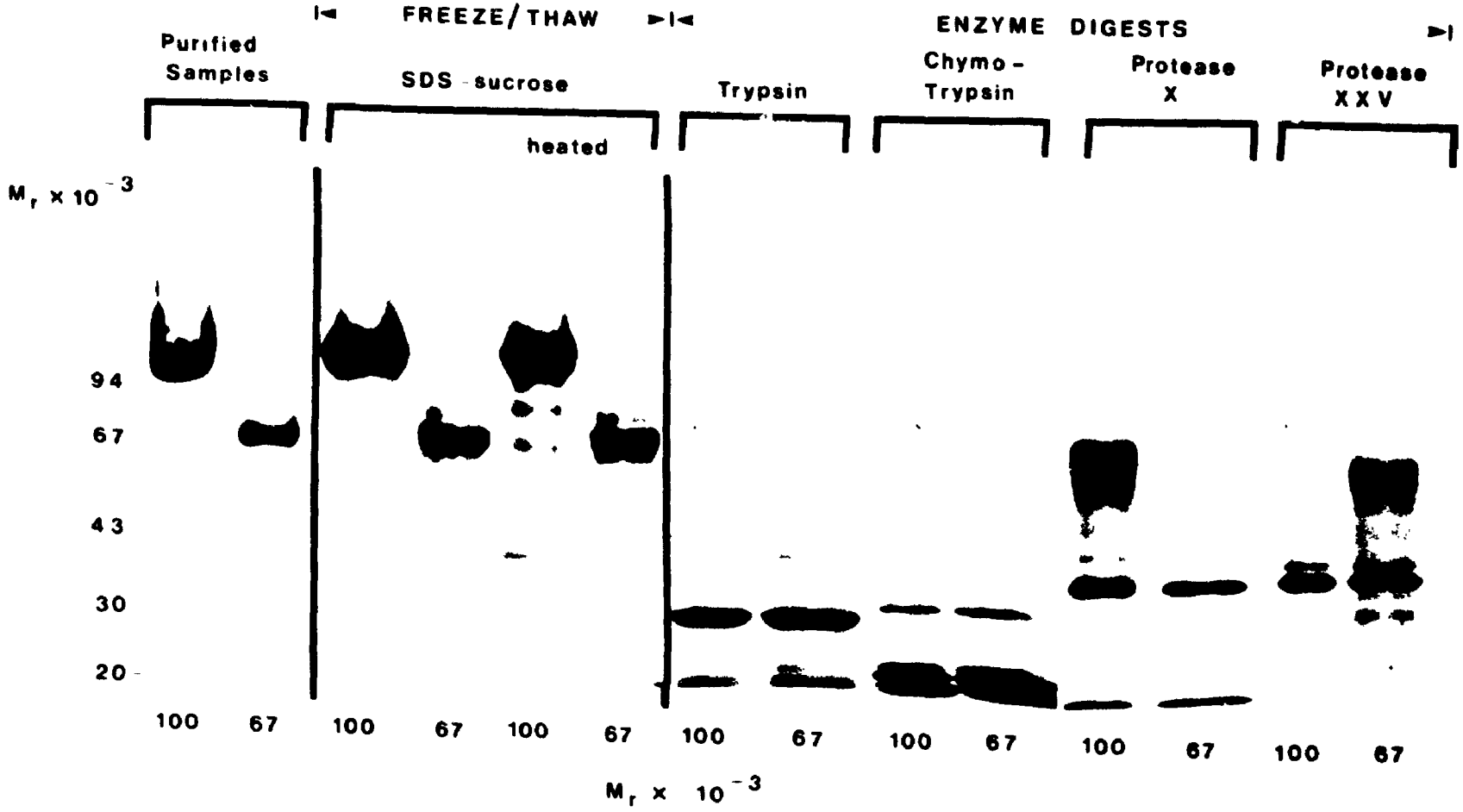
Figure 9:

The 100kD and 67kD polypeptides of the crystalline cone of *Calpodes ethlius* have similar denaturation products following freeze/thaw experiments and enzymatic digests.

Purified polypeptides (left panel): The 100kD and 67kD polypeptides were excised from SDS gels of total cone proteins, concentrated by electro-elution and visualized with Coomassie blue/silver double stain on 1D gels.

Freeze/thaw experiments (center panel): After numerous freeze/thaw cycles, samples of the natural degradation products of the 100kD and 67kD polypeptides were analyszed by 1D-SDS-PAGE (left). The two polypeptides have subunits of similar molecular weight. Heating the samples prior to electrophoresis promotes the natural degradation of the polypeptides (. .ght).

Enzymatic digests (right panel): The 100kD and 67kD polypeptides were digested for 5min with 5uL of four different proteases and the cleaved products were separated by 1D-SDS-PAGE. The molecular weights of the products from each enzyme digest are identical for the two polypeptides.


FREEZE/THAW
ENZYME DIGESTS
Purified Samples
SDS - sucrose
heated
Trypsin
Chymo - Trypsin
Protease X
Protease XXV
$M_r \times 10^{-3}$
94
67
43
30
20
100
67
100
67
100
67
100
67
100
67
100
67
100
67
$M_r \times 10^{-3}$

insects, the polypeptides of the crystalline cones of seven coleopteran species and two lepidopteran species were examined. The coleopterans consisted of the stag beetle (*Pseudolucanus placidus*; Lucanidae), the June beetle (*Phyllophaga spp.*; Scarabaeidae), and five dung beetles (*Onitis aygulus, O. alexis, O. caffer, O. pecuarius* and *Copris elphenor*; Scarabaeidae). The 2 lepidopterans were the skipper butterfly (*Calpodes ethlius*; Hesperiidae) and the tobacco hornworm moth (*Manduca sexta*; Sphingidae).

The crystalline cone polypeptides of the four *Onitis spp.* are similar (Fig. 10, lanes a-d). The major polypeptides of each have molecular masses of 125, 67 (although this polypeptide was not readily detected with Coomassie blue stain alone), 47, 43 and 38kD. However, there were interspecific differences. The polypeptide profile of *O. caffer* did not contain bands with molecular masses of 22 and 28kD (lane c), and the banding profile of *O. alexis* did not contain a 135kD polypeptide (lane b). Also, the polypeptides around 80-90kD were not as clearly defined in *O. alexis* (lane b), and *O. pecuarius* (lane d), as they were in *O. aygulus* (lane a), and *O. caffer* (lane c).

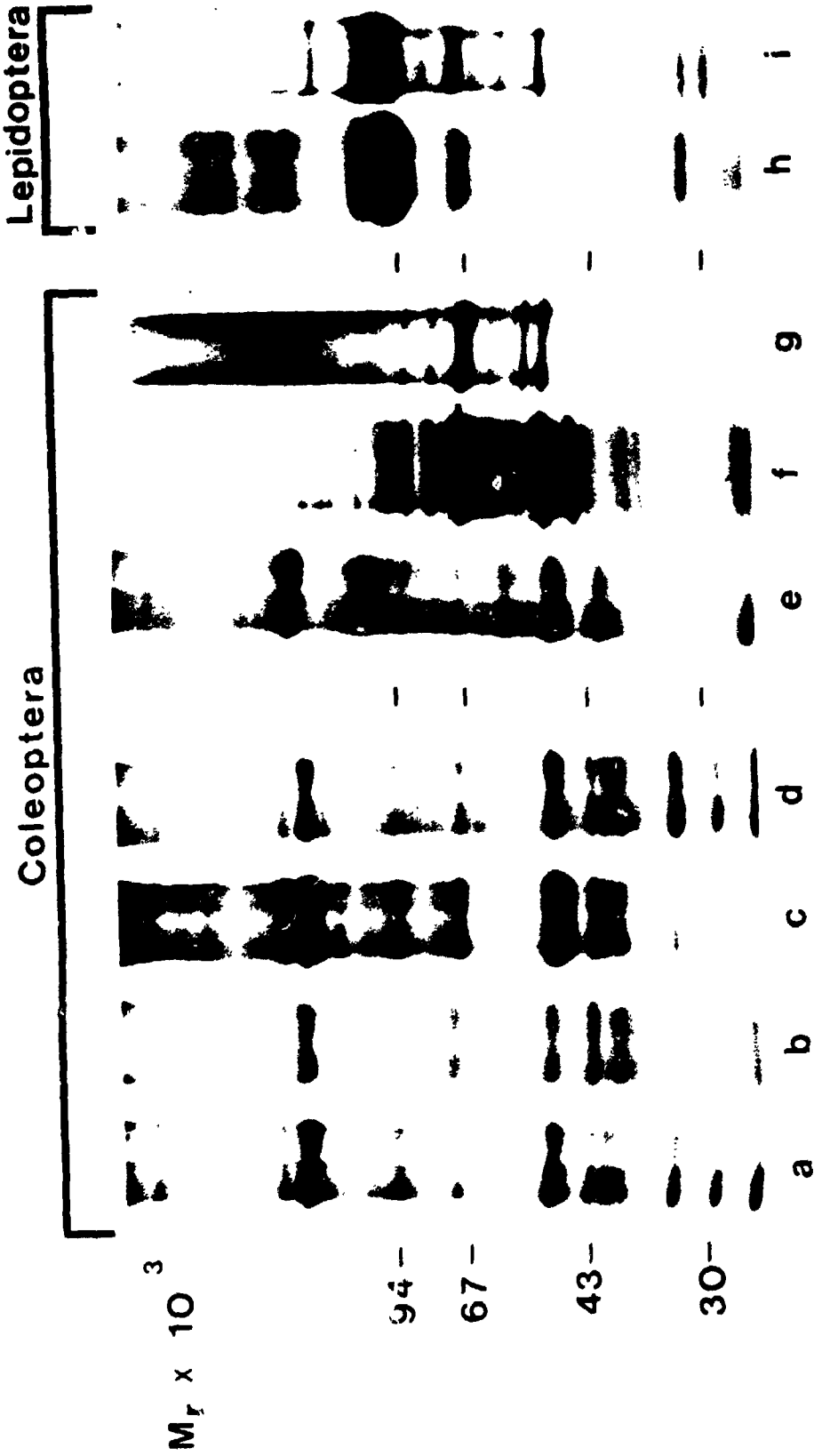
The polypeptides of the crystalline cone of the dung beetle, *Copris elphenor*, are similar to those of *Onitis spp.* (Fig. 10, lane e). The major polypeptides had molecular masses of 125, 47 and 43kD but this profile contained a more complex polypeptide pattern in the 50-60kD range and around 110kD.

The crystalline cones of the stag beetle *Pseudolucanus placidus* (Fig. 10, lane F) and June beetle *Phyllophaga spp.* (Fig. 10, lane G) have similar polypeptide patterns. The major polypeptides have molecular masses of 67 and 47kD. The cone of the stag beetle, however, is also rich in

Figure 10:

Crystalline cone polypeptides of seven coleopteran and two lepidopteran species.

Polypeptides were separated by 1D SDS-PAGE and stained with Coomassie blue. Dung beetles: (a) *Onitis aygulus*, (b) *Onitis alexis*, (c) *Onitis caffer*, (d) *Onitis pecuarius*, (e) *Copris elphenor*; (f) stag beetle (*Pseudolucanus placidus*); (g) June beetle; (*Phyllophaga spp*); (h) tobacco hornworn moth (*Manduca sexta*); (i) skipper butterfly (*Calpodes ethlius*). After Coomassie blue/silver nitrate staining, a 67kD polypeptide appears in the lenses of all species examined.


Lepidoptera
Coleoptera
i
h
g
f
e
d
c
b
a
94–
67–
43–
30–
$M_r \times 10^3$

a 45kD polypeptide which is not apparent in the June beetle. The crystalline cones of these 2 species do not appear to contain the high molecular weight polypeptides seen in samples from dung beetle crystalline cones.

The two lepidopteran species are also similar in their crystalline cone polypeptides. Both contain a large amount of 108, 100 and 67kD polypeptides (Fig. 10, lanes h, i). The protein profile of *Manduca sexta* cones also includes 4 high molecular weight bands which were absent from that of *Calpodes ethlius*.

## 2.4 DISCUSSION

The coleopteran and lepidopteran crystalline cone contains 3 groups of proteins which can be denatured into a small population of urea-soluble polypeptides. Although the cone polypeptides differ in molecular mass and/or pI in these two insect groups, and from the crystallins in the vertebrate lens (Clayton, 1974; Bloemendal, 1981; Pierscionek and Augusteyn, 1988), and the cephalopod lens proteins (Bloemendal, 1981; Siezen and Shaw, 1982) they do display similar characteristics. The crystalline cone polypeptides are present in high concentrations, are predominantly hydrophobic, are not glycosylated, and demonstrate some immunological cross-reactivity.

### 2.4.1 Lens Polypeptides Are Hydrophobic

Based on column chromatography, the crystalline cone proteins of *Calpodes* appear to be less hydrophobic than those of *Onitis* . As a consequence, the cone is more hydrated and less brittle. However, extraction with Triton X-114 shows that many of the cone polypeptides of *Onitis* are recovered in similar amounts in the aqueous phase and in the detergent phase. This suggests that the cone polypeptides may not be as hydrophobic as indicated by column chromatography. Pryde (1986) has reported similar findings using Triton X-114 extraction on integral membrane proteins of secretory granules, lymphocyte membranes and in

cytochrome b5. The partitioning of membrane proteins into the aqueous phase is caused by the formation of oligomers which remove their hydrophobic domains from the surrounding hydrophilic environment (Pryde, 1986). As a result, the aqueous phase of Triton X-114 is not considered as truly representative of hydrophilic proteins. The crystalline cone proteins may be acting in a similar manner.

### 2.4.2 Lens Polypeptides Are Conserved

The lens proteins of vertebrates (Alcala *et al.*, 1975; Piatigorsky *et al.*, 1978; Fu *et al.*, 1984) and insects (this study) are predominantly hydrophobic. The level of hydration found in a lens is believed to have evolved in response to the optical requirements of the species (Slingsby, 1985). This hypothesis is based on the correlation between the high concentration of delta-crystallins and the low concentration of gamma-crystallins in hydrated lenses found in species which must see well in both air and water (Slingsby and Miller, 1983; Stapel *et al.*, 1985). Relatively hydrated crystalline cones are present in diurnal insects, such as bees and butterflies (Warrant. 1988), which encounter varying light intensities. The degree of lens hydration may reflect the operative light intensity of the lens, but the functional significance of the different levels of lens hydration is unknown.

The most hydrophobic cone polypeptides of the beetle (125kD) and the skipper butterfly (100kD) do not readily enter or focus in an IEF gel, suggesting the presence of a high concentration of amino acids with nonpolar R groups (O'Farrell *et al.*, 1975). The gamma-crystallins are the

most hydrophobic vertebrate lens proteins. They are located in regions of the lens which are most dehydrated and are highest in refractive index (Slingsby and Miller, 1983). The gamma polypeptides bind with each other and other crystallins largely through hydrophobic side chains (Slingsby, 1985), and therefore are believed to control the hydration of the lens. The ability of the proteins to expel water assists in the closer packing of the proteins and allows for polymerization and transparency (Pierscionek and Augusteyn, 1988). It is reasonable to assume that the hydrophobic proteins of the insect lens function in a similar manner.

The 125kD and 100kD polypeptides not only exhibit similar solubility properties, but also have similar staining properties and immunological cross-reactivity. These reactions may result from similar amino acid compositions and/or conformational epitopes of the proteins. Most significantly, it shows that the two most abundant cone polypeptides of the dung beetle and skipper butterfly are immunologically related. This suggests that some of the crystalline cone proteins are conserved between the coleopterans and lepidopterans.

Conservation of the crystalline cone proteins between insect groups was indicated by the cross-reactivity of the 125kD and 100kD polypeptides with the anti-67kD lens antibody. This implies that all 4 polypeptides (125 and 67kD polypeptides of *Onitis aygulus*; 100 and 67kD polypeptides of *Calpodes ethlius*) are highly related. Regions of similar sequences are predicted to exist in the 67kD polypeptide and the higher molecular mass proteins because both freeze/thaw experiments and limited proteolytic digestion produce, in most cases, subunits with identical molecular weights. Possibly, the 67kD polypeptide binds together or with other cone proteins to form the high molecular weight polymer. Conversely, the

125kD and 100kD could be post-translationally processed or modified to give rise to different subunits, at least one with a molecular mass of 67kD. Processing of crystallin families has been observed in the vertebrate lens. The alpha-crystallins are deamidated, promoting the formation of polymeric structures. These alpha-crystallin aggregates become more insoluble and are thought to promote lens dehydration (Coopman *et al.*, 1984; Thomson and Augusteyn, 1988).

The 67kD family of proteins is the most highly conserved group of proteins in the arthropod lens. The 67kD families from both groups of insects contain a similar number of subunits, cross-react with the same lens antibodies, and have the same affinity for stains. The 67kD protein family has many characteristics of the beta-crystallins in the vertebrate lens. Both consist of several polypeptides which vary in pI and in solubility and are found in distantly related species.

### 2.4.3 Comparison of the Crystalline Cone Arthrocrystallins

The possibility that the 67kD polypeptides are glycoproteins, as suggested by their strong affinity for silver stain (Merril *et al.*, 1981; Dzandu *et al.*, 1984) is shown to be incorrect. Lectin binding with Concanavalin A and PAS staining showed that none of the cone polypeptides were glycosylated in the mature cone. The limited staining ability observed with Coomassie blue on the 67kD polypeptides is most likely due to the properties of the dye. Tal *et al.* (1985) showed that Coomassie blue binds proportionally to the number of positive charges on the protein and therefore displays an enhanced attraction for proteins rich

in lysine and arginine. Thus, the 67kD proteins probably do not have a high lysine and arginine content. This has also been suggested by the digest pattern obtained after treatment with trypsin, which specifically cleaves polypeptide chains at arginine and lysine sites. Only six subunit products were evident after digestion with trypsin for both the 67kD and 100kD proteins. The different staining properties of these two polypeptides, however, suggest that even though the 125kD and 100kD polypeptides are related to the 67kD family, they have a higher content of basic amino acids than the 67kD proteins. This cannot be verified until the amino acid composition of the crystalline cone polypeptides has been determined.

The 67kD family is the principle constituent of the arthropod lens and may possibly be regarded as a true 'arthrocrystallin'. The most abundant cone polypeptides of the skipper butterfly (100kD) and the dung beetle (125kD) are immunologically related to the 67kD family. The extent to which these 3 proteins are related is unknown.

### 2.4.4 The Importance of Polypeptide Homology in Lens Transparency

A lens must have a relatively high refractive power and must be transparent to be optically functional. A biological lens meets these requirements by containing structural proteins with few visible, closely packed chromophores and no abrupt discontinuities in refractive index (Slingsby, 1985).

A high concentration of proteins is necessary for protein-based lenses to obtain a high refractive index (Benedek, 1971). This criterion is met in insects, which have a large quantity of a few urea-soluble

polypeptides. The vertebrate lens, on the other hand, is composed of a combination of three to five families of crystallins. Close packing of particular combinations of macromolecules at critical water concentrations is dependent on the configuration, net charge and hydrophobicity of the side chains of the proteins (Slingsby, 1985). Therefore, it is the variation in lens protein composition and their concentration along the optical axis that determines the magnitude of refractive index (Piatigorsky, 1981; Slingsby, 1985).

Although concentrated protein solutions have a high refractive index, they are typically turbid. Benedek (1983) suggested that solution turbidity may be overcome when the protein density increases to the point that short-range inter-protein repulsion results in alignment of the components. This protein alignment is more readily accomplished with numerous copies of the same or similar proteins (Delaye and Tardieu, 1983). As the protein concentration increases, the relative distance between proteins decreases, causing the turbidity of the solution to decrease and transparency to increase (Delaye and Tardieu, 1983; Brewitt and Clark, 1988). The similarity in the subunits of the 100kD and 67kD crystalline cone polypeptides of *Calpodes ethlius* may be necessary for protein alignment and transparency.

### 2.4.5 Diversity of Biological Lens Proteins

The composition of the crystalline cone of insects suggests an evolutionary divergence in cone proteins similar to that seen in the vertebrate lens. The majority of the cone proteins, especially the most

prominent ones, appear to be conserved within closely related groups, such as the dung beetles. Differences in cone composition occur between species of dung beetles, but these differences are quantitative and not in the molecular mass of polypeptides present. More distinct differences occur between families of insects. For example, the 125kD polypeptide found in dung beetles is absent in the stag beetle. Similarities in lens proteins exist in vertebrates as distantly related as the Peking duck (*Anas platyrhynchos*) and the alligator (*Alligator mississippiensis*). At the same time, the protein profiles of closely related species may differ in both type and quantity of specific crystallins [e.g. Common Loon (*Gavia immer*) and Yellow-billed Loon (*Gavia adamsil*); Stapel *et al.*, 1985]. Similarities do exist, however, between coleopterans and lepidopterans. This was shown by the 67kD polypeptide family which was present in all the insect species examined. Possibly, this family of proteins will be found in all arthropod lenses. Many lens proteins are conserved between the vertebrates; some proteins also appear to be conserved among the insects.

# PART 3

# DEVELOPMENT OF THE CRYSTALLINE CONE

## 3.1 INTRODUCTION

In the previous chapter the protein composition of the mature crystalline cone was described. In this chapter the morphological and biochemical changes that occur during lens growth and maturation are described.

### 3.1.1 Refractive Index of the Lens

Biological lenses are unique in being graded refractive index lenses composed almost exclusively of hydrophobic proteins. The optical performance of a biological lens (which determines image quality at the level of the retina) depends both on its surface curvature and on the shape of its internal gradients of refractive index (RI). The refractive index of a graded-index lens is highest at the lens centre and lowest at the lens surface.

How is the graded refractive index established during lens development? Although the answer is still unclear, the most thorough

investigations have dealt with the developing crystalline lens of the vertebrate eye. The crystalline lens is composed of numerous cells which are rich in families of different crystallin proteins (Clayton, 1974; Bloemendal, 1977). The vertebrate lens grows by the addition of new cells at its periphery. This causes the differentiated (fiber) cells to compress into the core of the lens (Beebe and Piatigorsky, 1976). Thus, the lens proteins made first are found at the center of the lens. The lens apparently attains a graded distribution in refractive index due to molecular differences between core and peripheral crystallins rather than due to physical compression of the growth layers during lens development (Van Kamp and Hoenders, 1973; Pierscionek and Augusteyn, 1988). As a result, numerous attempts have been made to elucidate the radial distribution of different crystallins in the mammalian lens. Van Kamp and Hoenders (1973), tried unsuccessfully to separate the calf lens into its concentric growth layers by pushing lenses through a series of progressively smaller holes in a metal plate. Other physical methods applied to isolate individual growth layers have been only moderately successful (Maisel *et al.*, 1984; Li *et al.*, 1986).

The concentric growth layers of the mammalian lens have been successfully isolated by Pierscionek and Augusteyn (1988), who progressively dissolved the shells of the bovine lens away chemically. Each extract was examined for crystallin content. They showed that at the center of a mature bovine lens the alpha-crystallins constitute less than 5% of the total protein content, and that this concentration increases parabolically toward the lens margin, where it reaches a maximum of 40%. Similarly, beta-crystallins contribute less than 10% of the total protein at the center of the lens and approximately 35% in the peripheral

layers. Gamma-crystallins showed the opposite trend: a higher concentration in the core of the lens (10%) and a lower concentration in the final growth layers (<2%). The remaining crystallins were uniformly distributed throughout the lens.

The developing lens of the young calf did not show the same crystallin distribution (Pierscionek and Augusteyn, 1988). Alpha- and beta-crystallins each constitute about 35% of the total core protein. The majority of the betas at this stage are of the heavy-type (high molecular mass). The gamma-crystallins constitute 23% of the lens core. As the lenses age, there is a gradual transition toward the adult crystallin pattern. This transition involves the increased synthesis of light-type (low molecular mass betas) and a progressive decrease in synthesis of the gamma-crystallins. Alpha-crystallin synthesis remains constant but the core alphas become more insoluble with age. This is due to post-translational degradation and deamidation leading to polymer formation. This is thought to occur during dehydration of the lens and may assist in packing the proteins more tightly (Pierscionek and Augusteyn, 1988).

These results show that the refractive index gradient in the bovine lens is determined by the types of crystallin present, order of synthesis, processing, and their position in the lens. Similar results have been obtained in other mammalian lenses such as those of deer and gibbon (Chiou *et al.*, 1988), camel (Duhaiman, 1988), human (Fu *et al.*, 1984), rodent (Slingsby and Miller, 1983; Barron *et al.*, 1984) as well as those of birds (Narebor and Slingsby, 1985), and reptiles (Stapel *et al.*, 1985).

The relationship between the optical requirements of a lens and the crystallin distribution appears to be especially true for the gamma crystallins, which are always present in regions of low hydration and

where the RI is high. For example, nearly the entire rodent lens and the core of other mammalian lenses have comparatively high levels of gamma crystallins (Slingsby, 1985). Also, the refractive increment of purified and recrystallized gamma crystallins is higher than that observed for other proteins (Pierscionek and Augusteyn, 1988). Consequently gamma crystallins may function to shape the refractive index gradient of mammalian lenses by controlling lens hydration.

The optical properties of the crystalline cone of the insect compound eye are similar to those of the vertebrate crystalline lens; the crystalline cone has a graded internal refractive index. Because the cone is a tapered cylinder (and not biconvex like the crystalline lens), the RI of a cone is maximal along its axis and drops off radially along the cylinder and at its proximal ellipsoidal tip (Land, 1985; McIntyre and Caveney, 1985). The optical power of the crystalline cone lies almost exclusively in its graded-index optics, which together with its shape, position and size, optimizes the capturing, refracting, and focussing capacity of the lens system in the ommatidium. Although the RI gradients found in crystalline cones have been extensively documented (Caveney and McIntyre, 1981; McIntyre and Caveney, 1985; Land, 1985)), nothing is known about how these gradients are established.

Most of the information regarding the formation of the graded refractive index of the vertebrate lens has been obtained from analyzing the molecular components of the lens during its development. A similar approach may be followed to determine the formation of the refractive index profile in the insect crystalline cone.

alone, with the notable exception of the apparently silver-stain-specific 67kD polypeptide. The most notable difference between the protein profiles of the two lepidopteran species is the larger amount of the 100kD polypeptide in the lens of Manduca sexta (Fig. 15).

### 3.3.3 Protein Synthesis in the Developing Eye of *Calpodes ethlius*

Newly synthesized proteins labelled with L-[$^{35}$S]-methionine from *Calpodes ethlius* pupal eyes were separated as above and detected by fluorography. All the polypeptides of the pupal eyes seen in stained gels appear to be synthesized within 24 hours after pupation and this synthesis continues throughout the pupal period (Fig. 16). Most importantly, the major polypeptides of the adult cone are produced through the pupal period. These include the 100, 67, the poorly resolved group between 39-54, 21, and 19kD proteins.

The crystalline cones obtained after labelling eye tissue of newly emerged adults demonstrate preferential synthesis of the 100, 45, 21 and 19kD polypeptides. The synthesis of other cone proteins, such as the 67kD polypeptide, was not detected. Many polypeptides, however, are also being synthesized and incorporated into the cone at this stage in development (Fig. 16, CC). The synthesis of corneal proteins was not detected after adult emergence (results not shown).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### 3.1.2 Development of the Crystalline Cone

The first modern report on the development of the insect crystalline cone is that of Kim (1964) on *Pieris rapae* L. Cone development in *Pieris* begins approximately 35 hours after pupation. At this time, the cone cells are located at the distal end of the bundle of retinular cells and show an accumulation of PAS-positive substances. By 50 hours, the cone appears as a vacuole containing a large globule which also tests PAS-positive. At approximately 90 hours, the PAS-positive granules are no longer within the crystalline cone but are scattered at the margin of the newly forming cone. Just prior to emergence, the cones stain very readily with general protein stains and also show a homogeneous PAS-positive reaction. Kim (1964) concluded that in the early stages of development the PAS-positive granules were soluble polysaccharides which were eliminated during dehydration of the crystalline cone. He also suggested that later in development the polysaccharides polymerize and complex with proteins synthesized by the cone cells. This complex forms an insoluble mass which undergoes further dehydration to become the adult crystalline cone.

Gokan (1968) reported that the 4 crystalline cone cells of the Velvety Chafer (*Anomala sp.*) begin to secrete the cone-forming substance at pupation. Four days later, the secretions unite to form an immature cone, and the cones continue to grow as does the ommatidium. However, Gokan (1968) did not report further on how this might be accomplished or on the chemical composition of the cone-forming material

A more thorough examination of the development of the crystalline cone is necessary for a better understanding of how the refractive index of

these lenses is established. This chapter presents the first biochemical account of how the gradient of refractive index is formed in insect crystalline cones.

### 3.1.3 Models for the Formation of the Graded Refractive Index of the Crystalline Cone

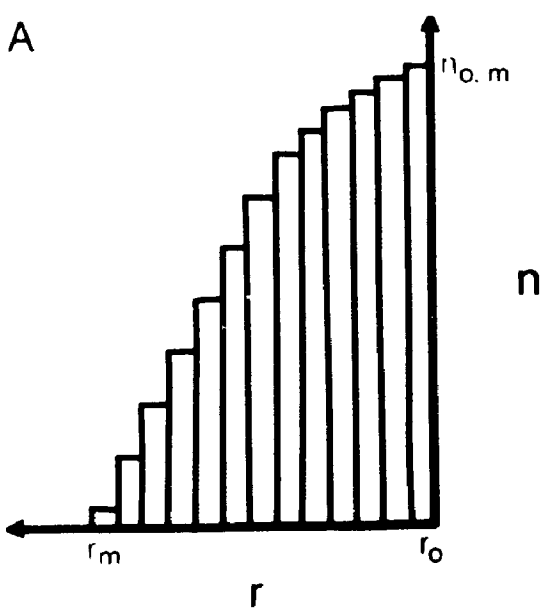
The graded refractive index of a crystalline cone could in theory be formed in several different ways. In order to help determine the actual mechanism it is useful to outline in general terms possible model mechanisms. This allows key differences between mechanisms to be identified and applied in the optical and biochemical analysis of the crystalline cone during development. All models must account for the striking near-parabolic drop in refractive index away from the lens axis, which theoretically gives the best optical performance to a cylindrical lens (Fletcher *et al.*, 1954; Caveney and McIntyre, 1981)

(i) The shell model (Fig. 11A). One obvious possible mechanism involves the deposition of layers of differing and final refractive index in successive shells around the cone axis. Each shell has a slightly lower refractive value (n) than that preceding it. This could be due to qualitative and/or quantitative differences in the protein composition of each shell. Each shell would thus be composed of a characteristic population of proteins and this should be evident when cones at different stages of growth are analysed biochemically. This mechanism requires that the synthetic sequence of lens proteins and the morphology of the cone are

Figure 11:

Models for the formation of the graded refractive index in the crystalline cone.

A; the shell model. B; the homogeneous model separating in time, cone growth and refractive index maturation. C; the constant parabola model. D; the homogeneous model in which gradient formation and growth occur simultaneously (see text for details).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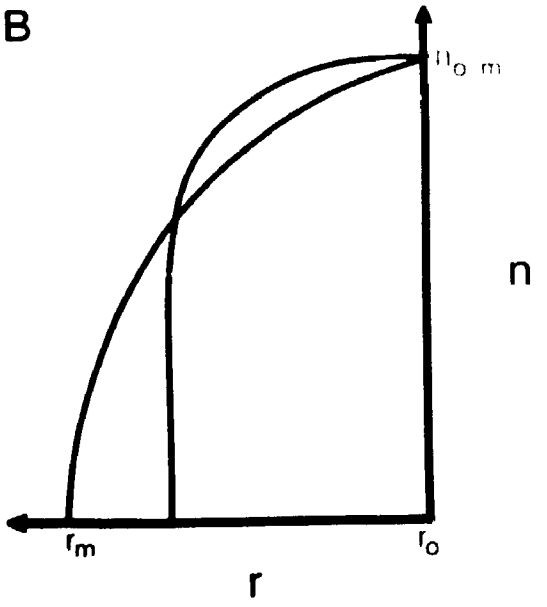
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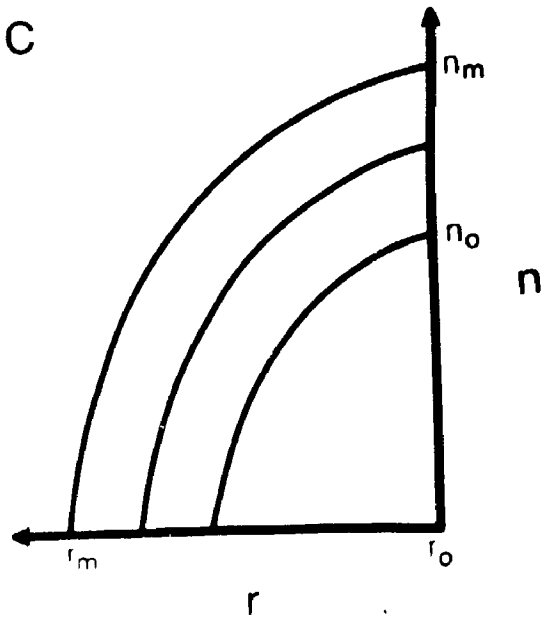
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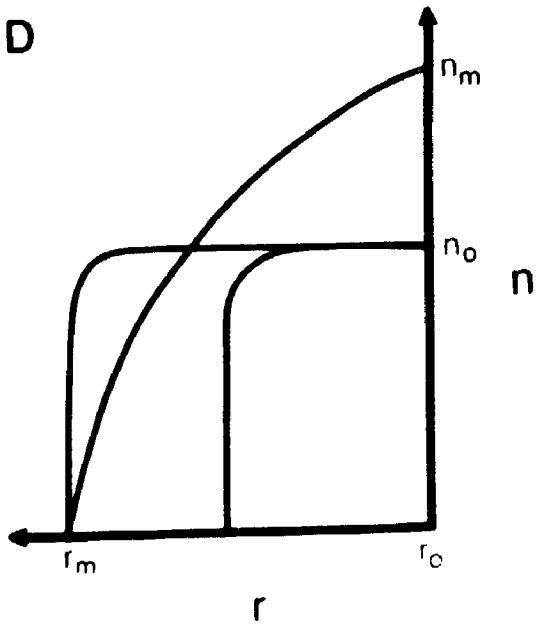
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precisely controlled throughout lens development. This model would account for the continuous growth of a miniature cone to a mature cone as observed in the chafer beetles (Gokan, 1968).

(ii) The homogeneous model, temporally separating cone growth and refractive index maturation (Fig. 11D). This model separates in time the deposition of the cone material from the formation of its graded internal RI. That is, the entire cone is first secreted as a hydrated homogeneous volume of low refractive index ($n_0$ in Fig. 11D), and the gradient forms only after the cone reaches its final volume (radius $r_m$). This could be a relatively spontaneous process that might only occur at a critical concentration of the hydrophobic lens proteins and would be associated with lens dehydration (which is known to occur). Due to the physicochemical nature of the lens proteins, the lens may self-assemble into a shell-like configuration of refractive indices. This reorganization would include the refractive index rising to a maximum value ($n_m$) at the cone axis and dropping to its minimal value ($n_0$) at the cone periphery (at $r_m$). The remaining RIs of the cone values would self-assemble to form a parabolic gradient of values intermediate between these two extremes.

Of the many possible models that are intermediates between the above two schemes for the formation of the graded refractive profile, the two below are worth mentioning:

(iii) Homogeneous model in which gradient formation and growth occur simultaneously (Fig. 11B). Here the cone proteins are initially deposited as a homogeneous mass with the axial refractive index at its

maximum ($n_m$) from the start, with a sharp drop-off in refractive index to a minimum value at the cone edge. As the cone continues to grow the proteins begin to interact with one another and reorganize to provide the appropriate spatial distribution of refractive indices. This model, then, involves the gradual appearance of the parabolic distribution of refractive indices during growth while the axial RI remains constant (differing from D above in both features).

(iv) The constant parabola model (Fig. 11C) This model requires that the refractive indices throughout the cone rise continually during cone growth to hold the parabolic GRIN distribution constant. That is, cone radius and axial refractive index climb in direct proportion so that when growth is complete ($r_m$), the maximum axial ($n_m$) and all intermediate values in refractive index are present and reorganization and index maturation in the cone is unnecessary.

### 3.1.4 Aims

What biochemical evidence is available to support any of the above models for the formation of the crystalline cone? At present, none. The aims of this study were to examine the developing crystalline cone with the following questions in mind:

i) What proteins are present in the developing crystalline cone?

ii) When during development are they synthesized?

iii) What is the morphology of the developing cone?

iv) Are there similarities between the development of the crystalline cone in the skipper butterfly and the scarab beetle?

v) What is the temporal relationship between development in the lens and the formation of the graded refractive index?

## 3.2 METHODS AND MATERIALS

### 3.2.1 Structural Analysis

#### 3.2.1 A) Fixation and Embedding

For structural analysis, pupal and adult eye tissue was fixed in a 5% glutaraldehyde / 0.2M phosphate buffer (Locke and Huie, 1980). Fixative was made by mixing 10mL of 50% glutaraldehyde with 10mL of distilled water and neutralized with several drops of sodium hydroxide. Fifty mL of 0.2M phosphate buffer (pH 7.3), and 2g of sucrose were added to the glutaraldehyde mixture, and the volume was brought up to 100mL with distilled water.

Tissue was fixed by injection and inflation according to Locke and Huie (1980). A short, gauge 20 needle was first inserted into the abdominal segment of the animal to allow blood and excess fluid to drain. The needle was then inserted into the thorax and extended just posterior to the head. Approximately 0.8mL of fixative was injected; this allowed complete inflation within seconds. The animals were placed at 4°C for 10 minutes, then the heads were removed and placed in fresh fixative overnight. Premature exposure to a large volume of fixative results in the extraction of soluble or slow fixing components (Locke and Huie, 1980).

Following fixation, the heads were washed 3 times in 0.1M phosphate buffer (pH 7.3) cooled to 4°C for 10 minutes each. The eyes were

then dissected from the heads and post-fixed in 1% osmium tetroxide in 0.2M phosphate buffer (pH 7.3) with 2% sucrose (Locke and Huie, 1980), for 3 hours at 4°C. The tissue was washed 3 times with a uranyl acetate rinse of 0.1M sodium acetate and 0.01M calcium chloride (pH 5.2), (Heuser and Reese, 1973), for 10 minutes each and stained overnight in uranyl acetate stain (0.5M sodium acetate, 5mM calcium chloride and 1% uranyl acetate, pH 5.2; Heuser and Reese, 1973).

When staining was complete, the eyes were washed 3 times in uranyl acetate rinse (pH 5.2) for 10 minutes each, dehydrated in a graded alcohol series of 70%, 95% and 100% ethanol for 10 minutes each, then washed twice in propylene oxide (15 minutes). Tissue was infiltrated with a 1:1 mixture of propylene oxide and araldite for 1 hour in a 45°C oven with occasional agitation. The 1:1 mixture was replaced with fresh embedding medium which contained 50mL of Epoxy Casting Resin A (Araldite 6005), 50mL of Epoxy Hardener DDSA (mixed thoroughly at 60°C) and 2mL of BDMA. The freshly embedded tissue was then placed in a 60°C oven for 20 hours (Locke and Huie, 1980).

3.2.1 B) Histology

For light microscopy, 1µm thick sections were cut using glass knives on a Reichert Ultramicrotome, placed on glass slides, and stained with 1% aqueous toluidine blue. Slides were viewed with a Zeiss microscope and photographed with technical pan film #2415 (Kodak Canada Inc., Toronto, Canada). A green #3 filter was used to enhance the contrast of the sections.

### 3.2.1 C) Electron Microscopy

Silver-gray sections were cut using a Du Pont diamond knife on a Reichert Ultramicrotome and placed on #400 copper mesh grids. The tissue was first stained with 10% uranyl acetate in a 45% methanol solution for 15 minutes at 22°C and then stained with 0.4% lead citrate in 0.15N sodium hydroxide for 1-2 minutes at 22°C (Heuser and Reese, 1973). Sections were viewed with a Philips 201 electron microscope and photographed using Kodak fine grain positive film.

### 3.2.2 Labelling with L-[35S]-Methionine

For labelling purposes, 8 eyes of specified stages and ages of *Calpodes ethlius* and *Onitis aygulus* were removed from the head, cut into halves or thirds and equilibrated in minimum essential medium minus methionine (MEM-met) for 30 minutes. Each preparation was then incubated with 0.1mCi of L-[$^{35}$S]-methionine (New England Nuclear (NEN), Boston MA; specific activity = 1000Ci/mmol.) for 3 hours at 27°C. The labelled samples were then placed on ice for 10 minutes, microfuged for 3 minutes and the supernatant was discarded. The pellet was resuspended in 1mL of MEM-met and the crystalline cones were isolated and treated in the same manner as previously described for gel electrophoresis.

### 3.2.3 Radioactivity Determination

Incorporation of L-[$^{35}$S]-methionine into lens proteins was measured after precipitation of eye tissue with trichloroacetic acid (TCA). The samples (2μL each) were precipitated in 0.5mL of 50% TCA, 0.1mL of 1mM methionine with 1 mg/mL BSA and 1.4mL of distilled water. The mixture was placed on ice for 90 minutes and filtered through a Whatman GF/c filter with 5% TCA. After air drying, the filter was placed into a scintillation vial (NEN) containing 5mL of a Triton X-100/toluene/Omnifluor (NEN) cocktail (Turner, 1968). All samples were counted in a Beckman LS-255 liquid scintillation counter.

### 3.2.4 Fluorography

Following electrophoresis and Coomassie blue R-250 staining and destaining, gels containing L-[$^{35}$S]-methionine labelled proteins were prepared for fluorography according to Bonner and Laskey, (1974) and Laskey and Mills, (1975). This was done by soaking the gels for 30 minutes in each of three separate baths of a 20-fold excess volume of dimethyl sulfoxide (DMSO). The gels were then transferred to a 22% (wt/vol) solution of 2,5-diphenyloxazole (PPO) in DMSO for 3 hours with mechanical agitation. After soaking, the PPO was precipitated in the gel by continuously rinsing each gel in water overnight. When all of the DMSO was removed from the gel, the gel was dried under vacuum (Bio-Rad drier Model 224) onto Whatman 3MM filter paper.

Fluorograms were obtained by apposing x-ray film (X-Omat R film

XR-1, Kodak Canada, Toronto) preflashed to an optical density of 0.15 next to the dried gel. The film was exposed to the gel in the dark at -70°C for a predetermined length of time based on the amount of radioactivity in the lysates applied to the gel. Finally, the x-ray film was developed and photographed.

## 3.3 RESULTS

### 3.3.1 Morphology of the Crystalline Cone of *Calpodes ethlius* During Their Development

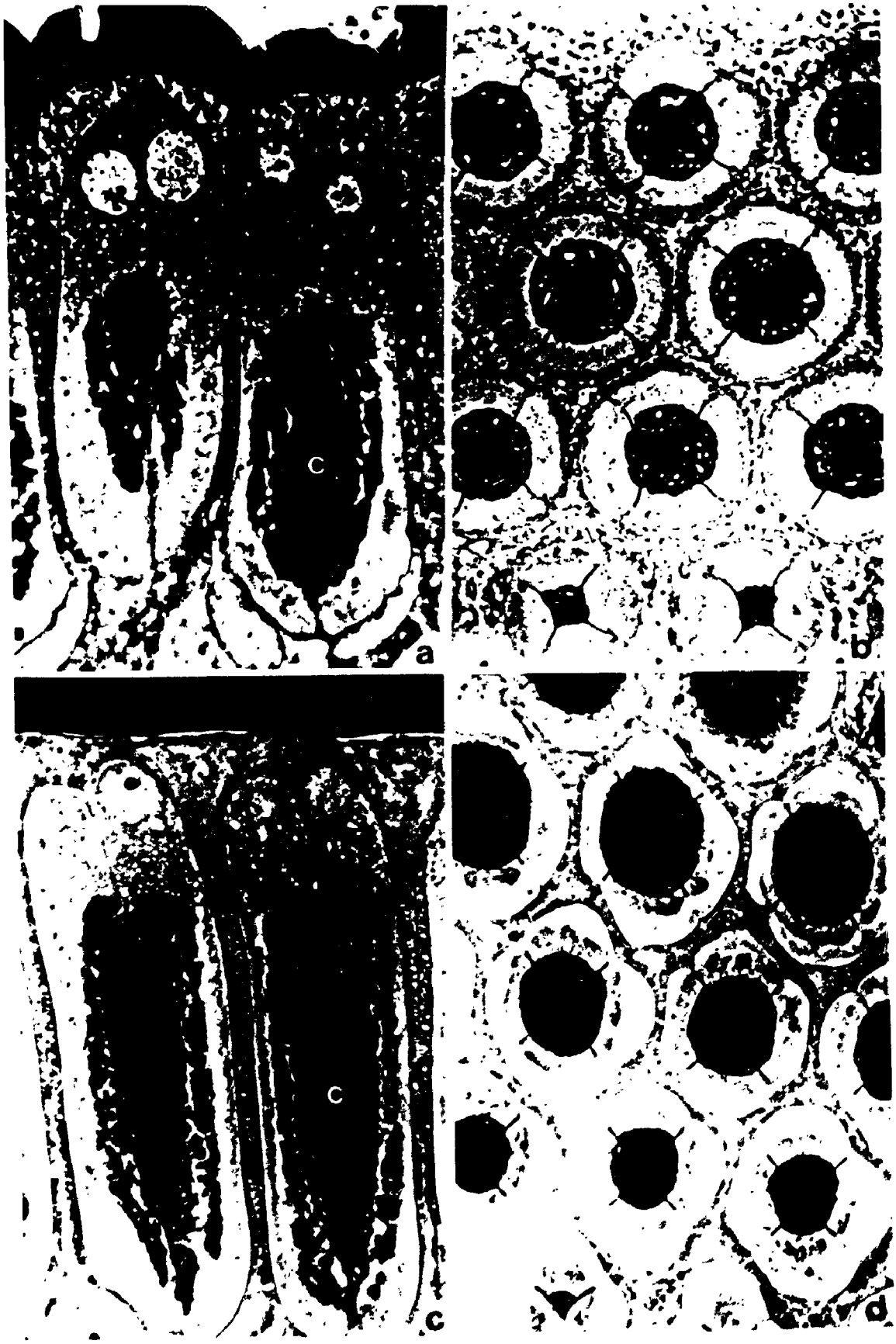
The skipper butterfly has a pupal period of approximately 10 days, depending on the rearing conditions. Until at least 3 days after pupation (P+3), the crystalline cone of *C. ethlius* is not detected ultrastructurally. By P+6, approximately 15% (by volume, see Appendix 1) of the cone material is deposited. This deposition is seen histologically as a darkly staining, granular mass. When viewed in longitudinal section (Fig.12a), the newly forming cone first appears in the mid to distal region of the cone cells while the cone cell nuclei are always in the most distal region of the cells. At this time in development, the cone is irregular in shape but is thickest in the middle and tapered at each end. The distorted shape of the cone is due to the presence of numerous longitudinal columns that at this time do not yet contain the dense, granular components of the cone material.

By scanning across a sagittal section of a compound eye, cones at the same stage in development may be seen at different levels of section along the cone axis (Fig.12b). Cone cells that in section appear closest to the cornea (top) are sectioned through the most distal region of the cone while those farthest from the cornea (bottom) include sections through the most proximal region of cone. Four darkly staining regions of plasma membrane radiate out from the axis of the newly deposited cone, which at this stage is circular and sponge-like in appearance. The sponge-like

Figure 12:

Growth of the crystalline cone of *Calpodes ethlius* during development.

(a) The crystalline cone (white c) of a P+6 day pupa consists of a dense granular material with no distinct shape. (b) In cross-section, the cone appears sponge-like with four darkly staining membranes radiating from the center. (c) The cones in a a P+8 day pupa are porous, more tapered and are surrounded by a flocculent substance. (d) In cross-section, the majority of the cone at P+8 is compact and distally, the deposition approaches the extent of the darkly staining membrane (top of (d)). (mag. 1000x).


c
a
b
c
c
d

appearance is due to the hollow columns mentioned earlier, which do not contain any of the cone material. Within this sponge, however, is a very dense deposit shaped as a 4-pointed star. Each point is bisected by the apposing plasma membranes of the 4 cone cells, only part of which is darkly stained. This densely staining membrane defines what appears to be the limits of the future cone and is also the site of initial cone deposition. Cross-sections through the more proximal regions of the cone are spongy and square in shape. This suggests that the cone material, which is initially deposited next to the plasma membrane, is not densely packed until a later stage in development.

By P+8, 60% of the cone material is deposited and the cone assumes a definite bullet-shape appearance (Fig. 12c). The distal region of the cone is no longer tapered but is now the site of greatest deposition. Although the cone material is still spongy, the hollow columns are not as pronounced. Immediately surrounding this deposit is a cloud of flocculent substance not evident in earlier stages of development. In cross-section (Fig. 12d), the majority of the cone is very compact and the deposition nearly extends the length of the specialized membrane. The proximal tips of the cone are less developed and hence more spongy in nature.

The development of the crystalline cone is complete by adult emergence and the compound eye is mature. The cone is transparent and bullet-shaped, with an average length of 80 microns and a distal width of 20 microns. The cone is easily dissociated from the surrounding pigment cells and may be removed intact and free of most of the cone cytoplasm. The cone is stable even when divested of the cell cytoplasm, but remains pliable, especially in its most proximal region.

In the mature eye, the crystalline cone occupies most of the volume

of the cone cells (Fig. 13a). The cone cell nuclei are condensed and displaced into a narrow space between the distal end of the cone and the cornea. The cone material in the mature eye is no longer sponge-like in texture but is uniformly electron-dense in consistency, with the exception of a less darkly staining margin along the periphery and at the proximal tip of the cone. Evidence of these two layers was more pronounced in cross-sections (Fig. 13b). The inner layer is granular while the outer layer appears more flocculent. Ultrastructural examination showed that this granularity is unevenly distributed in the cone. It is most dense at the center of the most distal region of the cone (Fig. 13c) and this density gradually decreases along the axis and toward the periphery. Cross-sections through the proximal region of the cone (Fig.13d) clearly showed a change in the density of the cone material with only a small fraction in the center being highly osmiophilic. This morphological change coincides with the pattern of refractive index within the crystalline cone (Caveney and McIntrye, 1981).

### 3.3.2 Pupal Eye and Adult Crystalline Cone Polypeptides of *Calpodes ethlius* and *Manduca sexta*

Proteins isolated from eye tissue at successive stages in pupal development were separated by SDS-PAGE and the gels were stained with Coomassie blue. These proteins include those of the cone cells and pigment cells, because in the pupa the two cell types could not be separated. At 24 hours, the pupal eye has numerous polypeptides ranging from 15kD to approximately 145kD (Fig. 14c, lane 1). The pattern remains

Figure 13:

Morphology of the adult crystalline cone of *Calpodes ethlius.*

(a) The tapered cylindrical cone of the adult (white c) occupies most of the cell cytoplasm and displaces cell organelles to the periphery (mag. 900x). (b) In cross-section, the central region of the cone is very granular and stains densely with toluidine blue. Surrounding this region is a less darkly staining margin which becomes more pronounced in proximal section (b, bottom; mag. 1000x).

(c and d) Ultrastructurally, the cone has numerous osmiophilic granules in the distal regions (c) and fewer in the proximal regions (d) which coincides with the distribution of refractive index (mag. approximately 3,300x).

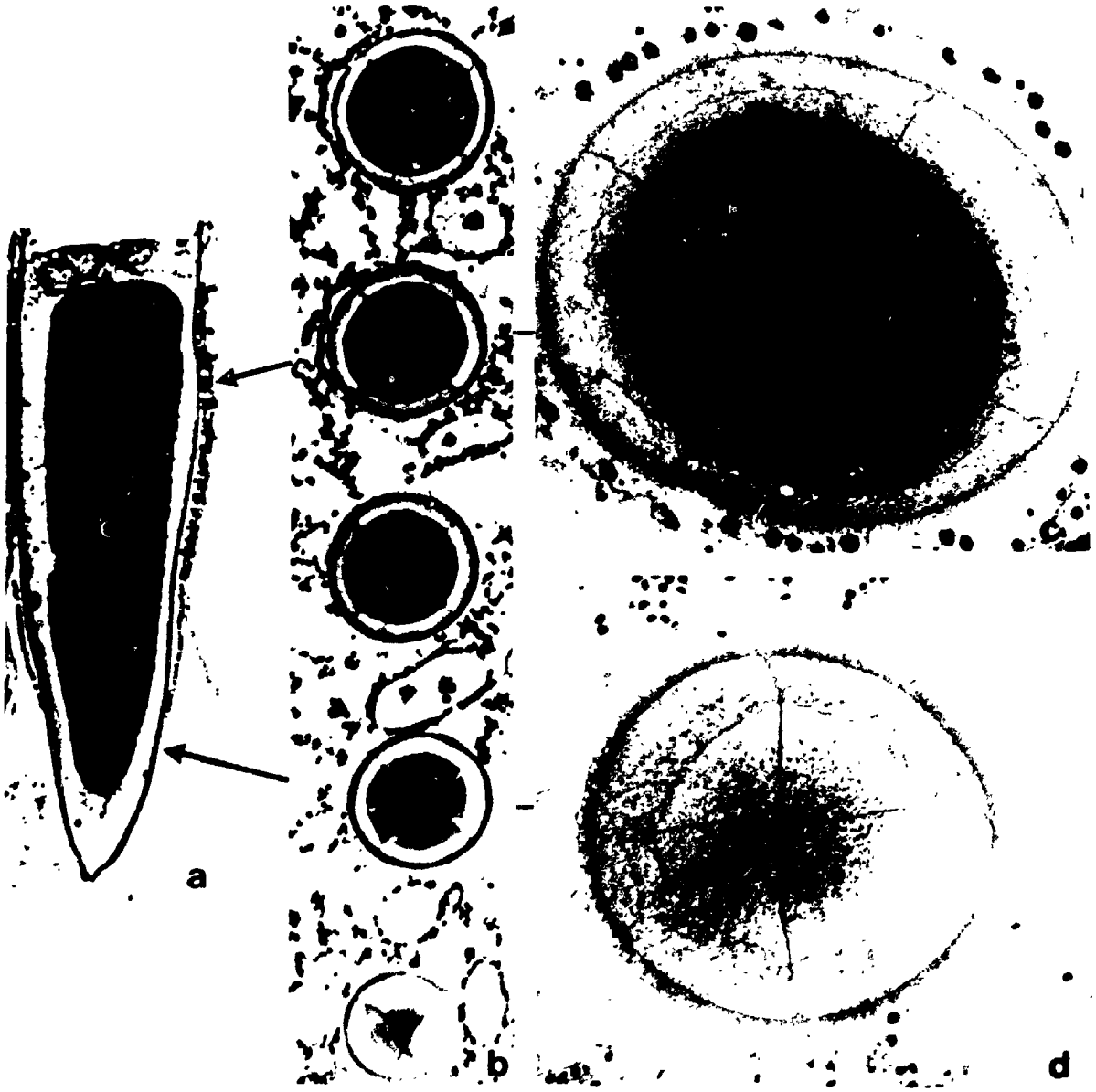

a
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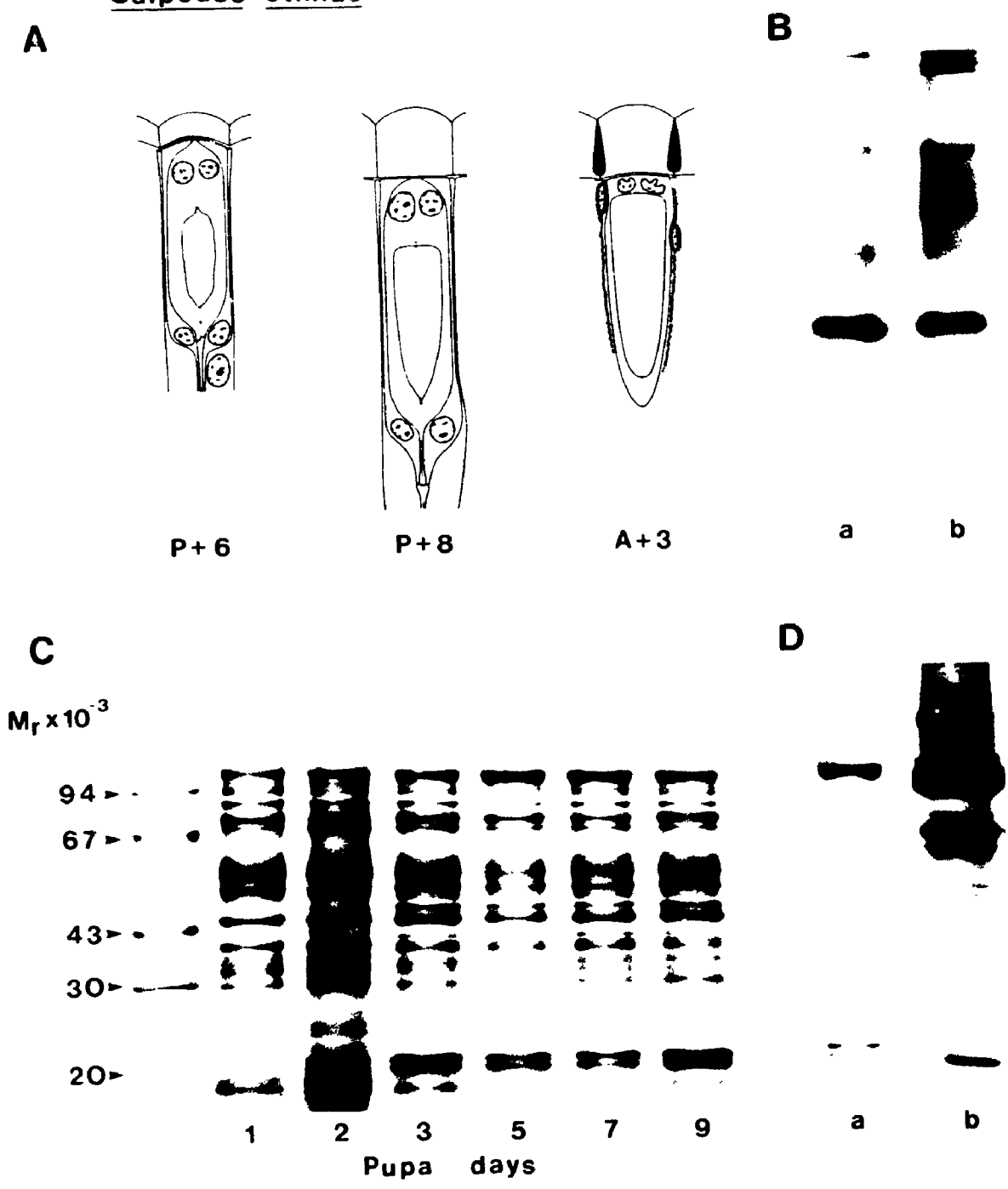
Figure 14:

Development of the crystalline cone of *Calpodes ethlius*.

A: schematic representation of cone development in the pupa (P+6, P+8) and early adult (A+3). B: native proteins of the adult crystalline cone stained with Coomassie blue (a) and double stained (b). C: polypeptides of the lens at various stages of pupal development. All the major cone proteins are present in the pupal eye within three days of pupation. D: polypeptides of the adult crystalline cone visualized with Coomassie blue (a) and double stained with silver (b). The 100kD and 67kD proteins are the most abundant cone polypeptides in this insect.


Calpodes ethlius
A
B
P+6
P+8
A+3
a
b
C
D
$M_r \times 10^{-3}$
94
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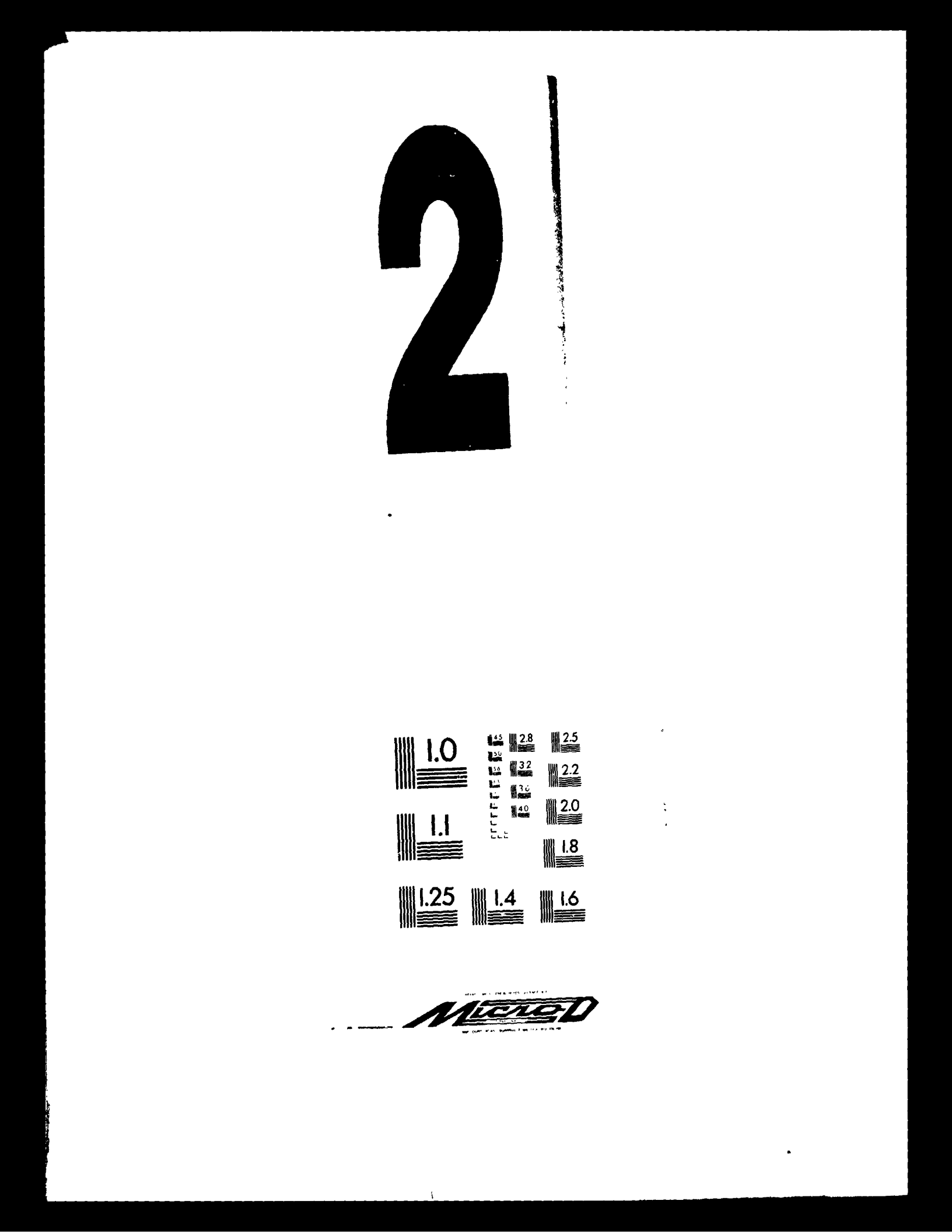
43
30
20
1
2
3
5
7
9
Pupa days
a
b

unchanged at 48 hours (Fig. 14c, lane 2). By day 3 of pupal development, when the cone first begins to form, two minor bands with molecular mass of 40kD and 47kD become apparent (Fig. 14c, lane 3). After this, the polypeptide banding profile remains constant throughout the rest of eye development in the pupa. The major cone proteins appear to have molecular masses of 108 and 100kD.

Double staining with silver nitrate followed by Coomassie blue of representative pupal eye stages of *Calpodes ethlius*, revealed additional polypeptides (Fig. 15). Most of the polypeptides previously discussed bind both stains. Some proteins, such as a 67kD polypeptide and several polypeptides between 42 and 46kD are more readily detected with silver stain. Although the protein profile appears to be conserved throughout pupal development, the 42 and 44kD polypeptides increase in relative amounts.

A similar polypeptide profile exists in the developing eye of *Manduca sexta* (Fig. 15). Like the skipper butterfly, the major crystalline cone proteins have molecular masses of 108, 100 and 67kD. The 100kD polypeptide appears early in development while the 67kD is the most prevalent polypeptide in the late pupal stage.

Isolated adult crystalline cones of both *Calpodes ethlius* and *Manduca sexta* have a smaller number of polypeptides when compared to the crystalline cones obtained from the eye of the pupal stage. The major cone proteins have molecular masses of 145, 108, 100kD, a group of polypeptides between 39-45kD, and a pair at 21 and 19kD. The 100kD protein is most abundant. Double staining of the gels of adult crystalline cone polypeptides also revealed more polypeptides. The most prominent proteins, however, were those previously visualized with Coomassie blue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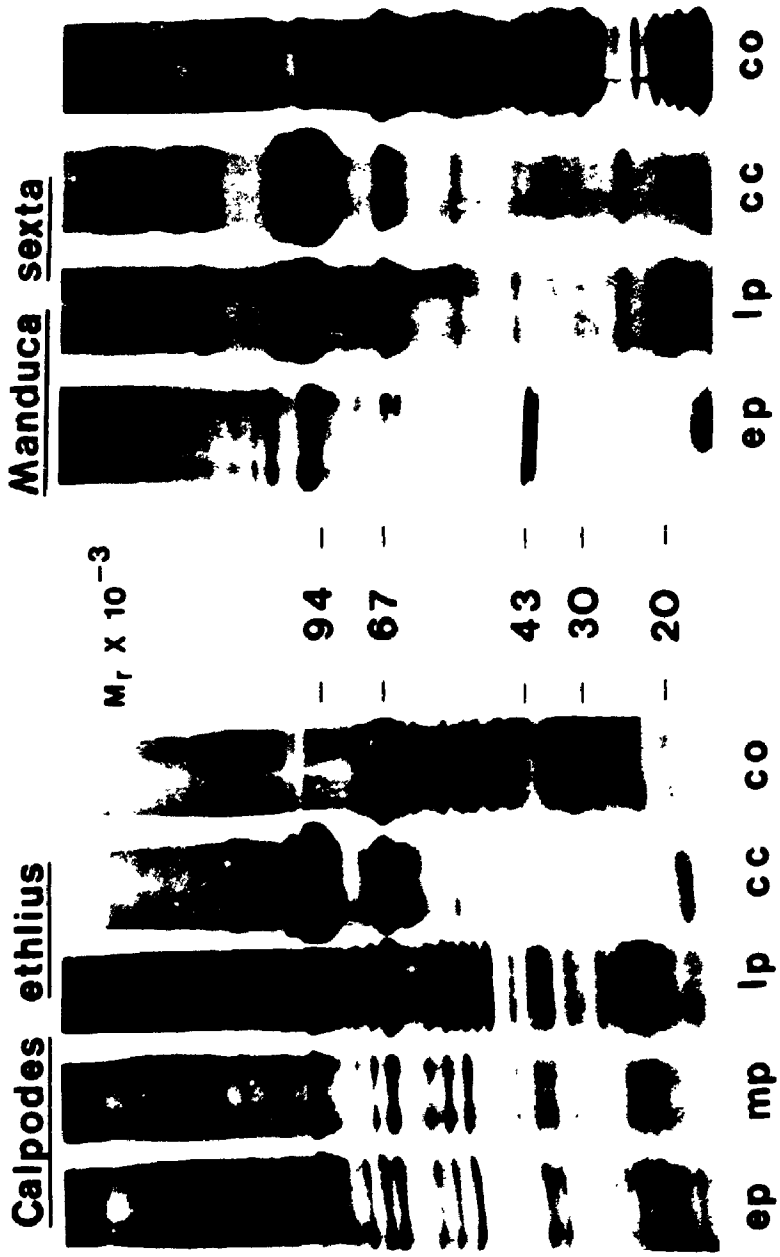

Calpodes ethlius
Manduca sexta
ep
mp
lp
cc
co
$M_r \times 10^{-3}$
94
67
43
30
20

alone, with the notable exception of the apparently silver-stain-specific 67kD polypeptide. The most notable difference between the protein profiles of the two lepidopteran species is the larger amount of the 100kD polypeptide in the lens of Manduca sexta (Fig. 15).

### 3.3.3 Protein Synthesis in the Developing Eye of *Calpodes ethlius*

Newly synthesized proteins labelled with L-[$^{35}$S]-methionine from *Calpodes ethlius* pupal eyes were separated as above and detected by fluorography. All the polypeptides of the pupal eyes seen in stained gels appear to be synthesized within 24 hours after pupation and this synthesis continues throughout the pupal period (Fig. 16). Most importantly, the major polypeptides of the adult cone are produced through the pupal period. These include the 100, 67, the poorly resolved group between 39-54, 21, and 19kD proteins.

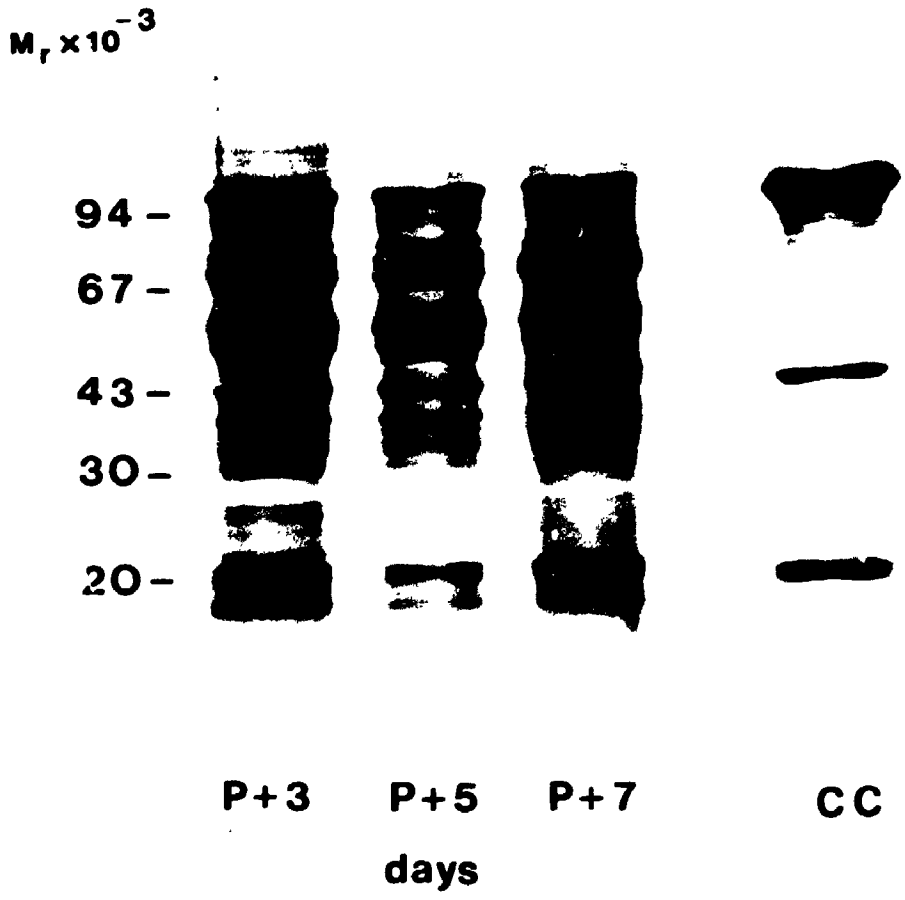
The crystalline cones obtained after labelling eye tissue of newly emerged adults demonstrate preferential synthesis of the 100, 45, 21 and 19kD polypeptides. The synthesis of other cone proteins, such as the 67kD polypeptide, was not detected. Many polypeptides, however, are also being synthesized and incorporated into the cone at this stage in development (Fig. 16, CC). The synthesis of corneal proteins was not detected after adult emergence (results not shown).

Figure 16:

Synthesis of crystalline cone polypeptides in *Calpodes ethlius.*

One-dimensional (SDS-PAGE) fluorogram of the cone polypeptides synthesized in the presence of $^{35}$S-methionine by the cone cells at 3, 5 and 7 days of pupal development (P+3, P+5, P+7) and after adult emergence (CC). All the major cone proteins appear to be actively synthesized in a coordinate manner throughout the pupal stage. The 67kD polypeptide does not appear to be produced in the adult cone (cc). Each lane was loaded with 50,000 cpm.

## C. ethlius



### 3.3.4 Morphology of the Crystalline Cone of *Onitis aygulus* Throughout Development

The pupal period of *O. aygulus* is about 15 days long at 28°C. Structural analysis showed that by P+8 only 4% of the total cone material has been deposited. Initial deposition occurs in the more distal region of the cone cells where the plasma membranes of the cone cells meet (Fig. 17a,b). Unlike in *C. ethlius*, the cone material is not sponge-like in the developing eye but is very granular prior to cone formation. Often several of these granules cluster together before fusing with the growing mass of cone material (Fig.18). This activity continues sequentially throughout the pupal stage.

At P+10, nearly 30% (by volume) of the cone is deposited. Growth at this stage is predominantly in cone length (Fig. 17c). In cross-section, the proximal region of the cones appears square while the distal regions, because of greater deposition, show pillow-shaped profiles (Fig. 17d). At the end of the pupal period, the crystalline cone remains less than 70% complete. It is at this time, however, that the cone first assumes a waisted, bullet-shape in longitudinal section (Fig. 17e). Cross sections show that extensive growth is still necessary for maturation, because the distal regions of the cone are not yet circular and the proximal regions still appear as inflated squares (Fig. 17f).

In constrast to the lens of the skipper butterfly eye, the crystalline cone of the dung beetle grows extensively during the first 3-4 days after adult emergence. Much of this growth occurs in the formation of the ellipsoidal proximal tip, although some growth occurs in shaping the distal region to give the precise bullet-shape found in the mature adult

Figure 17:

Growth of the crystalline cone of *Onitis aygulus* during development.

(a, b) The crystalline cone (white c) in the eye of 8 day old pupa is very granular and is first deposited in the distal region of the cone cells along their apposing cell membranes (a, b; mag. 750x). The cone appears tapered in longitudinal sections at 10 days after pupation (c) and square in cross section (d). By the end of the pupal period (P+12) the lens is bullet-shaped in longitudinal section (e) and barrel-shaped in cross-section (f)(c-f; mag. 650x).

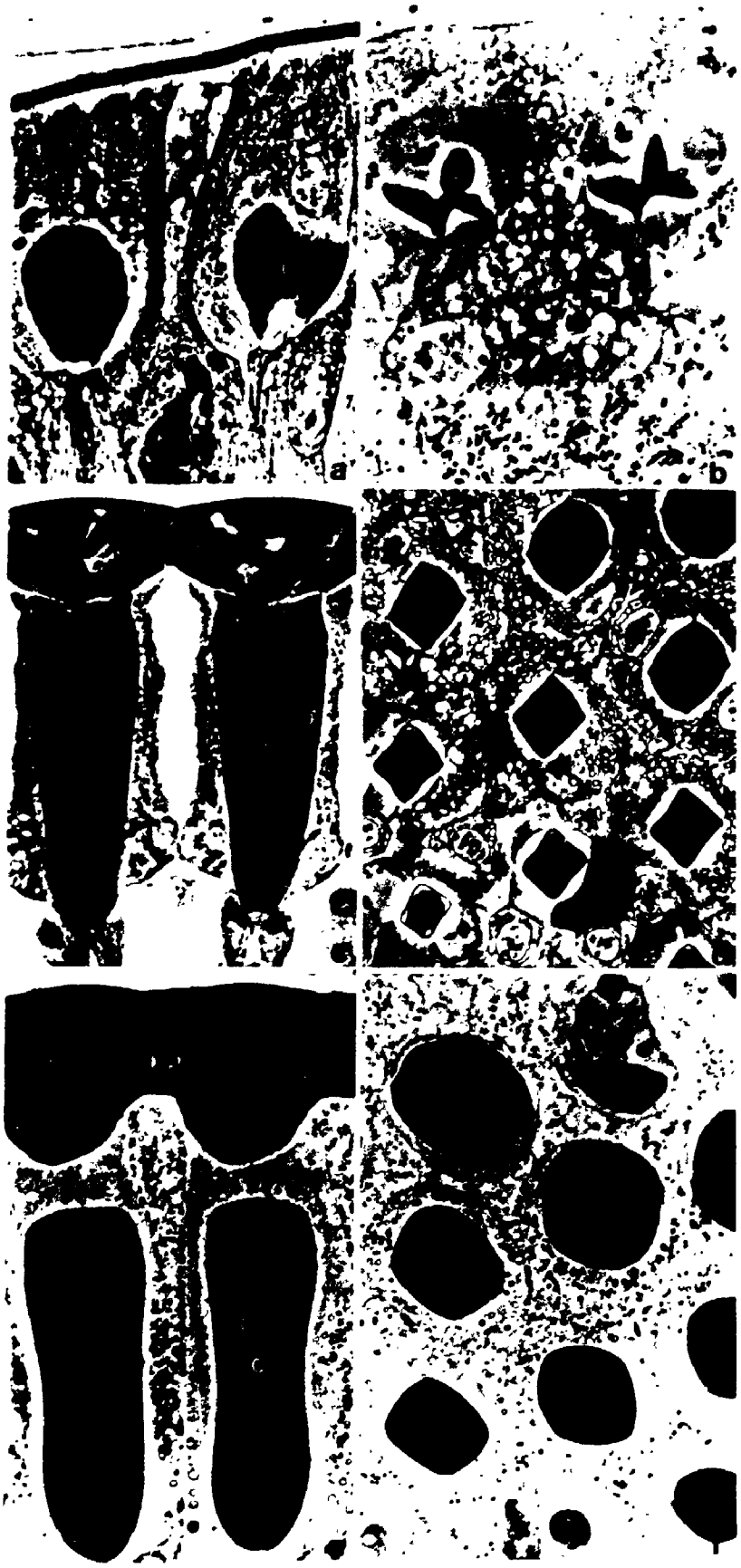
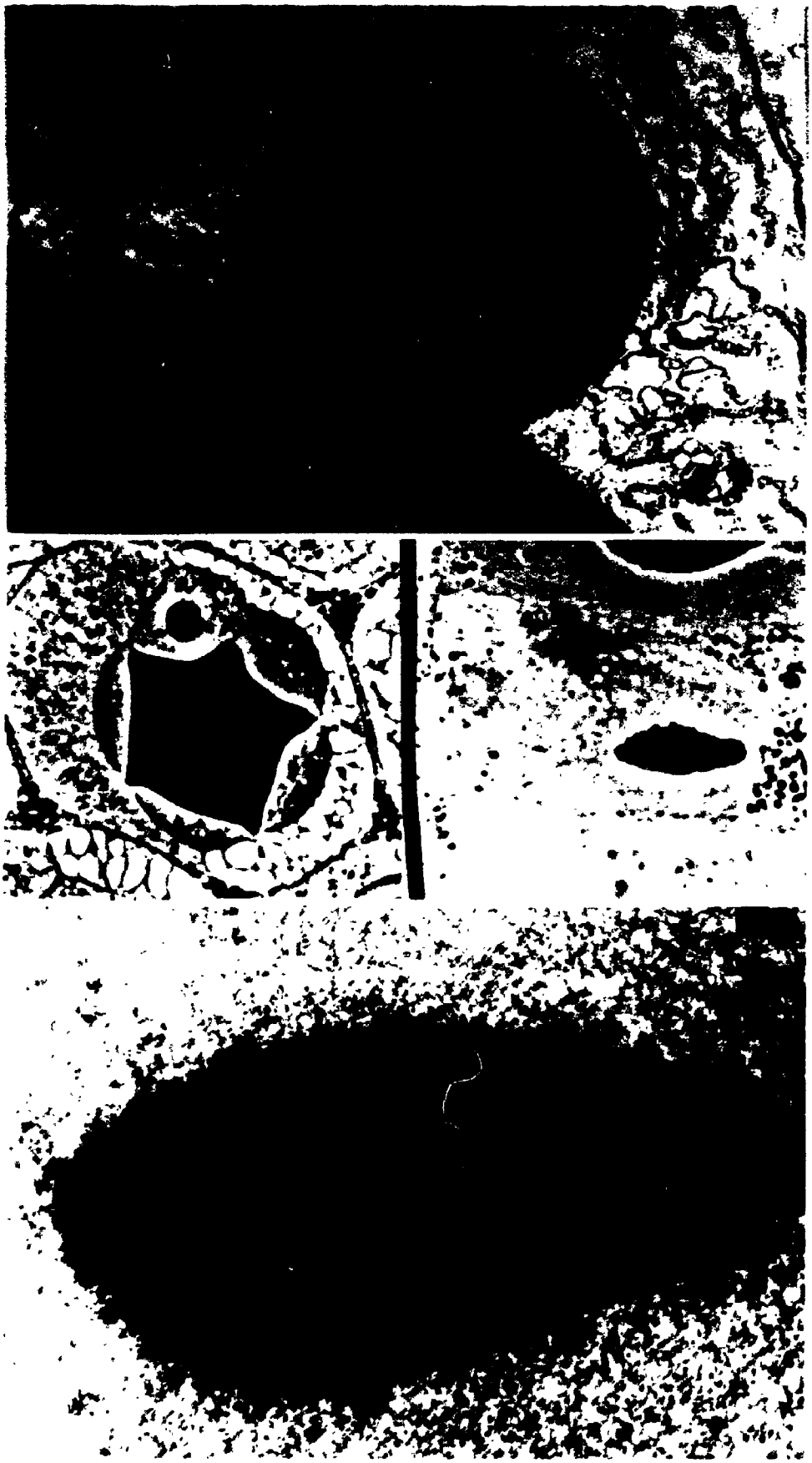

a
b

Figure 18:

Deposition of cone proteins in *Onitis aygulus*.

The crystalline cone proteins have a granular texture (left panel, mag 9,000x); bottom center panel, mag. 900x). Often these proteins aggregate into several of these granules to form large aggregations prior to deposition (top center panel, mag. 1,000x). The aggregates move through the cytoplasm and fuse with the growing cone (right panel, mag. 15,000x).



(Fig. 19). The crystalline cone reaches its final volume, but is not fully mature by 10 days after adult emergence.

### 3.3.5 Pupal Eye and Adult Crystalline Cone Polypeptides of *Onitis aygulus*

Separation of the total pupal eye proteins on SDS-polyacrylamide gels reveal a banding pattern consisting of numerous polypeptides when visualized with Coomassie blue (Fig. 20C). In the early pupal stage (6-8 days after pupation), two major bands of relatively high molecular mass (135 and 125kD) are apparent. Approximately 6 polypeptides between 43 and 53kD in molecular mass are also present. Of the lower molecular mass proteins, the most noticeable are a pair at approximately 30kD. The protein profile of eye tissue from 8-12 day old pupae has a very similar polypeptide pattern, but the relative amount of the 45kD polypeptide is increased. By the late pupal stage (>12 days) the polypeptides present in the greatest quantity were the 135, 125, 57, and 45kD polypeptides.

Double staining with silver nitrate and Coomassie blue of the same pupal eye stages reveals additional polypeptides. In the early pupal eye (<8 days), the notable difference is the appearance of silver staining bands at 67 and 26kD. The 67kD polypeptide is present in all later stages and is probably a lens protein. The 26kD band, however, becomes less prominent in the later pupal stages and absent from the adult crystalline cone and cornea.

Separation of the proteins from isolated crystalline cones of adult *Onitis aygulus* yields fewer polypeptide bands than in eye tissue from any of the pupal stages. The predominant polypeptides are the 135, 125, 57, and

Figure 19:

Growth of the crystalline cone of *Onitis aygulus* during development.

Left: Schematic representation of cone size and shape at different times during pupal and adult development.

Right: Increase in the volume of the crystalline cone (Appendix 1) during its formation. Note that 30% of the cone volume is deposited after adult emergence.

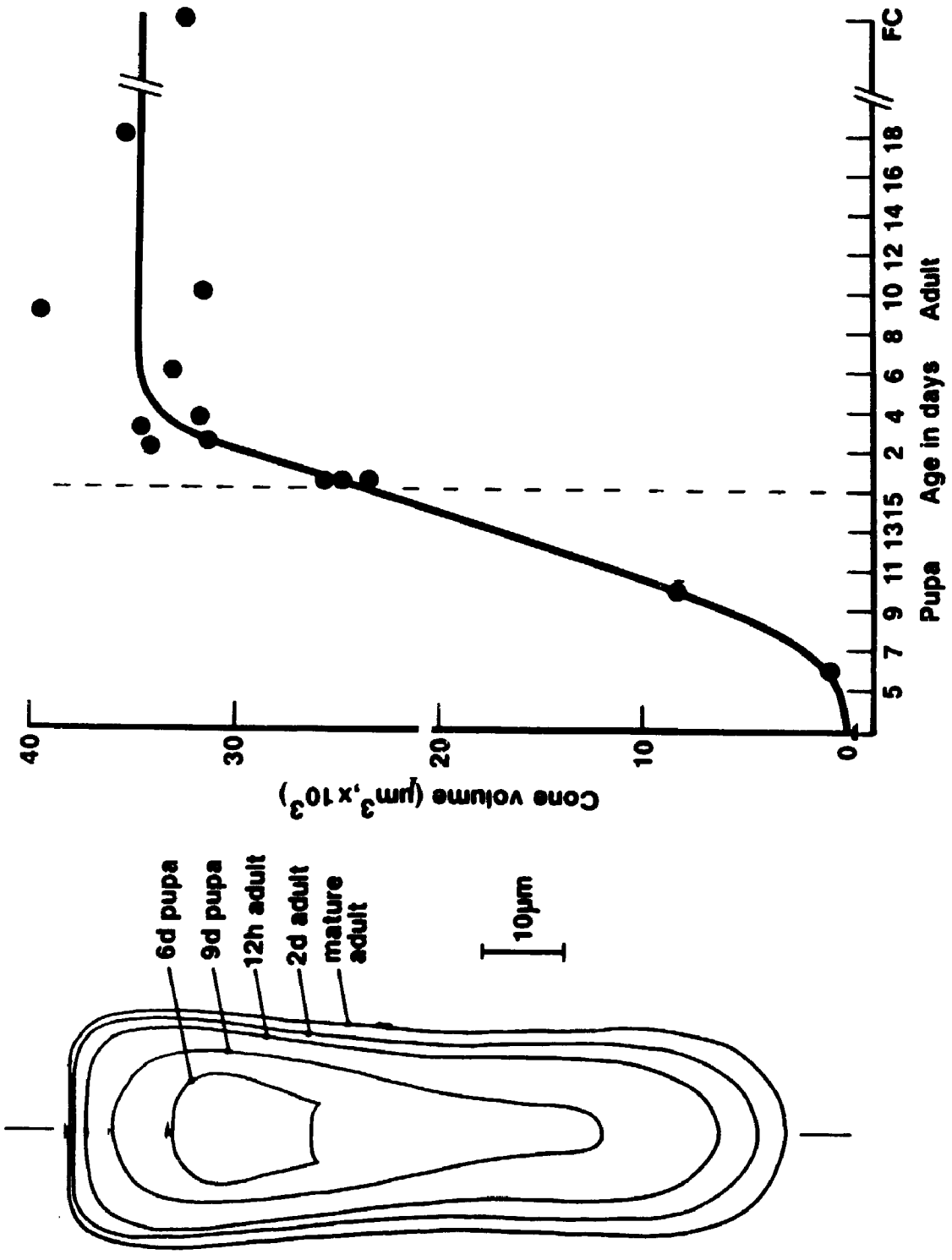
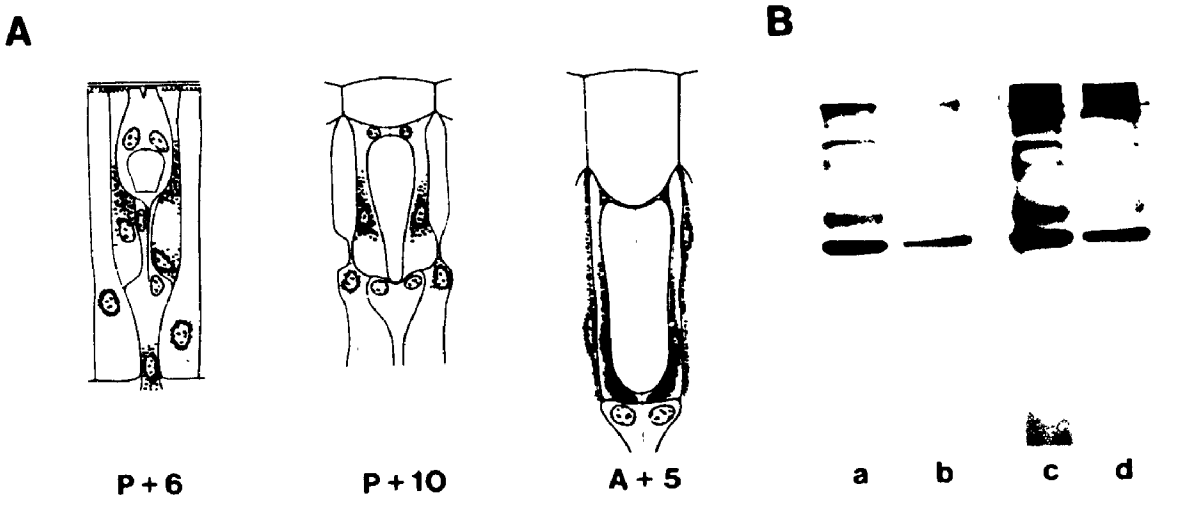

Cone volume ($\mu m^3 \times 10^3$)
0
10
20
30
40
5 7 9 11 13 15 2 4 6 8 10 12 14 16 18
Pupa
Age in days
Adult
FC
6d pupa
9d pupa
12h adult
2d adult
mature adult
10µm

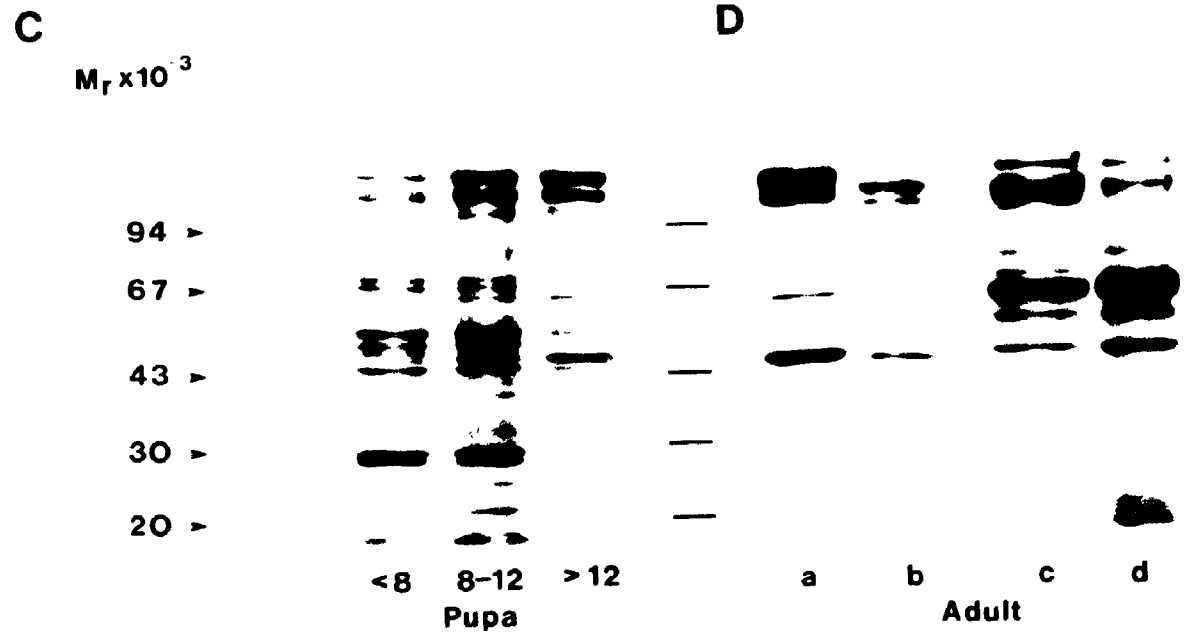
Figure 20:

Development of the crystalline cone of *Onitis aygulus.*

A: schematic representation of cone development in the pupa (P+6, P+10) and early adult (A+3). B: native proteins of the teneral (a, c) and mature (b, d) adult crystalline cone stained with Coomassie blue (left panel) and silver double stained (right panel). C: poly- peptides at various stages of pupal development (<8, 8-12, >12 days). The major cone proteins are not all present in the eye until 8 days after pupation. D; polypeptides of the teneral (a, c) and mature adult crystalline cone (b, d) visualized with Coomassie blue (left panel) and silver double stain (right panel). While the 67kD polypeptide is prominent in both the teneral and the mature lens, the 125kD polypeptide is the more abundant in the teneral adult lens.

Onitis aygulus



45kD, with a fainter staining band at 145kD (Fig. 20D). Because the crystalline cone continues to grow after adult emergence, the polypeptide composition of cones from teneral (<3 days) and mature (>10 days) adults were compared. The polypeptides of crystalline cones from newly emerged adults separate into seven major bands and several minor bands when double-stained with silver nitrate and Coomassie blue. The three most prominent bands had molecular masses of 125, 67, and 45kD. The 67kD polypeptide is predominantly seen after silver staining. The remaining major proteins had molecular masses of 135, 80, 70, and 57kD (Fig. 20D, lanes a,c).

The protein profile of the mature adult crystalline cone is similar to that just described. The most noticeable difference was an apparent reduction in the relative abundance of the 125kD polypeptide in the mature crystalline cone. The amounts of the 80, 67, and 45kD appear to remain constant (Fig. 20D, lanes b,d).

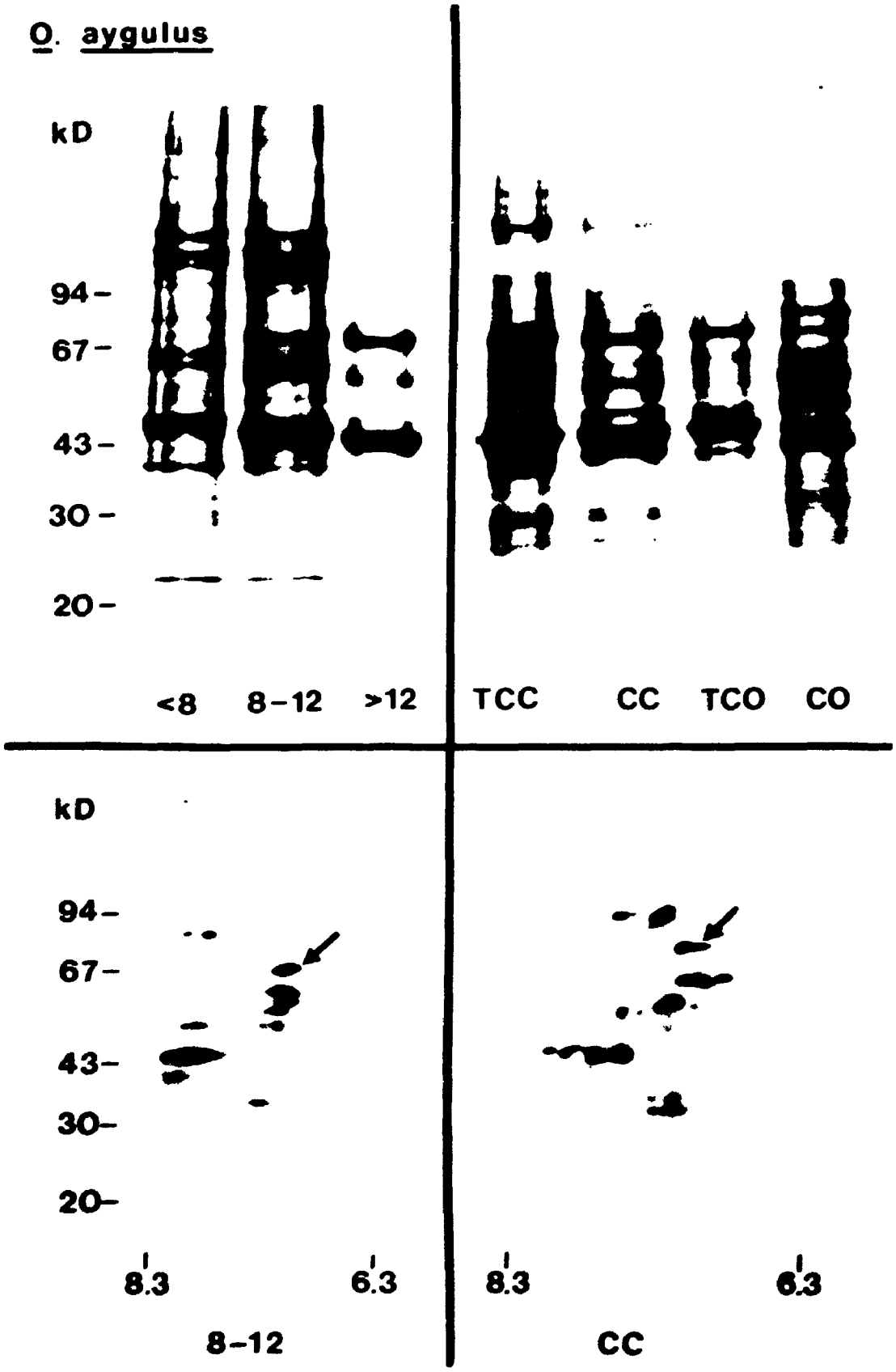
### 3.3.6 Newly Synthesized Polypeptides in the Developing Eye of *Onitis aygulus*

Pupal eyes were dissected into medium containing L-[$^{35}$S]methionine and its incorporation into newly synthesized proteins was detected by fluorography after one and two dimensional gel electrophoresis (Fig.21, left top panel). In the early pupa (6-8 days), the polypeptides most actively synthesized have apparent molecular masses of 145, 135, 60, 57 a pair at 45, 38 and 26kD. Other major polypeptides of the adult crystalline cone, such as the 125 and 67kD polypeptides, appear not

Figure 21

Synthesis of the crystalline cone proteins in *Onitis aygulus.*

One- and two-dimensional (SDS-PAGE) fluorograms of cone polypeptides synthesized in the presence of $^{35}$S-methionine by the pupal eye tissue (left panel) and adult crystalline cones and cornea (right panel). The high molecular weight polypeptides (125kD and 135kD) are synthesized predominantly during the early pupal stages (<8, 8-12 days) and early adult (TCC). These polypeptides do not appear to be produced in the late pupa stage (>12), mature adult (CC) or cornea (TCO, CO). Initial synthesis of the 67kD polypeptide is first detectable in the mid pupal stages (8-12) and resolves into a single isoform in the second dimension (arrow). The synthesis of two isoforms is detected in the adult cone (CC, arrow). 1D gels are loaded with 20,000 cpm/lane; 2D gels are loaded with 50,000 cpm. These fluorograms represent typical results for 5-6 separate experiments.


O. aygulus
kD
94-
67-
43-
30-
20-
<8
8-12
>12
TCC
CC
TCO
CO
kD
94-
67-
43-
30-
20-
8.3
6.3
8-12
8.3
6.3
CC

to be made at this time. Many minor polypeptides are being synthesized but are not clearly resolved. In the 8-12 day old pupa, synthesis of the 67kD and 45kD polypeptides is detectable. Also during this time, 4 other distinct proteins of molecular masses between 43, and 57kD are present. Synthesis of the 125kD polypeptide is also evident but not in the quantity expected based on the amount this polypeptide represents in the total protein of the lens. In the late pupa (>12 days), the synthesis of many of the polypeptides was greatly reduced, most notably the high molecular weight polypeptides. Of those synthesized, however, the most prominent were the 67kD, a group around 57kD and a 43kD polypeptide.

A representative two dimensional fluorogram of the newly synthesized polypeptides of pupal eye tissue (8-12 days) reveals that all detectable polypeptides have isoelectric focussing points (pI) between 6.3 and 8.3 (Fig. 21, left bottom panel). The newly synthesized 67kD polypeptide equilibrates at a single pI of 6.9. Those proteins with molecular mass between 53kD and 57kD, resolve at three separate spots each with pI's ranging between 6.8 and 6.9. Also detectable is a poorly resolved cluster of polypeptides of apparent molecular mass between 45 and 47kD and pI of 7.3 to 7.8.

The eye tissue of newly emerged adults (<3 days, TCC) was also labelled with 35-S-methionine *in vitro* to determine which of the cone proteins are being synthesized and deposited in the crystalline cones at this time (Fig. 21, top right panel). Numerous polypeptides are being actively synthesized and are therefore difficult to resolve. These proteins are the 145, 67, 57kD bands, many polypeptides between 35-47kD, and a cluster around 30kD. The 135 and 125kD polypeptides, however, were not synthesized at this time. Analysis of the teneral adult cornea (TCO) shows

incorporation of label into polypeptides ranging between 35 and 67kD. The synthesis of the 67 and 43kD polypeptides is most prevalent.

Proteins are still being synthesized and added to the adult crystalline cone 7 days after emergence (Fig. 21, CC). The synthetic profile in the cone cells appears similar to that found in the cone cells in the immature adult, although an apparent reduction in the synthesis of some polypeptides (145kD) was observed. Incorporation of label was observed into the 3 and 7 day old cornea. The majority of these proteins ranged between 40kD and 80kD.

Two dimensional fluorograms of this sample (Fig. 21, lower, right panel) reveal that all newly synthesized lens polypeptides detected have isoelectric points (pI) between 6.3 and 8.3. Also, the synthesis of the 125 and 135kD polypeptides is not detectable in the adult. Polypeptides with molecular masses between 80 and 85kD are being synthesized and resolve into 3 major and approximately 5 minor polypeptides with pI's ranging from 7.1 to 7.3. The 67kD polypeptide equilibrates into two subunits of pI 6.7 and 6.9. Proteins with a molecular mass of 57kD separate into 3 isoforms with pI ranging between 6.7 and 6.9. Polypeptides between 45-47kD focus into at least 5 subunits (pI 7.3-7.9) with the two most prominent having pI's of 7.3 and 7.5. Several other newly synthesized polypeptides could be identified by their isoelectric focussing points but are not regarded as major crystalline cone components.

## 3.4 DISCUSSION

### 3.4.1 The Crystalline Cones of *Calpodes* and *Onitis* are Formed Differently

The initial appearance of the crystalline cone during its formation is similar in the skipper butterfly and the beetle. In the pupa of both species, deposition of cone proteins occurs first in the distal region of the four cone cells along a densely staining membrane. This region corresponds to the area of highest refractive index in the mature cone (Caveney and McIntryre, 1981).

Based on the pattern of protein synthesis, however, different strategies are used in the subsequent development of the crystalline cone. The cone proteins of *Calpodes* are synthesized in a coordinate manner from about 24 hours after pupation and all are produced continually throughout the pupal period. The timing of the synthesis of the cone proteins in *Onitis* is staggered. The high molecular mass polypeptides (125kD) are first synthesized in the early pupa whereas other cone polypeptides (members of the 67kD group) are not synthesized until later in pupal development. The cone proteins are assumed to be deposited into the crystalline cone in the order in which they are synthesized.

This difference is also seen in the morphology of the developing crystalline cones. The cone proteins of *Calpodes* appear as 2 distinct deposits in the early pupal stage. Firstly, a dense granular deposit is present along the longitudinal axis of the cone next to the apposing cone cell membranes. In cross-section, this deposition appears initially as a 4-

pointed star and then becomes square as the cone grows. Secondly, a sponge-like deposit surrounds this dense accumulation of proteins. This sponge-like network gives the developing cone the characteristic cylindrical shape seen in the mature adult. In the late pupal stage, a flocculent material surrounds the already deposited cone proteins giving the impression that the sponge-like network is gradually being filled by cone material. Even though cone protein synthesis is coordinate, these observations suggest some partitioning occurs during protein deposition.

On the other hand, the cone material of *Onitis* has a grainy texture and has a uniformly dense consistency throughout cone formation. The spongy and flocculent layers are absent. In cross-section, the cone material looks the same as the dense deposit of the skipper butterfly cone and progresses through star- and square-configurations during its growth. In the late pupa and early adult, the square-sided cone transforms gradually from its distal to proximal end into the cylindrical structure seen in the mature adult.

The formation of the crystalline cone of *Calpodes* is complete shortly after adult emergence and is cylindrical in shape with a rounded proximal tip. Ultrastructural examination of the adult eye shows the crystalline cone to have undergone extensive development since the late pupal stage. Neither the sponge-like deposition nor flocculent material remain; instead the cone is completely granular in consistency. This granularity is unevenly distrubuted in the cone and appears to coincide spatially with the gradient in refractive index. That is, the granularity is most dense at the centre of the most distal region of the cone. From this point, the granularity gradually decreases along the length and width of the cone in parallel with its refractive index (Horridge, 1972). The cone

cells appear to be inactive within 3 days of adult emergence as their nuclei are condensed and other cytoplasmic components are reduced to a peripheral sheath around the cone.

The crystalline cone of *Onitis* is not complete at adult emergence but continues to grow, especially at its proximal tip, throughout the teneral period. The lens, when mature, has the same granular appearance observed throughout development and does not show any morphological features that correspond to its internal refractive index gradient.

### 3.4.2 The Graded Refractive Index in the Skipper Butterfly Crystalline Cone Forms During Its Growth and Appears to Involve Sorting of Coordinately Synthesized Polypeptides

As outlined in the Introduction, there are several possible mechanisms that could produce a lens with a strong internal gradient of refractive index. Since refractive index measurements have not been performed on the developing crystalline cone of *Calpodes*, the actual mechanism employed in forming the refractive index gradient is still unknown. The formation of this lens, however, does provide some insight into how this gradient may be obtained.

During the development of the crystalline cone in *Calpodes*, all of the cone constituents are synthesized by the cone cells in a coordinate fashion. This suggests that the lens is not deposited in successive shells of decreasing refractive index of differing protein composition. Morphologically, as the cone grows it does not appear as a miniature version of an adult cone. Instead, early in development the cone consists of

a sponge-like network. This also reinforces the idea that the refractive index is not established in successive shells by physical deposition. More significantly, it implies that some sorting may occur during cone formation. That is, the cone proteins are not simply synthesized and deposited as a homogeneous mass which is, at a later time, organized into a gradient of refractive indices. The cone proteins may self-assemble according to their intrinsic properties. Possibly, only certain cone proteins attach to the cone cell membrane and other proteins specifically form the sponge-like network. This would account for the coordinate synthesis of proteins and yet allow for formation of the graded index during development.

Good vision is essential for flight. The skipper butterfly flies shortly after adult emergence. Consequently, the crystalline cone must be structurally complete and optically mature within a few hours of adult emergence. Although there is no direct evidence to explain how the graded refractive index is established in the lens of *Calpodes*, examination of the morphology and development of the crystalline cone does reduce the range of possibilities. This study suggests that the refractive gradient is, at least partially, established during cone formation. It is possible that some cone proteins provide a unique cytoskeleton which physically organizes the bulk cone protein into zones of differing refractive index.

### 3.4.3 The Graded Refractive Index in the Beetle Crystalline Cone Only Forms During a Maturation Phase After Growth is Complete

The refractive index gradient in the crystalline cones of *O. aygulus* appears to develop differently than that observed in *Calpodes* . It is partly established as the cone material is being deposited, which according to growth measurements continues for several days after adult emergence (Fig. 22, Caveney, unpublished data). At adult emergence the crystalline cone is less than three quarters of its mature size. An incomplete cone at this time would not compromise vision in the beetle, since the teneral adult remains dormant underground in complete darkness for at least a week. During this time the body cuticle continues to be deposited as well (Zelazny and Neville, 1972).

The biochemical and morphological observations that suggest that the refractive index of the crystalline cone is partly established during its growth phase are as follows. Firstly, cone proteins show a temporal pattern in their synthesis and therefore are assumed to be added to the cone at different times during development. The high molecular mass proteins (125 and 135kD) which are most hydrophobic are synthesized first and are likely localized in the central region of high refractive index in the lens. The less hydrophobic proteins (the 67kD family) are synthesized later in the pupal stage and are likely positioned more peripherally. A similar mechanism is seen in the synthesis of the vertebrate crystallins, where the high molecular alpha-crystallins are produced first during lens development. These very hydrophobic crystallins are found at the core of the vertebrate lens (Pierscionek and Augusteyn, 1988).

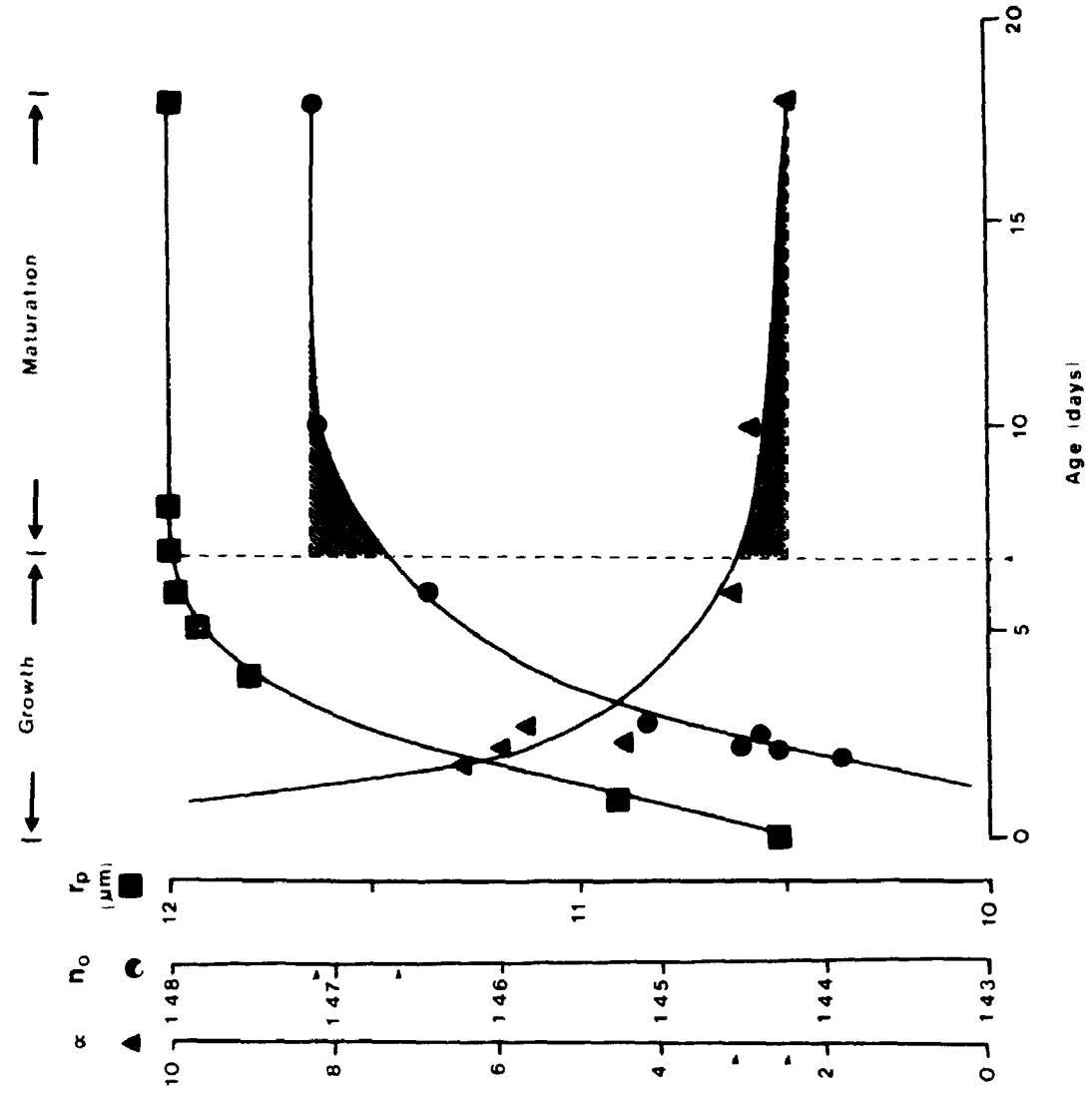
Secondly, while the uniform granular appearance of the beetle cone

Figure 22:

Growth and maturation of the adult crystalline cone of *Onitis aygulus.* (based on Caveney and Warrant unpublished data)

Growth phase: Deposition of cone proteins continues for several days after adult emergence seen here from the measurements of the radius of the proximal cone tip ($r_p$). (This curve [ ■ ] is derived from the same set of data shown as volume changes in Fig. 19) During this time, the gradient of refractive index is partially established and the optical quality of the lens is improved. This is shown by the axial refractive index ($n_0$) climbing from 1.44 to over 1.46 and the alpha value ($\alpha$) dropping from over 6 to approximately 3 by the time growth is complete (dotted line).

Maturation phase: The magnitude and slope of the gradient in refractive index is modified after growth of the crystalline cone is complete. This is shown by a further rise in axial refractive index to over 1.47 ($n_0$, arrows along axis) and a drop in the alpha value to less than 2.5 ($\alpha$, arrows along axis). Based on these two optical parameters, lens maturation occurs for several days after growth is complete
(hatched areas).


Growth
Maturation
Age (days)
$r_p$ (μm)
$n_o$
α
0
5
10
15
20
10
11
12
143
144
145
146
147
148
0
2
4
6
8
10

offers no clue that shells of different polypeptides exist in the lens, the ordered growth of the beetle cone and the phased pattern of lens protein synthesis imply that regions of differing refractive index contain different proteins. In the vertebrate eye, gamma-crystallins have the highest refractive index and are believed to control the hydration of the lens. Thus, they are found in abundance in the nucleus of mature mammalian lenses and are generally present where lens refractive index is high (Slingsby, 1985; Chiou et al., 1988).

Growth of the crystalline cone continues for at least 7 days after beetle emergence. As growth slowly tapers off, several molecular processes continue in the lens which suggests that the completion of the gradient of refractive index requires that the lens proteins mature in some way.

Direct measurement of lens refractive index shows that the gradient continues to mature after the radial growth of the proximal tip is complete (Fig. 22, $n_0$, Caveney, unpublished data). The optical performance of a cylindrical graded-index lens is determined primarily by two parameters, (i) the magnitude of the gradient and (ii) its slope. The graded-index profile is described by the equation:

$$n(r) = n_0 (1\text{-}ar^{\alpha})$$

where $n_0$ is the refractive index at the lens axis, $n(r)$.is the refractive index at radial distance $r$, $a$ is a constant, and a describes the slope of the gradient (McIntyre and Caveney, 1985). Fletcher *et al.* (1954) have shown mathematically that an alpha value of 2 (representing a near-parabolic drop off in refractive index with radial distance for the lens axis) is optimal for image formation.

The refractive index of the bulk cone material is low during the

pupal stage but increases progressively throughout development of the teneral beetle (Fig. 22, Caveney, unpublished data). The axial refractive index of the proximal tip of the teneral lens is only 1.46 when its radial growth is complete but increases to over 1.47 in the mature lens (Fig. 22, $n_0$ arrows along axis, Caveney, unpublished data). Also, by the end of the growth phase, the alpha value has dropped to approximately 3 and only approaches the optimal value ($\alpha$=2) several days later. Warrant (unpublished data) has shown electrophysiologically that the performance of the eye increases gradually over several weeks of adult life.

A maturation phase has been shown to occur in the developing vertebrate lens (Chiou, 1984; Slingsby, 1985; Pierscionek and Augusteyn, 1988). Although packed crystallins have an intrinsically high refractive index their deamidation and cross-linking with other crystallin sub-types further decreases their solubility and raises the refractive index of the lens (Pierscionek and Augusteyn, 1988; Chiou *et al.*, 1988). It is very likely that proteins of the crystalline cone also undergo similar modifications in the early adult beetle. Post-translational processing of the proteins would account for the apparent decrease in the relative amount of the 125kD polypeptide between the teneral and mature crystalline cone seen in this study. Possibly the 125kD polypeptide forms insoluble aggregates and therefore is not extracted in similar quantities. Or, this polypeptide may undergo degradation giving various polypeptides differing in molecular weight.

In summary, the mechanism that establishes the graded refractive index profile in the crystalline cone of *Onitis* appears to be a combination of the models described earlier. The refractive gradient is uniformly low in the lens during pupal development. During the later stages of growth,

which in this case includes at least the first 7 days after adult emergence, the cone proteins continue to be synthesized but may also be post-transcriptionally modified before becoming optically functional. By analogy with the vertebrate lens, these alterations could possibly involve modifications which increase dehydration, protein interaction and insolubility. This could result in an increase in the axial refractive index and a reorganization of the cone refractive gradient to a near-parabolic distribution. Following this growth phase in the teneral adult, there is a further maturation phase. Maturation probably involves the completion of protein processing which fine tunes the optics to produce the precise refractive gradient characteristic of the mature crystalline cone.

# PART 4

# SHAPING OF THE CRYSTALLINE CONE IN ARTHROPOD EYES: EVIDENCE FOR A TEMPLATE MEMBRANE

## 4.1 INTRODUCTION

The previous chapter discussed the morphology and protein synthesis in the developing crystalline cone of the beetle and skipper butterfly. In this chapter, I present evidence that the shape and size of the crystalline cone in arthropods is determined by a template formed by the plasma membrane of the cone cells, in association with a small number of cone polypeptides.

### 4.1.1 Shaping of Vertebrate Lenses

The mammalian lens is a multicellular structure which undergoes extensive cellular differentiation during development. The shape of an embryonic lens is controlled by an elaborate submembranous skeleton of the lens cells. The contraction of an actin-based cytoskeleton is thought to be responsible for the shaping of the lens placode, while microtubules are

responsible for the elongation of the lens fiber cells (Piatigorsky, 1981). The shaping of the mature lens is controlled by interactions between actin, intermediate filaments, and the crystallins (Bloemendal, 1981). These cytoskeletal components are present in both the water-soluble and in the highly polymerized insoluble fractions of the lens (Benedetti *et al.*, 1981). The majority of the actin is concentrated throughout the lens along the plasma membranes of the epithelial and fiber cells and contributes to the formation of a plasma membrane-cytoskeletal complex (Ireland *et al.*, 1983). Vimentin is found mainly in the epithelial and cortical fiber cells. It gradually decreases in concentration towards the inner layers of the lens and is absent in the nuclear fibers; its function is unknown (Ellis *et al.*, 1984). The intermediate filament, fodrin, is also found in a submembranous position in all the lens cells and binds directly to actin. Fodrin regulates the shape of the plasma membrane and controls the interaction between the membrane and the cytoskeleton (Green and Maisel, 1984).

The lenticular plasma membranes are the most significant components in establishing and maintaining the shape of the lens (Benedetti *et al.*, 1981). The majority of the membranous organelles of the lens disappear as the cortical fibers terminally differentiate. The plasma membranes undergo extensive reassembly to form junctional domains which provide sites of nucleation and attachment for the cytoskeleton (hence the name plasma membrane-cytoskeletal complex). Most importantly, in the absence of cellular organelles, the plasma membrane serves as an attachment site for the polyribosomes which actively synthesize the crystallins (Benedetti *et al.*, 1981).

Morphologically, the crystallins stabilize microfilaments. Actin and

alpha-crystallin remain together even after urea extraction (Bloemendal, 1981). The relationship between the crystallins and the plasma membrane-cytoskeletal complex, however, is still unclear. Nearly 5% of the alpha-crystallins remain associated with the complex after extraction with urea. Even following prolonged exposure, thin microfilaments of the complex are heavily decorated with globular entities, presumably macromolecular crystallins (Benedetti *et al.*, 1981). The plasma membrane, cytoskeleton and crystallins form a unique structure which functions in shaping the vertebrate lens.

### 4.1.2 Morphology of the Crystalline Cone

Unlike the vertebrate lens, the crystalline cone is made by only 4 cells, each of which provides an equal quadrant to the complete lens. Nevertheless, the cone exists in a wide variety of shapes and sizes in different insect species (Caveney, 1986) suggesting cellular control of the cone shape. The crystalline cone may be divided into 3 separate regions along its optical axis based on structure and function. The most distal region lies just proximal to the cornea in the eye and has either a flat or slightly concave surface. This allows for the proper on-axis alignment with the inner surface of the corneal lens to provide the optimum performance of both lenses (Caveney and McIntyre, 1981). The mid region comprises the cylindrical body of the cone and is the site of the intermediate focal plane of the lens. The shape of this region, which varies in the extent to which a waist is developed, is related to the time of day an insect species is active (Land, 1985; Caveney, 1986). Shapes can

range from skittle shapes found in crepuscular and nocturnal species of beetles and moths, to hour-glass shapes found in diurnal dung beetles, to the extremely tapered shape of diurnal butterflies (Caveney and McIntyre, 1981). The most highly conserved region of the crystalline lens in the superposition eye is the proximal tip because of its importance in forming the image on the rhabdom. It is typically ellipsoidal in shape and is the most pliable of all the regions of the cone (Caveney and McIntyre, 1981).

### 4.1.3 Aims

The aim of this study was to determine how the shape of the crystalline cone is determined. More specifically:

i) Are cytoskeletal components, such as actin and tubulin, involved internally or externally in shaping the crystalline cone?

ii) Does specialized plasma membrane play a role in shaping the cone? If so, how does this membrane influence the shape of the lens?

## 4.2 METHODS AND MATERIALS

### 4.2.1 Immunofluoresence

#### 4.2.1 A) Fixation

Adult eye tissue of *Calpodes* and *Onitis* was fixed in 0.5% glutaraldehyde, 3.0% paraformaldehyde, 0.1M sodium cacodylate (pH 7.2) for 1 or 2 hours. Following fixation, the samples were washed in PBS (20mM Tris (pH 8.2), 0.9% saline and 1% BSA) for 30min and dehydrated in a series of ethanol washes (70%, 80%, 95%, 100% for 30 minutes each).

#### 4.2.1 B) Embedding with Diethylene Glycol Distearate (DGD)

The procedure for embedding tissue in DGD is basically that of Capco *et al.* (1984) with some modifications. Following fixation and dehydration, the eye tissue was transferred into a 2:1 mixture of 100% ethanol and n-butyl alcohol, followed by a 1:2 mixture and finally into 100% n-butyl alcohol. Each transfer was for a duration of 30 minutes. Transfers from n-butyl alcohol to DGD (Polyscience, Inc., Warrington, Pa.) were for 1 hour each, under vacuum, at 60°C with the final incubation overnight. Occasionally, 0.5% DMSO was added to the DGD for better penetration.

The eye tissue was embedded in fresh DGD and allowed to harden.

After solidification, the blocks were cut with glass knives and sections were collected on coverslips pretreated with poly-l-lysine for adhesion.

The DGD was removed by immersing coverslips in 100% n-butyl alcohol for 2 hours, followed by a 1:1 mixture of n-butyl alcohol and ethanol (15 minutes) and finally into 100% ethanol for 75 minutes. The eye tissue was prepared for immunofluorescence through a descending ethanol series into PBS and sodium borohydride (0.5mg/mL in 50% ethyl alcohol).

4.2.1 C) Primary and Secondary Antibodies

Prior to primary antibody treatment for immunofluoresence, the eye tissue was briefly rinsed in fresh PBS and then incubated for 1 hour in 1% BSA in PBS at 37°C. Primary antibodies used in this study were monoclonal anti-tubulin (Sigma Chemical Co.), and monoclonal anti-actin (Sigma Chemical Co.). The monoclonal antibodies were used in a 1:5 or 1:10 dilution with a rhodamine conjugated secondary antibody. The sections were exposed to the primary antibody (prepared in PBS containing 1% BSA) for 2 hours at 37°C, then rinsed 3 times for 5 minutes in PBS buffer. The rinsed tissue sections were incubated in secondary antibody (prepared in PBS containing 1% BSA) for 1 hour at 37°C. The sections were washed again in PBS and mounted for viewing.

Western transblots of lens polypeptides separated by SDS-PAGE were challenged with the polyclonal antibodies raised against the 125kD and 67kD lens polypeptides of *Onitis aygulus*. The lens antibodies were used in a 1:1, 1:10, 1:50, or 1:100 dilution with a fluorescein isothiocyanate

(FITC) conjugated secondary antibody. For further details, see methods section in Part 1.

### 4.2.2 Agents used in Solubilization of the Crystalline Cone Components

In order to examine the 'electron dense' membrane more thoroughly, a procedure to isolate the cone membrane from the cone proper was necessary. Consequently, a series of solubility experiments was undertaken.

Freshly dissected cones from mature adult eyes were placed on a clean slide in a drop of M10 medium and one drop of the following solutions was added:

A) M10: M10 was used as a control solution. Medium contains M199 (Connaught labs, Toronto, Canada) supplemented with 30mM potassium chloride, 20mM magnesium chloride, 50mM trehalose and buffered with 20mM Pipes (pH 6.8). Medium was filtered using a Nalgene 0.2 micron filter.

B) 10mM NaOH:

C) SBA: SBA solution contains 2% SDS, 5% 2-mercaptoethanol, 20% glycerol, 80mM Tris and PMSF.

D) 10% SDS:

E) 5% Triton X-100:

F) RIA: RIA mixture contains 20mM sodium phosphate (NaP), 200mM NaCl, 2% Triton X-100, 1% Na-deoxycholate, and 1mM PMSF.

G) 4.5M urea:

H) 9M urea:

The preparation was viewed under a Zeiss light microscope as each solution was added. The effect the solutions had on the crystalline cones, cone cell cytoplasm and pigment cells was then recorded.

### 4.2.3 Template Membrane Extraction

Based on the solubility experiments, the following protocol was used to isolate the proteins of the template membrane from the proteins of the cone body.

Mature crystalline cones were dissected into M10 medium. The suspension was centrifuged for 2 minutes (table top microfuge, 14,000 x g) and the supernatant was discarded. Two hundred µL of 4.5M urea, which partially extracts the cone proteins, was added to the pellet of isolated cones. The samples were thoroughly vortexed and then put on ice for 10 minutes. The samples were then centrifuged as above, and the supernatant (1A) was removed. Another 200µL of 4.5M urea was added to the pellet, the procedure was repeated and the resulting supernatant was designated 1B.

The pellets obtained were thoroughly washed with M10 and the supernatant was discarded. To extract all cone proteins, 200µL of 9M urea was added to the pellet. The sample was thoroughly vortexed, set on ice for 10 minutes, and briefly centrifuged. The supernatant (2A) was removed and the procedure was repeated. After removal of supernatant 2B, the pellet was again washed in M10.

To solubilize the remaining membrane fragments, 200µL of SBA

was added to the final pellet. After vortexing, the sample was briefly sonicated and then boiled for 3 minutes. The mixture was put on ice for 10 minutes and the supernatant removed. The procedure was repeated if any pellet remained. Each sample was examined by one- or two-dimensional SDS-PAGE.

## 4.3 RESULTS

### 4.3.1 Involvement of Cytoskeletal Elements in Shaping the Crystalline Cone

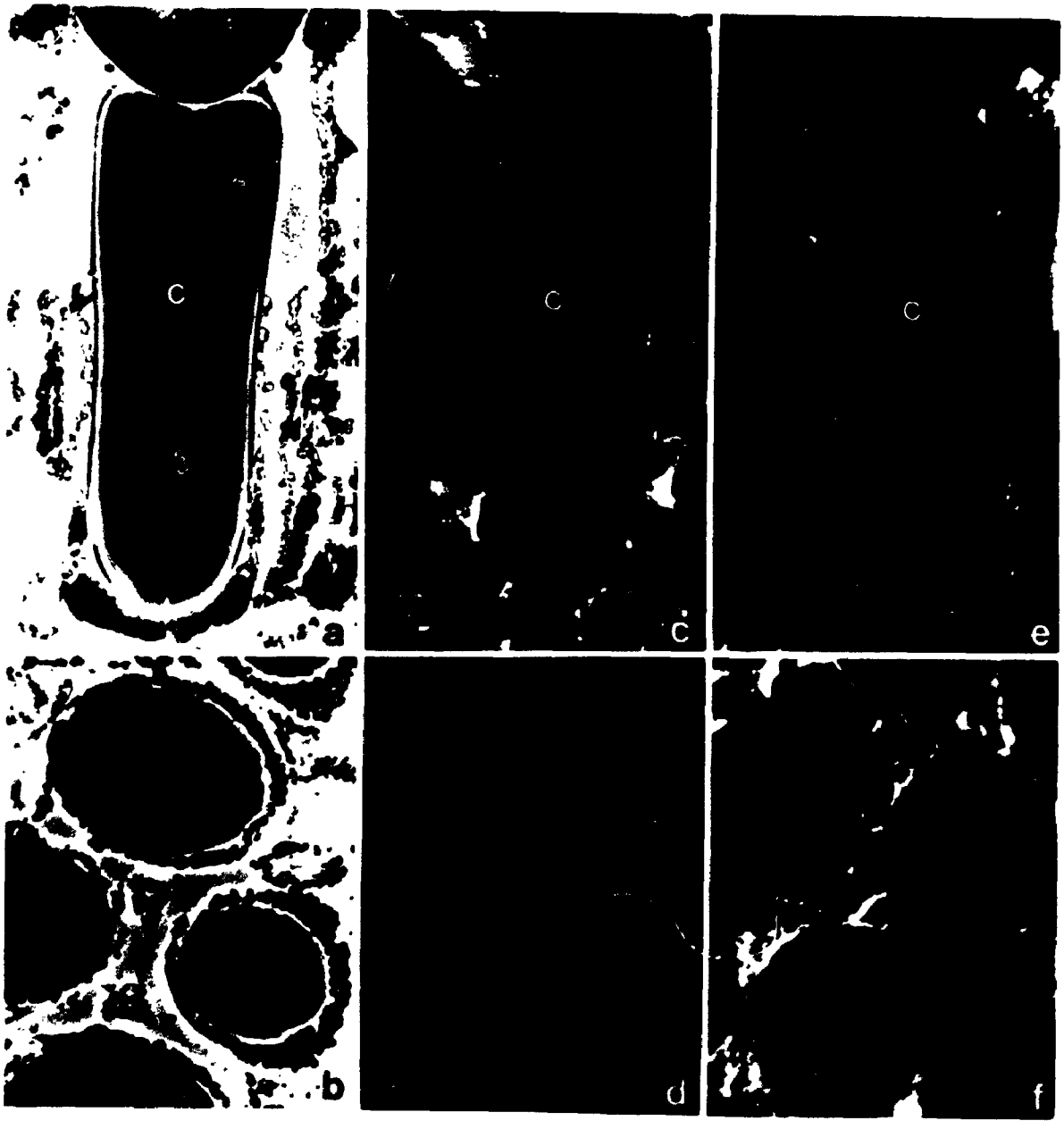
Sections through peripheral eye tissue of *Calpodes*, which include the crystalline cones, were treated with fluorescently-tagged antibodies directed against either actin or tubulin. In both instances, there was no evidence that actin or tubulin was present in the body of the crystalline cone. positive reaction was observed along the periphery of the cones and the primary and secondary pigment cells (Fig. 23, panels c-f). Although the fluorescence after anti-actin labelling was weak, the major response was noticed near the proximal tip of the cone and in the surrounding pigment cells (Fig. 23, panels c,d). A stronger fluorescent reaction was observed with anti-tubulin, especially in the cone cell cytoplasm surrounding the crystalline cone (Fig. 23, panels e,f).

Polypeptides from mature crystalline cones were isolated by 1D-SDS-PAGE, transferred onto nitrocellulose and challenged with antibodies raised against actin and tubulin. Both reactions with anti-actin and anti-tubulin were negative (results not shown). This reinforces results obtained by the immunofluorescence experiment; actin and tubulin are not present within the crystalline cone.

Figure 23:

Actin and tubulin are absent from the crystalline cone of *Calpodes ethlius.*

Crystalline conea (white c) are viewed by phase contrast and immunofluoresence in longitudinal section (top) and cross-section (bottom). Indirect immunofluorescent localization of actin (c,d) and tubulin (e, f) with monoclonal anti-actin and anti-tubulin antibodies. Note that actin and tubulin are not present in the crystalline cone, although they are found in the cone cell cytoplasm (mag. approx. 1,000x).


c
c
c
a
c
e
b
d
f
ANTI-
ACTIN
ANTI-
TUBULIN

### 4.3.2 Morphology of the Template Membrane

In analysing the growth and maturation of the crystalline cone histologically, one region of the cone cell membrane consistently stained more densely with toluidine blue than any other membrane in the eye. This densely staining membrane formed a portion of the apposing cone cell membranes and appeared to be the initial site for the deposition of cone proteins (Fig. 24).

Longitudinal sections through the early pupal eye (Fig. 24, a), showed that the specialized membrane extends, proximal to the nuclei, with a length approximately one half that of the cone cell. This membrane, however, is only present along the inner plasma membrane of the cone cell tetrad and was never observed in any region of membrane confronting the surrounding pigment cells. In cross-section (Fig. 24, b), the specialized membrane appears as spokes radiating from the center of the 4 cone cells. These spokes, however, never extend the full length of the membrane between apposing cone cells (Fig. 24).

Sections through the eye of an early pupa showed that newly synthesized cone proteins preferrentially aggregate along this membrane (Fig. 24, a and b). This aggregation or deposition is most evident in cross-sections where the newly forming cone appears as a 4-pointed star. Each arm of the star results from cone proteins being deposited along the specialized membrane. At the point of each star and along the longitudinal axis, there is always a region of this membrane that is not yet associated with cone proteins. Similarly, in eyes of older pupae (Fig. 24, c and d), the deposition of cone proteins does not extend along the entire length of this membrane. The relative amount of this 'naked' membrane

Figure 24:

Template membrane of the developing crystalline cone of *Calpodes ethlius.*

Top panel: The crystalline cone template (arrows) in the eye of 6 day old pupae seen in longitudinal (a) and cross-sections (b) (mag. 650x).

Bottom panel: The template membrane in the eye of 8 day old pupae seen in longitudinal (c) and cross-section (d)(mag. 650x).

decreases, however, as the eye continues to develop. This suggests that as the cells of the ommatidia grow, so does the specialized membrane. The template membrane growth appears to be completed before the crystalline cone reaches its final size and hence provides a guideline for cone growth.

Crystalline cone growth is complete by the time the adult emerges (Fig. 25). At emergence, the deposited cone proteins extend the entire length of the specialized membrane on the transverse axis as well as the longitudinal axis (Fig. 25, a and b). Regardless of the age or maturation of the eye, the crystalline cone was never observed to exceed the limits defined by this membrane. Thus, it appears that the specialized membrane functions as the site for initial deposition of the crystalline cone, and serves as a template for the size and shape of the mature cone.

### 4.3.3 Function of the Template Membrane

Clearly the template membrane has a high affinity for newly formed cone proteins. But is the template membrane necessary for the formation of the crystalline cone? To answer this question, ommatidia from around the periphery of the eye were examined. Some ommatidia in this region never develop fully into functional facets. In the eye of late pupae, the peripheral ommatidia appear to have crystalline cones of various stages of development. The cells housing the most fully developed cones appear similar to those described previously. That is, the template membrane is evident in both longitudinal (Fig. 26, a) and transverse (Fig. 26, b) sections. Other cone cells in the same sections lack template membranes. In these cells, although small amounts of cone protein are

Figure 25:

Template membrane of the mature crystalline cone of adult *Calpodes ethlius.*

The template (arrows) of the crystalline cone is seen in longitudinal (left) and cross-section (right). Note that the deposition of the crystalline cone proteins never exceeds the limits defined by the template membrane (mag. 800x).

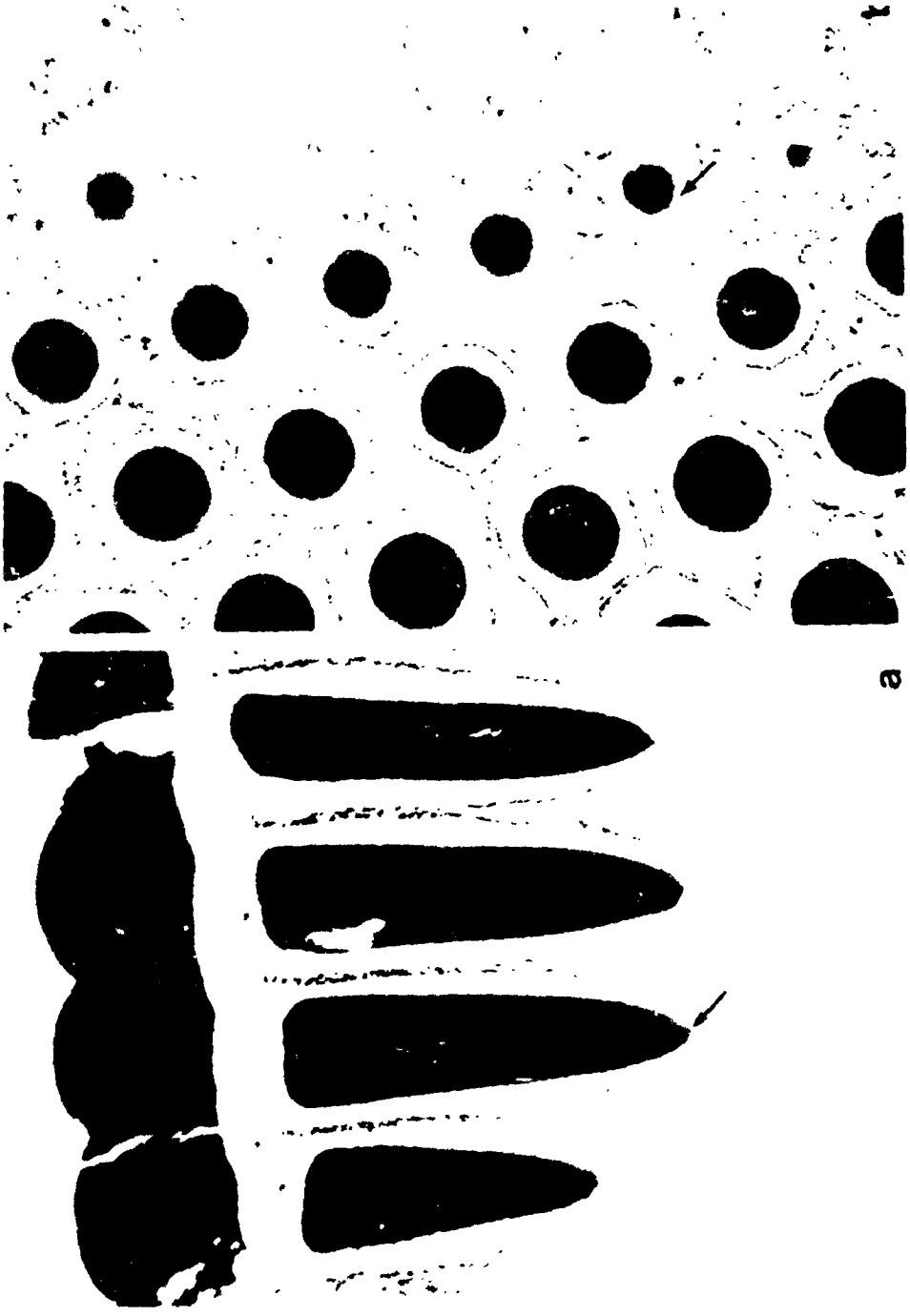
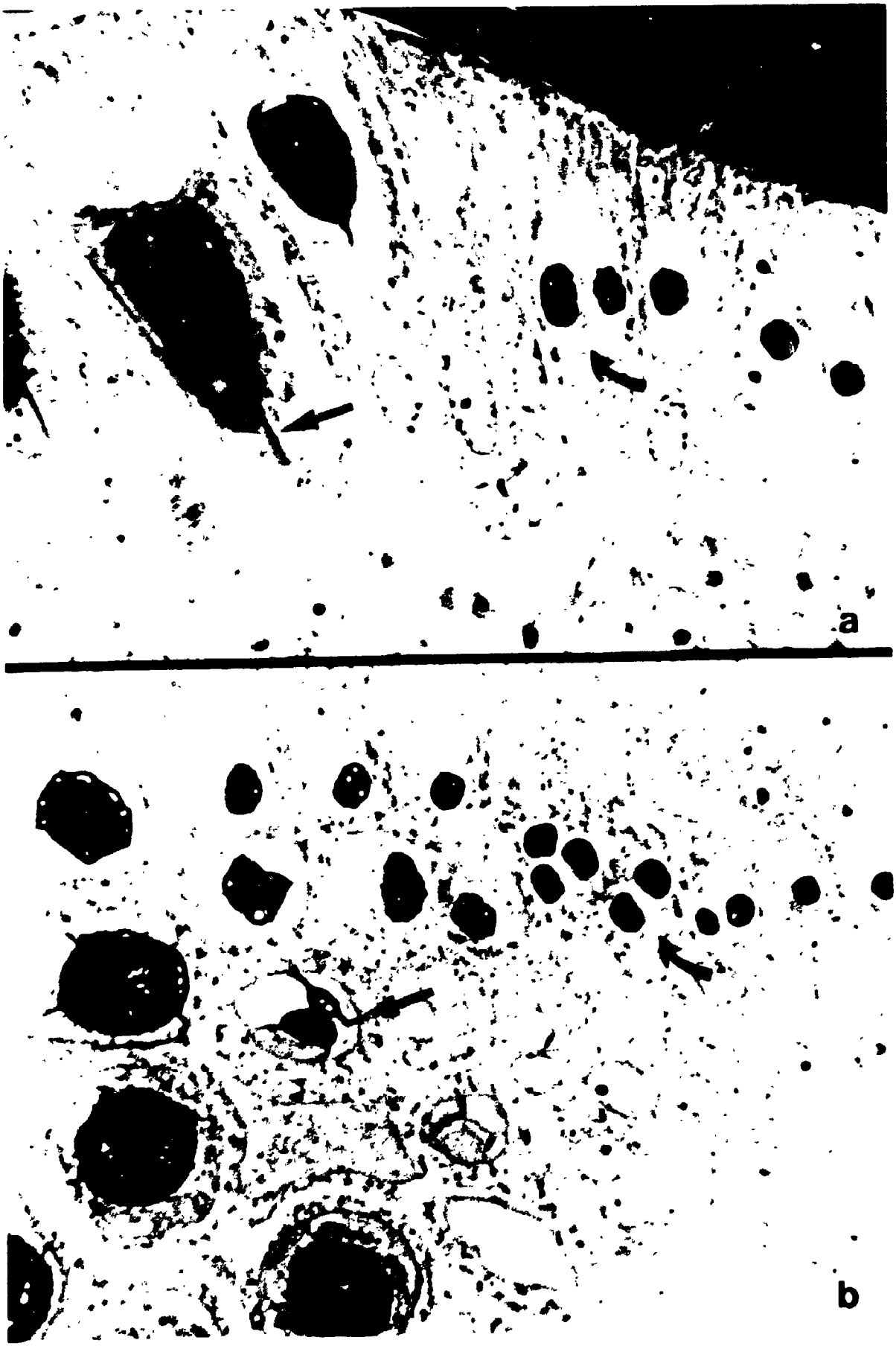


Figure 26:

The crystalline cone fails to form in the absence of the template membrane in the eye of *Calpodes ethlius*.

Histological examination (a: longitudinal sections; b: cross sections) of the peripheral eye regions in the 8 day old pupa. In the presence of the template membrane (straight arrows), the proteins are deposited to form a crystalline cone. In the absence of the template membrane (curved arrow), the cone proteins persist as aggregates in the cytoplasm and cone formation is arrested (mag. approx. 1,000x).


a
b

synthesized, it is not deposited in an ordered fashion. Instead, the cone material accumulates as granules in the cytoplasm. Any further development is apparently arrested (Fig. 26).

### 4.3.4 Template Membrane Extraction

In an attempt to isolate the template membrane, the crystalline cones were extracted with several solubilizing agents (Fig. 27 through 30). Table 2 summarizes the effects of these solubilizing agents on the crystalline cone and surrounding cellular components.

The template membrane of the crystalline cone was isolated from proteins making up the bulk of the cone by a selection of the above series of extraction procedures (Fig. 30). Partial extraction of the cone proteins was achieved with 4.5M urea. The major cone polypeptides solubilized in this fraction had molecular masses of 145, 100, 67, 43 and 21kD (Fig. 31, lane a). Other polypeptides were also apparent but are not considered major cone polypeptides. There were only 2 notable differences between the protein profiles obtained after the first and second wash with 4.5M urea. The first wash extracted a larger quantity of the 67kD polypeptide than did the second. Conversely, the second wash extracted more of the 100kD polypeptide than did the first. This implies that at least one member of the 67kD group is more accessible to initial solubilization than is the 100kD polypeptide.

The remaining cone proteins were extracted with two washes of 9M urea. The polypeptides solubilized in the first wash resolved into bands with molecular masses of 100kD and 108kD. The second wash produced a

Figure 27:

Cone cell membrane fragments are resistant to NaOH treatment which solubilizes cone proteins.

Time lapse series showing the morphology of the crystalline cone during solubilization with 10mM NaOH: 1. Isolated crystalline cones in M10 medium. 2. Crystalline cones immediately begin to solubilize upon the addition of NaOH. 3. The cone is completely solubilized except for fragments of membrane within 30sec. 4. No change is apparent by 3min. 5. Membrane fragments (arrow) remain present after 30min.

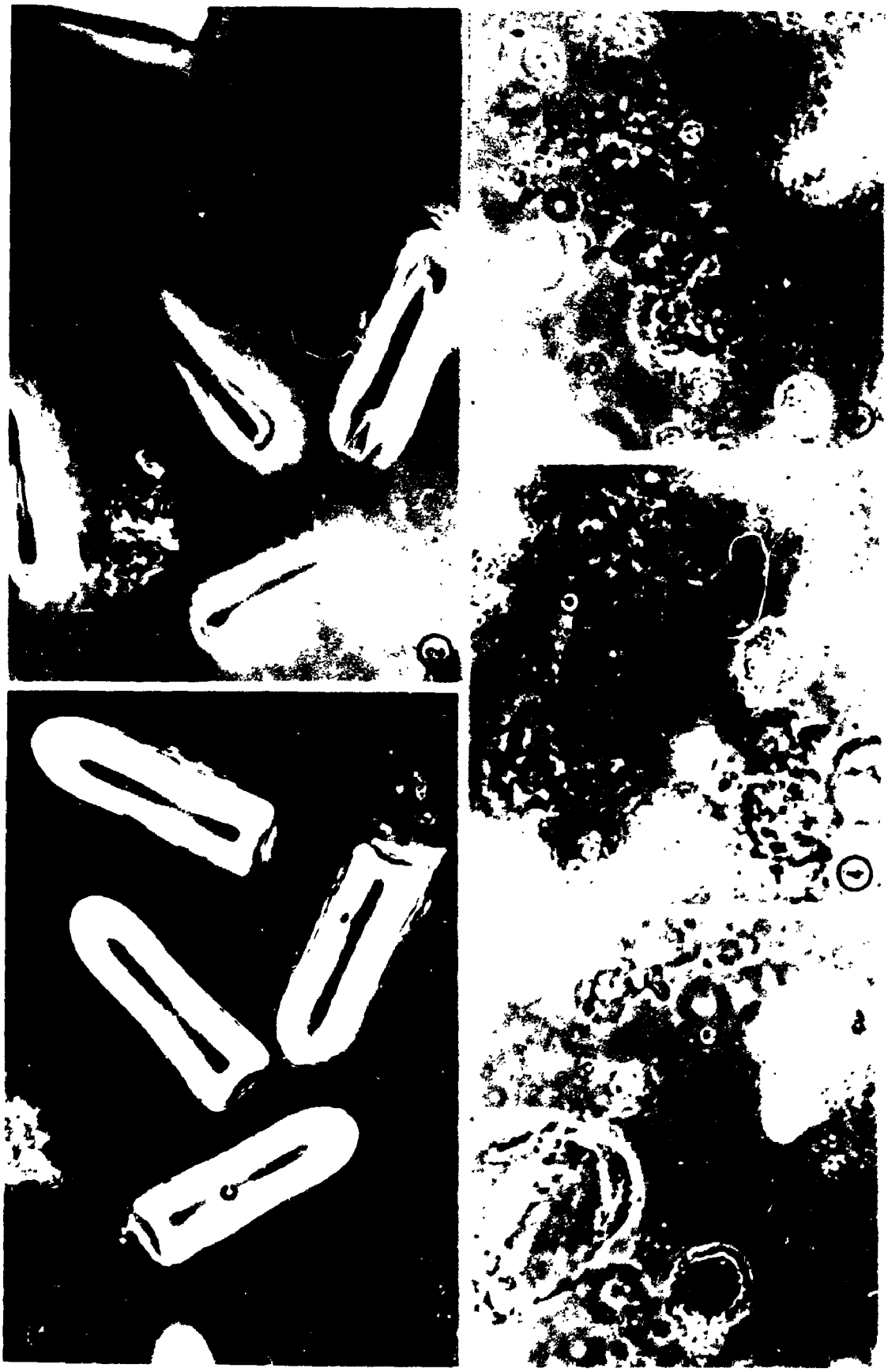


Figure 28:

Complete solubilization of the crystalline cone with SDS.

Time lapse series showing the morphology of the crystalline cone during solubilization with 10% SDS. Within 225 sec., the entire cone has solubilized.

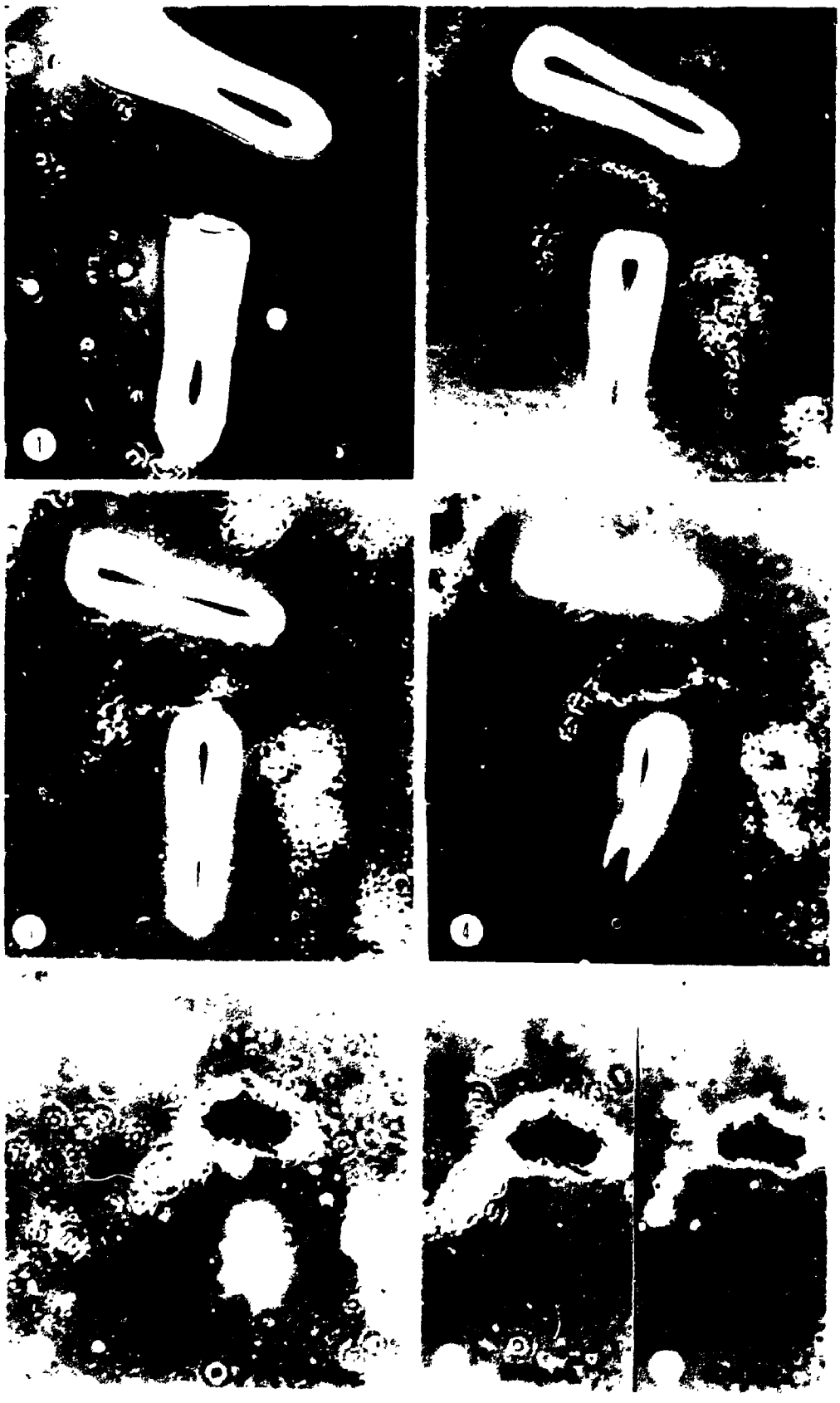


Figure 29:

Isolation of the template membrane using 9M urea.

Time lapse series showing the morphology of the crystalline cone during solubilization: 1. Isolated crystalline cones prior to the addition of urea. 2. The cone has partially solubilized by 15 sec. 3. Further solubilization is apparent at 1min. 4. The entire cone has solubilized by 2min. with the exception of membrane fragments. 5. Membrane fragments (arrow) are present after 50 min.

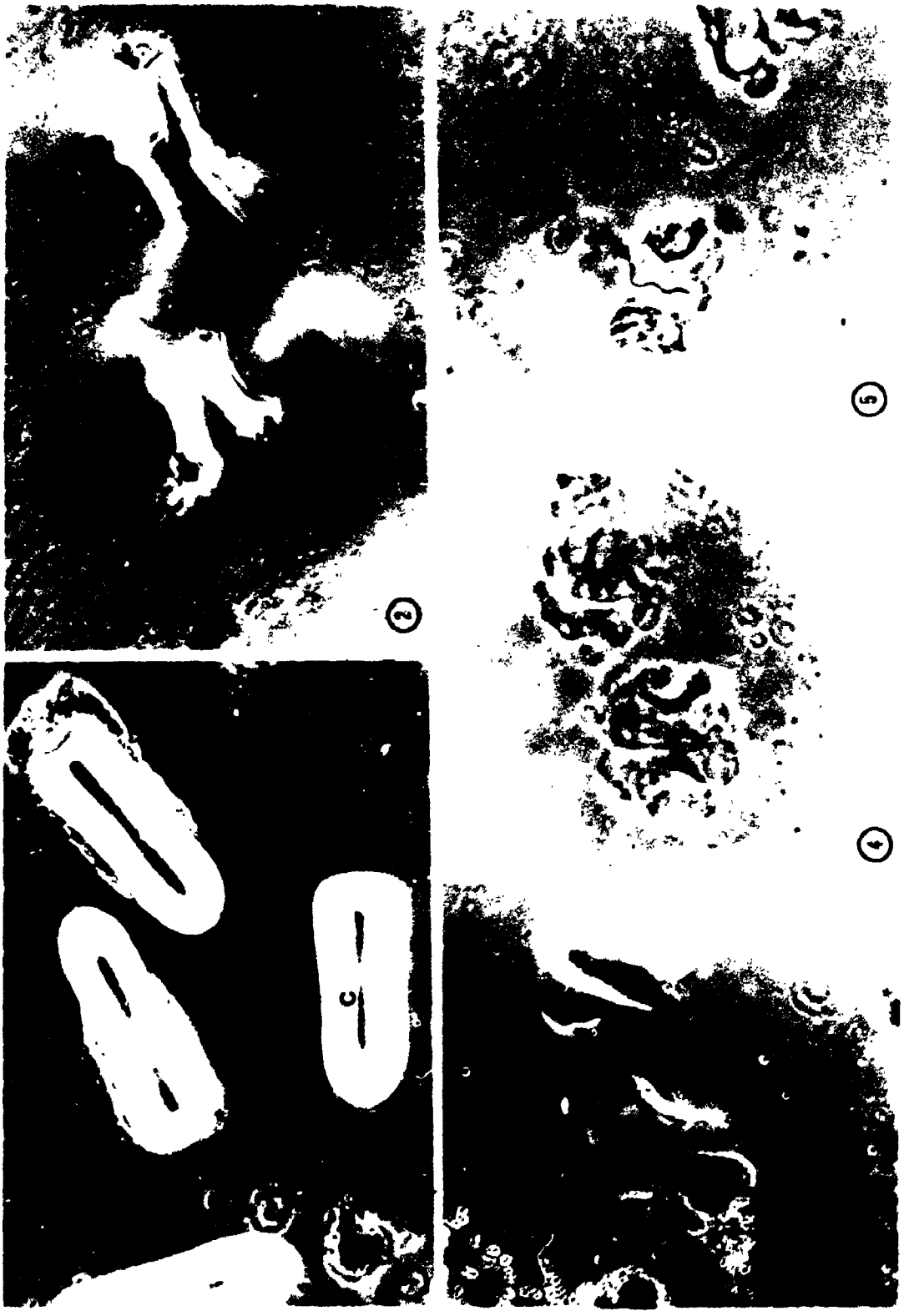

2
3
4
5

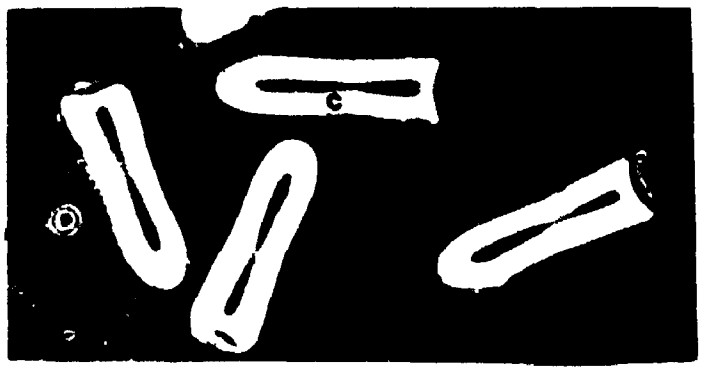
**Table 2. Properties of Solubilizing Solutions Used in Isolating the Template Membrane**

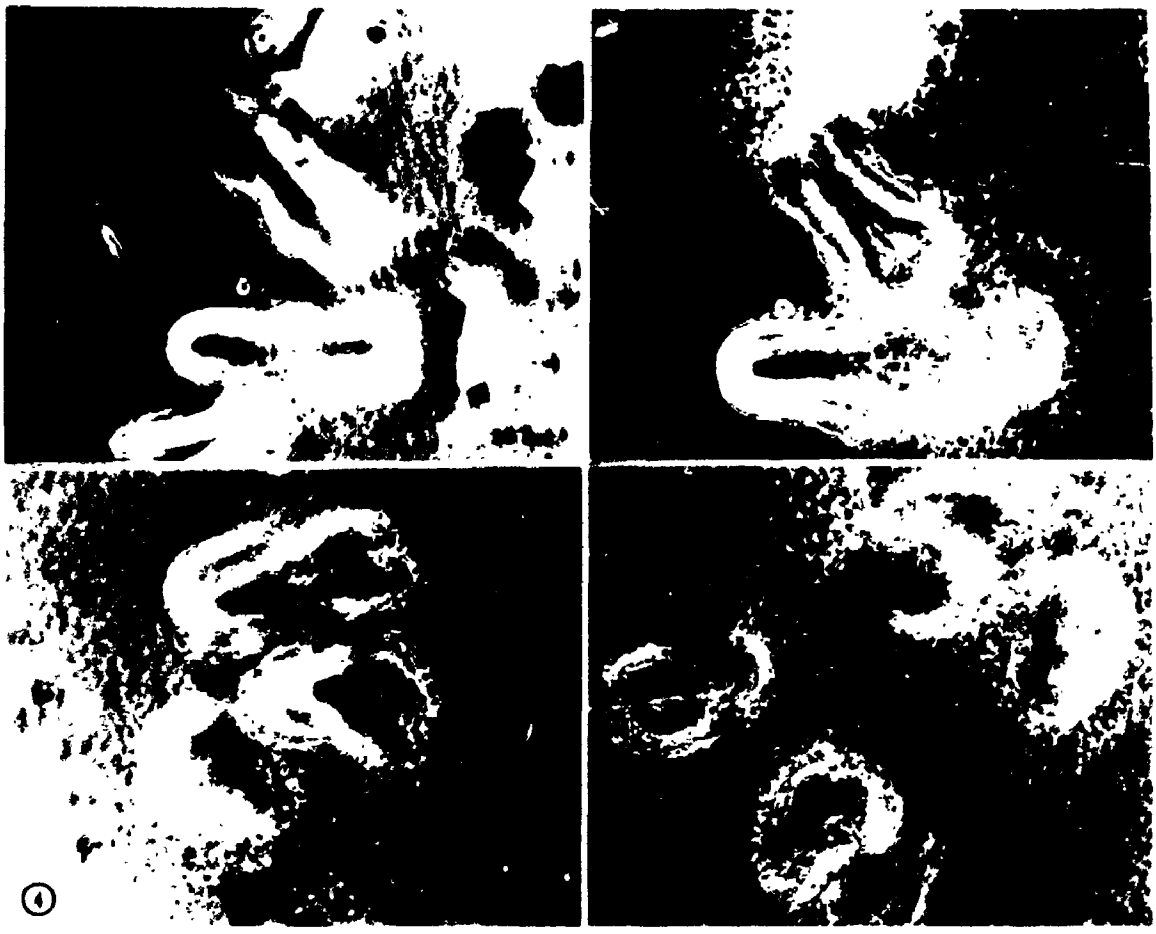
| | Minimal Solubilization | | | Selective Solubilization | | | Complete Solubilization | |
|---|---|---|---|---|---|---|---|---|
| Cellular Components: | M 10 | 5% Triton X-100 | RIA Buffer | 10 mM NaOH | 4.5 M Urea | 9.0 M Urea | SBA | 10% SDS |
| Cytoplasm | Stable | Stable | Partial Solubilization 2 min. | Solubilized Instantly | Solubilized 1 min. | Solubilized 1 min. | Solubilized Instantly | Solubilized Instantly |
| Cone, First Sign of Solubilization | Stable | Stable | Stable | 15 sec. | 15 sec. | 15 sec. | 15-30 sec. | 30 sec. |
| Time to Complete Solubilization | Stable | Stable | Stable | 30 sec. | Solubilization Not Complete | 2 min. | 3 min. | 3 min. |
| Cone Template | Stable | Stable | Stable | Vesicular Remnants | Intact | Intact | Solubilized | Solubilized |
| Pigment Granules | Stable | Stable | Dispersal of Pigment Granules | Stable | Stable | Stable | Solubilized | Solubilized 1 min. |

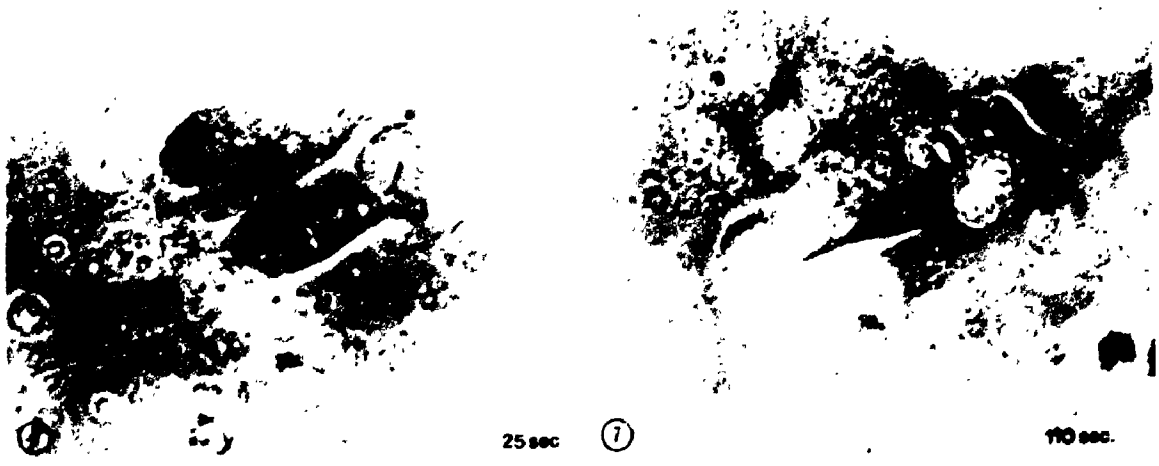
Figure 30:

Sequential extraction method used to isolate and characterize of the template membrane.

Time lapse examination of the morphology of the crystalline cone during partial solubilization with 4.5M urea (panels 1 through 5) followed by complete solubilization with 9M urea (panels 6 and 7). Only plasma membrane fragments of the cone (arrow) are present following this two-step extraction.







25 sec

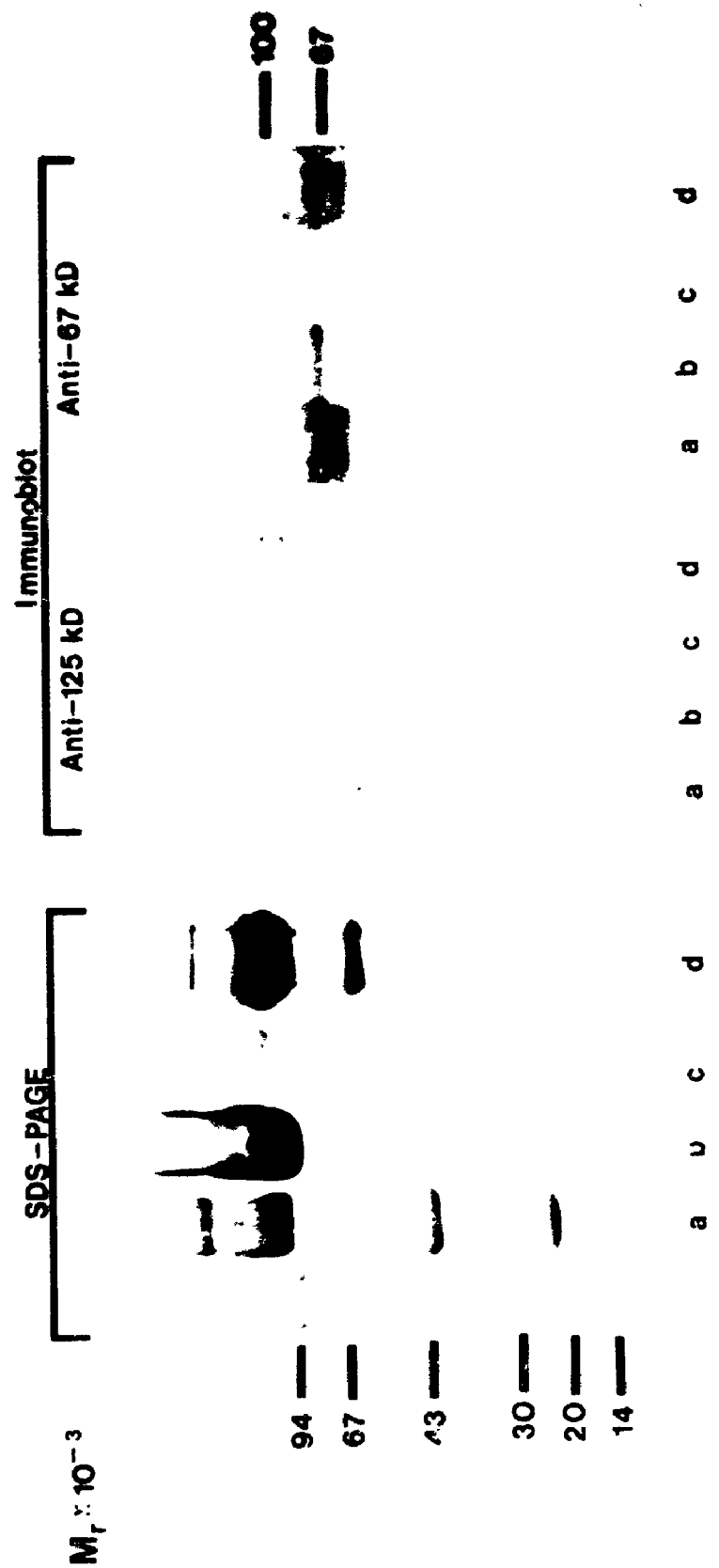
110 sec.

Figure 31:

Identification of the template membrane proteins of *Calpodes ethlius.*

Left panel: One-dimensional SDS-PAGE analysis of the supernatant during the 2-step extraction of the crystalline proteins by 4.5M urea (lane a) and 9M urea (lane b), followed by the solubilization of the template membrane in SDS (lane c). Total crystalline cone lysate (lane d).

Right panel: The polypeptide patterns, identical to that shown in the left panel, were transferred to nitrocellulose and challenged with the anti-125kD (left) and anti-67kD (right) lens antibodies of *Onitis*. The anti-125kD antibody identified a 100kD polypeptide (left lane c) and the 67kD identified a 67kD polypeptide (right lane c) in the template fraction of *Calpodes*.


SDS-PAGE
Immunoblot
Anti-125 kD
Anti-67 kD
$M_r \times 10^{-3}$
94
67
43
30
20
14
100
67
a b c d
a b c d
a b c d

banding pattern quantitatively similar to that obtained from the extraction of total protein using 9M urea followed by sonication (Fig. 31, lane d). The difference between these profiles, however, was the reduction in the amount of the 67kD polypeptide in the second wash in comparison to that found in the total protein extracts. The two 9M urea washes were pooled and a typical separation pattern of the extract is shown in Fig. 31, lane b.

To obtain the template membrane fraction, the insoluble fraction remaining after the above treatment was treated with two washes containing 2% SDS. The solubilized polypeptides from both washes were pooled and visualized with silver nitrate on 1D SDS-PAGE. Although little protein was present in the sample, one polypeptide of 100kD was clearly resolved (Fig. 31, lane c).

### 4.3.5 Polypeptides of the Template Membrane

Due to the limited resolution of the template membrane extract on stained gels, proteins were transferred to nitrocellulose and challenged with lens antibodies raised against the 125kD and 67kD polypeptides of the lens of *Onitis aygulus*. The anti-125kD and anti-67kD specifically recognized the 100kD and the 67kD polypeptides respectively in the lens of *Calpodes ethlius*.

As expected, a positive reaction with the 125kD antibody was observed in every step of the extraction procedure (Fig.31, center panel), including the fraction of template membrane (lane c). The anti-67kD recognized 2 bands at 67kD from the total lens extract (Fig. 31, final panel, lane d) and after partial extraction with 4.5M urea (lane a). Only 1 band

with a molecular mass of 67kD was present in the fraction extracted with 9M urea. In the template membrane fraction, 2 bands, with the same molecular mass as observed in the total extraction, reacted positively but were very faint (lane c).

4.3.6 Polypeptide Isoforms in the Template Membrane

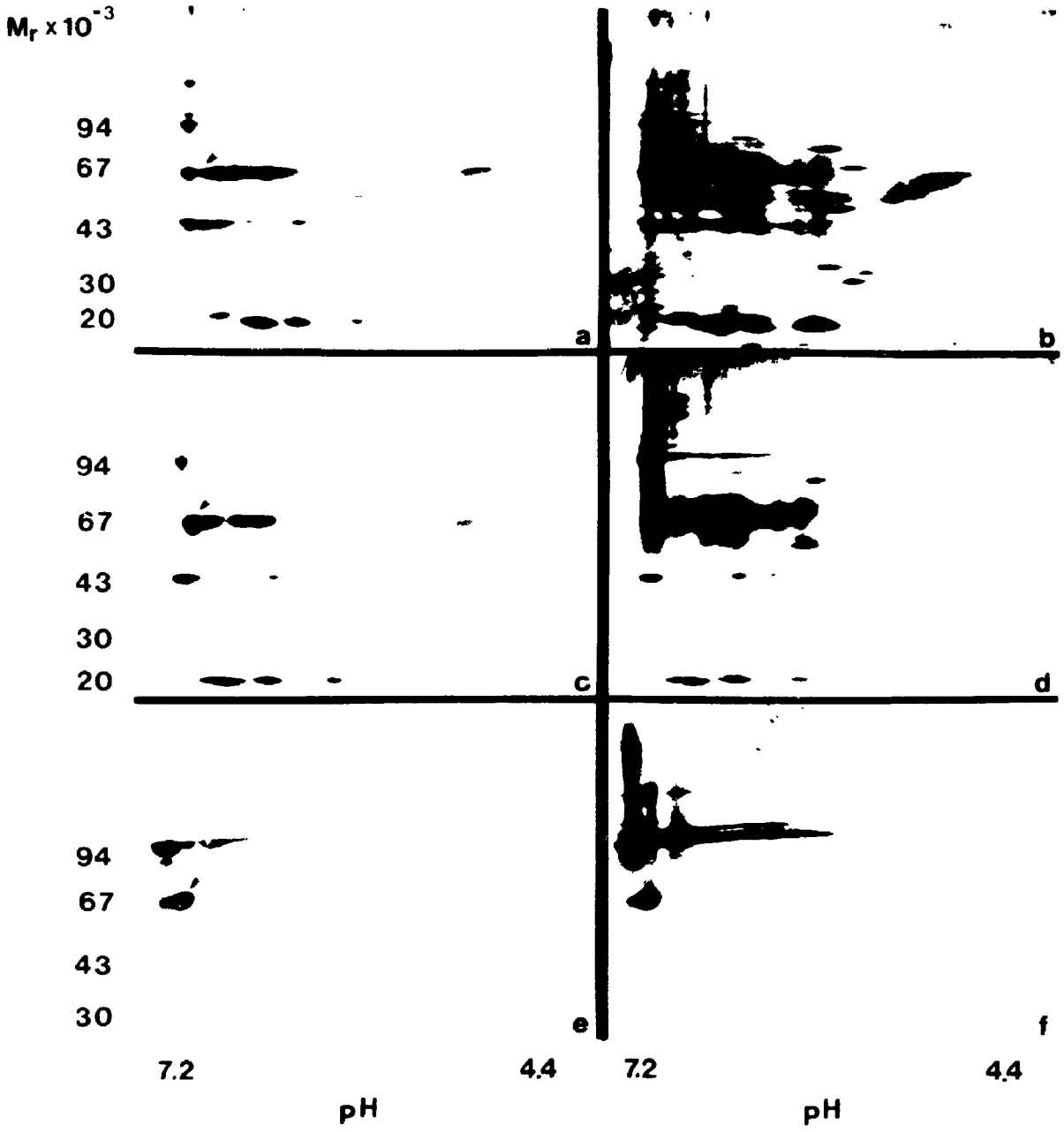
Two-dimensional analysis of each of the fractions used in the isolation of the template membrane (Fig. 32), showed that different isoforms of the major crystalline cone polypeptides were located in specific regions of the lens. The 45, 21 and 19kD polypeptides have numerous isoforms which occur in the body of the crystalline cone. All of these polypeptides were extracted, to varying degrees, with 4.5M (Fig. 32, panels a and b) and 9M urea (Fig. 32, panels c and d). The 45kD protein consists of approximately 9 isoforms. The 21kD protein has approximately 4 isoforms and the 19kD polypeptide resolves into approximately 5 isoforms. Since none of these isoforms are associated with the template membrane, they will not be discussed further.

The 67kD polypeptide family consists of 8 isoforms when stained on gels with Coomassie blue. Seven isoforms were extracted from the cone with 4.5M urea (Fig. 32, panel a). Six of these isoforms had relatively neutral isoelectric focussing points (pI's) of 7.2, 6.9, 6.7, 6.6, 6.4, and 6.3. The final isoform appeared as a smear with a pH ranging from 4.3 to 4.6. The isoelectric point of the isoform of pI 7.2 may not be accurate because the polypeptide only entered the most proximal region of the IEF gel and may not have focussed properly.

Figure 32:

Polypeptides associated with the template membrane.

IEF-SDS-PAGE analysis of the sequential extraction of the cone proteins in 4.5M urea (a,b), 9M urea (c,d) and SDS (e,f). Gels stained with Coomassie blue (left panel) and double stained with Coomassie blue and silver nitrate stain (right panel).Three isoforms of the 100kD polypeptide and one isoform of the 67kD polypeptide are associated with the template membrane. The arrowheads in panels a, c and e indicate the position of the 67kD isoform associated with the template membrane. Note, this isoform is not extracted with 4.5M urea (a).


$M_r \times 10^{-3}$
94
67
43
30
20
a
b
94
67
43
30
20
c
d
94
67
43
30
e
f
7.2
4.4
pH
7.2
4.4
pH

When the polypeptides extracted with 4.5M urea are stained with both Coomassie blue and silver stain, many regions of the gel were so heavily stained that individual polypeptides were difficult to discern. This, unfortunately, was the situation with the family of 67kD polypeptides. It is possible, however, that two additional isoforms with pI's between 5.0 and 6.0 exist (Fig. 32, panel b).

Subsequent extraction with 9M urea, yielded 6 isoforms of the 67kD family on Coomassie blue stained gels (Fig. 32, panel c). Five of the isoforms had the same pI's seen in the 4.5M urea fraction (6.9, 6.6, 6.4, 6.3, and the smear at 4.3-4.6). A previously undetected isoform with pI of 7.0 was also present (arrowhead). This isoform was the most abundant of all the 67kD polypeptides. The other 67kD polypeptides were reduced in quantity compared to the previous fraction. The isoforms with pIs of 7.2 and 6.7 were not detectable.

Staining the above gel with silver (Fig. 32, panel d), again resulted in a polypeptide pattern which was difficult to interpret. The staining pattern, however, suggested that two other isoforms with pI of approximately 5.5 and 6.1 exist in this fraction. These polypeptides may.be present in very low amounts and therefore only detectable by silver nitrate staining. Alternatively, these isoforms may be extremely partial to silver staining as are many other members of the 67kD family.

The final extraction with SDS produced a fraction containing very few polypeptides when visualized with Coomassie blue (Fig. 32, panel e). Only 3 isoforms of the 67kD family were detected. Of these, the isoform with a pI of 7.0 (arrowhead) was predominant. The other polypeptides with pI of 7.2 and a smear between 5.0 and 5.3 occur in only trace amounts. None of the 43kD or 20kD isoforms appear in this fraction.

No significant difference in the protein pattern was observed when the SDS fraction was visualized with silver stain (Fig. 32, panel f). Although the pH 7.0 isoform of the 67kD polypeptide stained more readily with silver, the other isoforms did not. By elimination then, one isoform (pI of 7.0) of the 67kD polypeptide family contributes exclusively to the template membrane.

The 100kD polypeptide has several isoforms that are associated with the template membrane. Because of the extremely hydrophobic nature of this polypeptide, it focusses very poorly in IEF gels. Often the protein fails to enter the gel, or remains in the uppermost regions of the gel giving misleading pI values. Similar difficulties were encountered when nonequilibrating pH gel electrophoresis was used.

Fractions produced after extraction with 4.5M and 9M urea produced similar two-dimensional separations for the 100kD polypeptide when visualized with Coomassie blue. In both instances, the 100kD polypeptide was detectable but was not present in large quantities. It barely entered the IEF gel and had a pI value of 7.2 under these experimental conditions (Fig. 32, panels a,c). Staining the same gels with silver showed that in the 4.5M urea extraction (Fig. 32, panel b), the 100kD polypeptide resolved into possibly three isoforms with pIs of 7.2, 7.0 and 6.8. These spots occasionally foccussed as far as pH 6.4. The separation pattern obtained following 9M urea was less defined (Fig. 32, panel d). Only a 7.2 isoform was recognized as a distinct polypeptide. The rest of the profile consisted of a streak from pH 7.2 to approximately 6.0.

The template membrane fraction contains a substantial proportion of the 100kD polypeptide family. When stained with Coomassie blue (Fig. 32, panel e), 3 distinct isoforms with pIs of 7.2, 7.0 and 6.9 were detected.

Streaking of the polypeptides was evident from pH 6.9 to 5.5. Staining the identical gel with silver nitrate did not reveal any differences in the spot pattern but did enhance all aspects of the previously observed profile (Fig. 32, panel f). The template membrane, therefore, contained or was closely associated with at least 3 isoforms of the 100kD polypeptide family.

## 4.4 DISCUSSION

### 4.4.1 Function of the Template Membrane

The size and shape of the crystalline cone in the compound eye of *Calpodes ethlius* (skipper butterfly) is determined by a specialized region of cone cell plasma membrane. This membrane is easily identified by its staining properties. It stains readily with toluidine blue in histological preparations and is highly osmiophilic in comparison to the plasma membranes of the other cells of the ommatidium when viewed with the electron microscope.

This membrane is detectable in eye tissue as early as 3 days after pupation, which coincides with the initial formation of the crystalline cone. The primary function of the membrane appears to be to provide a preferred site for the deposition of the cone proteins. As the cone proteins are synthesized, they are transported through the cytoplasm and adhere to the specialized template membrane. Fusion of the cone proteins with this membrane during the early stages of development results in the cone having a 4 pointed star configuration when viewed in transverse section. In the absence of the specialized membrane, the newly synthesized cone proteins still travel through the cytoplasm but appear to be unable to attach to the existing plasma membrane. As a result, the cone proteins aggregate in the cytoplasm. These aggregates appear in numerous sizes and shapes but apparently are never organized into a structure that can

successfully function as a lens.

The template membrane appears to grow as the crystalline cone grows. This may be a consequence of plasma membrane growth or because the template can be only visualized after a component of the cone material is added. Growth of the membrane, however, proceeds at a faster rate than the actual deposition of the cone proteins. This is evident throughout the pupal stage, especially when the cone cells are examined in cross-section. As the cone grows from its star configuration into a square then into the characteristic circular shape, the peripheral regions of the specialized membrane remain free of any cone proteins. A similar portion of membrane is also present along the longitudinal axis of the crystalline cone. This 'naked' section of membrane appears to act as a guideline for the growth of the cone. Once the growth of the specialized membrane is complete, however, cone proteins are continually deposited until none of the naked membrane remains exposed. Deposition of cone proteins beyond the limits defined by the specialized membrane was never observed.

The function of the specialized membrane, then, is two-fold. Firstly, it provides a specific site for the initial attachment of newly-synthesized cone proteins to the plasma membrane. In its absence, cone proteins are synthesized but fail to deposit onto the cone cell membrane. Secondly, the specialized membrane serves as a blueprint for the ultimate size and shape of the mature lens, in the latter case by controlling precisely the radius of the cone at all levels along its longitudinal axis. Based on these two functions, the specialized membrane acts as the template for the formation of the crystalline cone. Species-specific differences in cone shape result from differences in template membrane topography.

### 4.4.2 Involvement of Cytoskeletal Elements in Shaping the Crystalline Cone

In determining the overall size and shape of the lens, the template membrane contributes to the normal optical functioning of the crystalline cone. But, how is the precise shape of the lens controlled when the template membrane only provides a guideline for 2 of the 3 sides (the internal 2) in each quadrant of the crystalline cone? The third side, which contributes to the circumference of the lens, is directly exposed to the cone cell cytoplasm and has no association with the template membrane. The curvature of this cytoplasmic face must be uniformly cylindrical in all four of the cone cells to enhance the optical performance of the lens. What controls the shaping of the cytoplasmic face of the crystalline cone?

Cytoskeletal elements, such as actin, vimentin, tubulin, and intermediate filaments play a major role in vertebrate vision by controlling the shaping of the lens fibers during development and during accommodation (Ireland *et al.*, 1983; Ellis *et al.*,1984; Scholz and Rafferty, 1988). The crystalline cone, however, does not contain either actin or tubulin. The internal cytoskeleton, if one exists, is due to the intrinsic properties of the cone proteins and not due to cytoskeletal elements commonly found in other lenses.

It is possible that cytoskeletal elements external to the lens are involved in shaping the cone. Although actin and tubulin are not components of the crystalline cone, they are present within the cone cell cytoplasm. Immunofluorescent studies show that both actin and tubulin are concentrated around the periphery of the crystalline cone, especially around the most pliable region of the lens, the proximal tip. These

components alone, or in conjunction with other filaments, could restrain or compress the proteins into their appropriate position. Cytoskeletal elements, therefore, may maintain the surface curvature of the exposed faces of the developing crystalline cone after its overall size has been outlined by the template membrane.

Finally, other researchers have observed extensive layers of endoplasmic reticulum bordering the cytoplasmic face of the crystalline cone (Horridge *et al.*, 1972). They postulated that this network of membranes may provide a physical barrier which aids in the shaping of the cone. Similar stacks of endoplasmic reticulum, although much less developed, have been observed in the cone cells of *Calpodes ethlius* and *Onitis aygulus*,. The stacks of membrane, however, are only evident in adult eyes and are not consistent in their alignment or location. The location of the endoplasmic reticulum may be a consequence of the crystalline cone displacing all cytoplasmic components towards the periphery of the cone cells during its growth rather than contributing to its shaping. It is possible, however, that the cytoplasmic membranes are exerting some physical force or tension around the crystalline cone.

### 4.4.3 Solubility of the Crystalline Cone Components

Purified membrane preparations were obtained by partial extraction of cone proteins in 4.5M and 9M urea followed by complete solubilization in 2% SDS. The majority of the crystalline cone proteins were extracted with 4.5M urea. Unlike that observed during the solubilization with SDS or NaOH, the crystalline cone does not shrink in

size, but becomes very bubbly in appearance and then ruptures. The undissolved components remain suspended in solution with no apparent organization. This mode of solubilization negates the possibility that these are peripheral cone proteins which are readily solubilized because of their accessibility to chemical denaturation. It is conceivable that these polypeptides are distributed throughout the crystalline cone, and have equal exposure to solubilization by the urea. This group of polypeptides can be regarded as the components forming the bulk or body of the cone.

The proteins extracted with 9M urea are probably also constituents forming the body of the crystalline cone. Possibly because this group of proteins is more hydrophobic in nature or has a more stable configuration, they are more difficult to solubilize. After exposure to 9M urea, the proteins which remain unsolubilized are components of, or are tightly associated with, the template membrane. This fraction consists solely of a 100kD polypeptide and one isoform (pI of 7.0) of the 67kD family.

### 4.4.4 Possible Interaction of the Crystalline Cone Proteins with the Template Membrane

How does the template membrane control the deposition of the cone proteins to obtain the precise shape and size of the mature crystalline cone? Although the complete answer to this question is still unknown, an hypothesis can be made based on the results obtained in this study.

In the absence of this membrane the cone proteins are synthesized and aggregate in the cytoplasm. This implies that a component of the template membrane serves as a recognition site for the attachment of the

cone proteins. The recognition between the membrane and cone proteins is dependent on one isoform (pI of 7.0) of the 67kD family and/or the 100kD polypeptide. These polypeptides may either be intrinsic membrane proteins or be cone proteins with the appropriate charge, side groups or configuration to be attracted to and bind to a component of the membrane. It is unlikely that either of the polypeptides is an intrinsic membrane protein. The basis for this assumption is two-fold. Firstly, both polypeptides are present in significant amounts in other fractions of the extraction procedure. Secondly, the quantity of both polypeptides seems to be excessive for the area of membrane involved considering that other membrane components were not detected.

Although the 67kD isoform and the 100kD isoforms in the template membrane fraction are unlikely to be intrinsic membrane proteins, their close association with the template would classify them as peripheral membrane proteins. The 67kD isoform is found predominantly in the SDS fraction and is probably the cone polypeptide which could bind to a receptor protein in the template membrane and provide a link between the membrane and other cone polypeptides. The 100kD polypeptide, however, is found in large amounts in all fractions and must be considered as one of the polypeptides forming the bulk of the cone. Because of its close association with the membrane, the 100kD polypeptide must either bind to the 67kD isoform or directly to the template membrane.

Possibly the 100kD polypeptide is the sponge described earlier in the development of the crystalline cone. This sponge could act as a scaffold by attaching to the membrane and providing numerous sites for other cone proteins to attach. This would account for the quantitiy of the 100kD polypeptide, its close association to the template membrane, the presence

the proteins attach to a specialized region of cone cell membrane (the template membrane). As the cone grows, the protein deposit appears sponge-like and may serve as a scaffold. Other proteins attach to the scaffold (fill in the sponge), and the cone becomes a dense granular deposit.

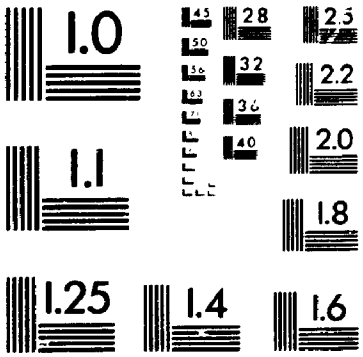
b) Unlike *Calpodes ethlius*, the crystalline cone proteins of *Onitis aygulus* are synthesized in a temporal sequence. The newly synthesized proteins are transported through the cytoplasm and deposited as granules or aggregates along the template membrane to give a star configuration. The cone grows by the addition of newly synthesized proteins to its length and girth. As a result, the cone advances through several shape changes before obtaining its characteristic bullet-shape.

7) The formation of the graded refractive index is not completely established by accretion during the development of the crystalline cone. A post deposition maturation model, in which the properties of the cone proteins (hydrophobicity, packing, hydration) control the GRIN formation after the cone proteins have been synthesised and incorporated into the cone, is more plausible.

8) A specialized region of the cone cell membrane is regarded as a template membrane because of its function in the formation of the crystalline cone:

a) It serves as the initial site of cytoplasmic deposition for the cone proteins. In the absence of this membrane, synthesized cone proteins form random aggregates in the cytoplasm.

b) It determines the size and shape of the mature cone by defining



of this polypeptide in all fractions of the extraction and the sponge appearance during development. The other isoforms of the 67kD family, the 45kD family and the rest of the cone proteins can be perceived as the flocculent material which fills in the sponge during the development of the lens.

Starting with the template membrane as a blueprint, the crystalline cone proteins successfully organize themselves into a precisely shaped, functional lens.

# PART 5

## 5.1 CONCLUSIONS

This is the first report of the protein composition of the crystalline cone and the first detailed analysis of the structure and formation of this lens in the superposition compound eye. The following conclusions can be made:

1) The adult crystalline cones from the coleopteran and lepidopteran species examined consist of a small population of native proteins made up of predominantly hydrophobic polypeptides.

2) The major cone polypeptide in the beetle (125kD) is very similar to the major cone polypeptide in the moth (100kD) in being very hydrophobic, abundant and immunologically related. Both of these polypeptides appear to be related to a 67kD family of polypeptides in having similar protease digest products, denaturation products and a immunological cross-reactivity.

3) The proteins of the crystalline cones examined are not glycosylated. Glycogen is absent from the body of these cones. The cone cell cytoplasm does however, contain varying amounts of glycogen depending

on the species. The crystalline cone of *Onitis aygulus* is wrapped in a thin sheath of glycogen. In *Calpodes ethlius*, the glycogen sheath surrounding the cone is less defined and thicker, especially around the proximal tip.

4) The composition of the crystalline cone shows phylogenetic diversity:

a) Some of the crystalline cone polypeptides are common to all of the insects examined. Examples of this are the 57kD and 67kD polypeptides. The 67kD polypeptide was the only one that appeared as a major crystalline cone constituent in both coleopterans and lepidopterans and may be considered as an arthrocrystallin.

b) Other major polypeptides are specific to one insect order. The 100kD and 108kD polypeptides are only found in moths and butterflies; a 45kD polypeptide is found in significant amounts only in beetles.

c) The distribution of some polypeptides is restricted to more closely related groups. The 125kD and 135kD polypeptides are found only in the dung beetles.

d) Finally, relative quantities of certain polypeptides appear to be species specific.

5) Most of the major polypeptides of the crystalline cone are also found in the cornea of the same species.

6) The crystalline cone is formed by different mechanisms in *Calpodes ethlius* and *Onitis aygulus*.

a) The crystalline cone proteins of the skipper butterfly are synthesized and apparently deposited in a co-ordinate manner. Initially

the proteins attach to a specialized region of cone cell membrane (the template membrane). As the cone grows, the protein deposit appears sponge-like and may serve as a scaffold. Other proteins attach to the scaffold (fill in the sponge), and the cone becomes a dense granular deposit.

b) Unlike *Calpodes ethlius*, the crystalline cone proteins of *Onitis aygulus* are synthesized in a temporal sequence. The newly synthesized proteins are transported through the cytoplasm and deposited as granules or aggregates along the template membrane to give a star configuration. The cone grows by the addition of newly synthesized proteins to its length and girth. As a result, the cone advances through several shape changes before obtaining its characteristic bullet-shape.

7) The formation of the graded refractive index is not completely established by accretion during the development of the crystalline cone. A post deposition maturation model, in which the properties of the cone proteins (hydrophobicity, packing, hydration) control the GRIN formation after the cone proteins have been synthesised and incorporated into the cone, is more plausible.

8) A specialized region of the cone cell membrane is regarded as a template membrane because of its function in the formation of the crystalline cone:

a) It serves as the initial site of cytoplasmic deposition for the cone proteins. In the absence of this membrane, synthesized cone proteins form random aggregates in the cytoplasm.

b) It determines the size and shape of the mature cone by defining

the outer limits of the cone and by providing a blueprint of the lens shape.

9) The template membrane in *Calpodes* contains, or is tightly associated with, a 100kD polypeptide and one isoform of the 67kD family of proteins. Although their function is unknown, the 67kD isoform may initiate the deposition of the crystalline cone by providing a link between the template membrane and the other cone proteins. The 100kD polypeptide may also bind directly to the template membrane or interact with the 67kD polypeptide. Because of its abundance, the 100kD polypeptide probably forms the sponge-like network seen in the developing cone and is providing a scaffold for the attachment of other cone proteins.

## 5.2 APPENDIX 1:

### Calculation of Crystalline Cone Volumes Using Simpson's Rule

1. One half of the longitudinal cone contour is placed onto graph paper. The X-axis is divided into small intervals of delta x. The more intervals, the more accurate the volume calculation.

2. Where the lines dividing the intervals cross the contour, this is where a point occurs. Along the X-axis these points are separated equally by a distance of delta x. The first point occurs at the start of the contour, i.e. at X=x0, then successive points at $X=x_1, x_2, x_3,.....$

3. Tabulate the Y-coordinates at all of these points by $Y=f(x_0), f(x_1), f(x_2),.....$, then square them:

| n | $x_n$ | $f(x_n)$ | $[f(x_n)]^2$ |
|---|---|---|---|
| 0 | $x_0$ | $f(x_0)$ | $[f(x_0)]^2$ |
| 1 | $x_1$ | $f(x_1)$ | $[f(x_1)]^2$ |
| 2 | $x_2$ | $f(x_2)$ | $[f(x_2)]^2$ |
| etc. | | | |

4. Simpson's Rule is a technique for numerical integration. For reasons soon to be apparent, you must calculate:

$$\int_a^b [f(x)]^2 dx \qquad \text{equation A}$$

f(x) is the equation of the cone contour. Since only the points along the contour are known, equation A can be calculated numerically with Simpson's Rule:

$$\int_a^b [f(x)]^2 dx = 1/3 \text{ delta } x[f(x_0)^2+4[f(x_1)^2+f(x_3)^2+...+f(x_n-1)^2] +[f(x_2)^2+f(x_4)^2+ \quad +f(x_n-2)^2]+f(x_n)^2$$

i.e.,

$$\int_a^b [f(x)]^2 = 1/3 \text{ delta } x[f(x_0)^2+4(\text{odds})+2(\text{evens})+f(x_n)^2]$$

this is equation B

5. Now, volume is given by:

$$V=\pi\int_a^b [f(x)]^2 dx$$

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