THE ROLE OF SOME PHOSPHATASE ENZYMES IN INVERTEBRATE PHOTORECEPTORS

STEPHEN CHARLES TROWELL

A THESIS SUBMITTED FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY OF THE

AUSTRALIAN NATIONAL UNIVERSITY

CANBERRA

JANUARY 1987
DECLARATION

Most of the ultrathin sections that are shown in Chapter 2 were cut by Claudia de Couet. Dr. Robin Irvine prepared the $^{32}$P-labelled inositol trisphosphate that was used in the experiments described in Chapter 3.

I declare that the rest of the material described in this thesis is my own original work.

STEPHEN TROWELL


I would like to thank everyone who has helped and supported me throughout this work.

Above all, I would like to thank Dr. David Blest for his careful and patient supervision. Dr. Gert de Couet and Dr. Sally Stowe provided invaluable advice throughout. Claudia de Couet and Margrit Carter gave expert technical assistance. Mira Dumancic proofread the manuscript and helped in numerous other ways.

I have been allowed to use equipment and facilities throughout R.S.B.S. Particular thanks are due to: Professor Brian Gunning and the members of the Department of Developmental Biology; George Weston and the staff of the A.N.U. Transmission Electron Microscopy Unit; and Neal Gowen of the former Department of Genetics.
ABSTRACT

1. Selected aspects of the phototransductive biochemistry of vertebrates and invertebrates are reviewed and compared with particular reference to the role of the two classes of phosphatases that are discussed below.

2. A magnesium-dependent phosphatase was localized by ultrastructural cytochemistry to the photoreceptive microvilli of a crab (Leptograpsus variegatus) using the non-specific substrate 4-nitrophenylphosphate (4-NPP); it was identified biochemically in soluble extracts of the crab retina.

3. The activity showed considerable specificity for 4-NPP over other general phosphatase substrates. It was isolated in the supernatant after high-speed centrifugation of retinal homogenates. It was uninhibited by specific alkaline phosphatase inhibitors but was highly sensitive to broad spectrum phosphatase inhibitors such as tartrate and fluoride. The pH optimum of the activity is between 7 and 7.5. It was stabilized by calcium, magnesium and manganese (II) ions. Millimolar calcium inhibited the activity but micromolar calcium appeared to be synergistic with magnesium in stimulating the activity. It was also stimulated by manganese (II) and cobalt (II) ions.

4. The activity is distinct from a number of cellular phosphatases including non-specific acid and alkaline phosphatases and the Na+/K+ ATPase. The activity was separated from the ATPase and GTPase activities of crab retina by column chromatography.

5. The retina of the local squid Sepioteuthis australis, was demonstrated to possess a similar magnesium-activated 4-NPPase activity to that localized in crab retina. A method for the partial purification of the squid...
enzyme is described. The squid and crab enzymes were shown to have apparent native molecular masses of 70-80 kDa and a homodimeric structure is proposed for them.

6. The divalent cation dependency, activity towards 4-NPPase and calmodulin sensitivity of the activity resemble those of mammalian calcineurin but the subunit compositions are different.

7. Proteins with the characteristics of rhodopsin were shown to be phosphorylated in a light-dependent manner in both the species studied. Squid rhodopsin-bearing membranes were not dephosphorylated by crude or partially purified preparations of the 4-NPPase from crab retina. A 48 kDa membrane-associated phosphoprotein of crab retina, whose phosphorylation is also light-dependent, was dephosphorylated by a crude 4–NPPase containing extract of crab retina.

8. The possible physiological role of the 4–NPPase is discussed, with particular reference to the photoreceptor's requirement for a rhodopsin phosphatase.

9. Inositol trisphosphatase and inositol bisphosphatase activities were also identified in extracts of the crab retina. These enzymes, acting in concert, were shown to degrade inositol 1,4,5 trisphosphate; removing at least one of the pair of vicinal phosphates that are believed to be responsible for the biological activity of the molecule when pressure-injected into Limulus photoreceptors. The enzymes are present in high activity, as would be required to fulfil the kinetic requirements for termination of excitation in photoreceptors. The inositol trisphosphatase was purified separately from the 4-NPPase on a DEAE-cellulose column.
CONTENTS

CHAPTER 1 – General Introduction .................................................. 1
  1.1 The visual pigment................................................................. 4
  1.2 Photoreceptor function.......................................................... 7
  1.3 The cGMP cascade................................................................. 8
     The vertebrate pathway of excitation........................................... 8
     Vertebrate inactivation processes.............................................. 9
     Excitation and inactivation in the invertebrate photoreceptor...... 10
  1.4 The role of calcium ions in photoreceptors............................. 13
     Calcium in vertebrate photoreceptors........................................ 13
     Calcium in the invertebrate photoreceptor.................................. 15
  1.5 The role of inositol phosphates in photoreceptors.................... 17
     Inositol trisphosphate in the invertebrate photoreceptor.............. 18
     Inositol trisphosphate in vertebrate photoreceptors.................. 19
  1.6 Light-dependent phosphorylation in photoreceptors................ 20
     The characteristics of light-dependent rhodopsin
     phosphorylation.......................................................................... 21
     Possible functional roles of photoreceptor protein
     phosphorylation........................................................................... 22
     Dephosphorylation and the phosphatase..................................... 30

CHAPTER 2 – Cytochemical Distribution and Biochemistry of a Novel
Phosphatase in the Photoreceptive Microvilli of a Crab.................. 33
  2.1 Summary.................................................................................. 33
  2.2 Introduction.............................................................................. 34
  2.3 Materials and Methods............................................................ 35
     Crabs......................................................................................... 35
     Materials.................................................................................... 35
     Cytochemistry............................................................................ 36
     Electron microscopy..................................................................... 37
     Biochemical fractionation of crab retina..................................... 37
     Enzymatic assays........................................................................ 38
     Protein determination................................................................... 39
  2.4 Results..................................................................................... 39
     Distribution and nature of cytochemical deposits....................... 39
     There are magnesium-dependent and -independent 4-NPPase
     activities in crab retinal homogenates......................................... 42
4-NPPase activity does not sediment with membrane fractions...

The effects of divalent cations...

Effects of phosphatase inhibitors and alternative substrates...

2.5 Discussion...

The specificity of 4-NPPase cytochemistry...

What is the physiological role of the 4-NPPase?

CHAPTER 3 - Inositol Trisphosphatase and Inositol Bisphosphatase Activities of Crab Retina...

3.1 Summary...

3.2 Introduction...

3.3 Materials and Methods...

Crabs...

Materials...

Preparation of inositol trisphosphate...

Specificity of inositol trisphosphate hydrolysis...

Determination of total phosphate in retinal extracts...

3.4 Results...

Hydrolysis of $^{32}$P-labelled 1,4,5-inositol trisphosphate...

Inhibitor studies...

Fractionation of inositol trisphosphate activity of crab retina...

3.5 Discussion...

CHAPTER 4 - Partial Purification from Squid Retina of a 4-NPPase Previously Localized to the Photoreceptive Microvilli of a Crab...

4.1 Summary...

4.2 Introduction...

4.3 Materials and Methods...

Crabs...

Squid...

Materials...

Column chromatography procedures...

Non-denaturing polyacrylamide gel electrophoresis...

SDS-polyacrylamide gel electrophoresis...

Molecular weight estimation by gel permeation chromatography...

4.4 Results...

Activity of 4-NPPase towards alternative phosphoester substrates...
1. GENERAL INTRODUCTION

Collectively, the processes by which a photoreceptor cell transduces and amplifies the energy of an incident photon into a form that can be processed by the nervous system are described as phototransduction. Many biochemical pathways may be involved besides the principal excitatory pathways, such as those subserving sensitivity control, down-regulation, diurnal variations and membrane regeneration.

The biochemistry of phototransduction has only been investigated in any detail in one type of photoreceptor cell, namely the vertebrate rod. This is because it is easy to obtain large quantities of fresh retinal tissue from slaughterhouses and relatively simple methods exist for the purification of rod outer segments (R.O.S.) (for example, Hamm & Bownds, 1986). These studies have been enormously successful in establishing the enzymology of the cGMP pathway of excitation (see, for example, Lamb, 1986). The task now confronting those working on the rod outer segment is to detail the control mechanisms which regulate the photoreceptor's response to light.

The eyes and the photoreceptors of many invertebrates, particularly those of some arthropods and cephalopod molluscs, are as highly developed and specialised as those of any vertebrate.

The reasons for wishing to investigate invertebrate phototransduction are the same as those that stimulated research on the vertebrate rod: to understand the mechanism of a supremely sensitive yet adaptable system. Despite their extreme morphological specializations photoreceptors are cells that can be supposed to have elaborated basic signalling mechanisms in the course of their evolution: for example, research on the rod outer
segment phototransductive cascade has given us the best characterized example of a cGMP second messenger system in cell biology (Stieve, 1986). This has not come about by chance; allied to the unique degree of specialization of photoreceptive segments is the ease with which light stimuli can be manipulated. Unlike a hormone or neurotransmitter, light is available in unlimited quantities at almost no cost.

Another reason for studying invertebrate photoreception is that of comparative evolution. It is the implicit belief of most workers in the field that, despite the demonstrated differences between them, invertebrate and vertebrate phototransduction schemes are closely related and have evolved by emphasising different parts of an ancestral system. This belief is supported by the fact that all the visual pigments so far characterized are highly conserved in both the chromophore and protein moiety. Major differences are thus more likely to be found at later stages of the transductive cascade (Applebury et al., 1986) where interaction with the visual pigment is not a constraining factor. However, because the phototransductive biochemistry of the rhabdoms of arthropod and cephalopod species does apparently differ in some respects from that of the rod outer segment, research in the two systems should be complementary and research on the invertebrate may suggest new areas for investigation in the vertebrate system.

Traditionally, biochemical investigations require that pure preparations of photoreceptive segments be available in quantity. This requirement has limited biochemical work on invertebrate photoreceptors but neither electrophysiological nor ultrastructural investigations of rhabdomeric photoreceptors have lagged significantly behind those using the rod outer segment. In future the techniques of molecular biology may partly circumvent this problem. At present cephalopods are the
invertebrates of choice for biochemical study whereas the most productive invertebrate systems from the electrophysiologists' point of view continue to be the arthropods (particularly the decapod crustaceans, higher insects and, of course, *Limulus.*) It is tempting to try to capitalise on the elegant electrophysiological experiments that have been performed using arthropod rhabdoms, which have included the microinjection of nucleotides and other pharmacological agents (e.g. Bolsover & Brown, 1983; Corson & Fein, 1983a & 1983b).

The approach that was adopted here was the novel one of examining the distribution, at the ultrastructural level, of phosphatase enzymes within the crab retina using a non-specific substrate in a cytochemical screening procedure. Activities that were detected by this procedure were subsequently further characterized using biochemical techniques. This reverses the traditional order in which cytochemical and biochemical techniques have been applied to such problems (see for example the work of Ueno *et al.* on the vertebrate retina (1983; 1984a; 1984b) but it has the following distinct advantages:

1. Enzyme activities that are important in photoreceptor physiology but have not so far been investigated may be revealed.
2. This might allow us to move beyond the limitations of current models of phototransductive biochemistry in the invertebrate.
3. The problem of insufficient quantity and/or purity of biochemical preparations is partly circumvented. Minimally, a precise *in vivo* subcellular localization may be obtained for an enzyme that otherwise may only be studied in crude cell-free extracts. In practise this can be an invaluable pointer to physiological role, particularly in a cell-type with such a high degree of subcellular specialization as the photoreceptor.
This approach has led to the identification of two retinal phosphatase enzymes that are presumed to be important in phototransductive biochemistry. The first has been investigated in some detail, and is present in crab and squid, having the characteristics of a phosphoprotein phosphatase. It is magnesium- and calcium-dependent. The second, for which there is no cytochemical localization, is an inositol trisphosphate degrading activity. This activity has been investigated in far less detail and has only been identified in crab.

It therefore seems appropriate to review the current state of knowledge of the following topics as they apply to the invertebrate photoreceptor:

1. The visual pigment molecule.
2. The cyclic nucleotide cascade of phototransduction.
3. The role of calcium in phototransduction.
4. The role of phosphoinositide metabolism in photoreceptors.
5. Photoreceptor protein phosphorylation.

This account will also necessarily draw on a good deal of research that was conducted in vertebrate systems either because little or no data has been obtained in any invertebrate system or because comparison with the vertebrate case may provide useful insights for the invertebrate. The intention is to avoid reiterating the details of rod outer segment biochemistry per se which are readily available in a number of reviews.

1.1 The Visual Pigment

The principal requirement of any photoreceptor is a light-sensitive pigment. This role is filled by a family of closely related molecules in all the animals studied to date. Each consists of a protein moiety, opsin, covalently linked to a carotenoid chromophore. The similarities seem to
place limits on the degree of divergence of the early steps of phototransduction.

Minimal variation is observed in the chromophore. For the majority of vertebrates and some invertebrates $11\text{-cis}$ retinal is the chromophore (Wald, 1936). In some freshwater fishes and amphibians $11\text{-cis} 3\text{-dehydroretinal}$ (Wald, 1937) is the chromophore as is also the case in a decapod crustacean (Suzuki et al., 1984). Vogt & Kirschfeld (1984) and Vogt (1984) report that in some insect orders the photopigment is $11\text{-cis} 3\text{-hydroxyretinal}$. 

In a series of relatively recent advances, the nature of the protein moiety has been clarified. Bovine opsin was initially amino-acid sequenced by Ovchinnikov et al. (1982) and Hargrave et al. (1983). In conjunction with earlier protein modification studies this revealed opsin as being a 39 kDa protein with its mass divided equally between seven hydrophobic transmembrane segments and the eight connecting hydrophilic loops and tails. When the amino-acid sequence of ovine opsin was determined (see Pappin et al., 1984) it was found to conform to the same general model as bovine opsin. Subsequently the genes for opsins from bovine rods (Nathans & Hogness, 1983), human rods (Nathans & Hogness, 1984), fruit-fly (O'Tousa et al., 1985; Zuker et al., 1985; Cowman et al., 1986) and all three types of human cones (Nathans et al., 1986) were cloned and sequenced.

The picture that has emerged from these studies is summarised in Figure 1.1. The overall predicted structure of all the opsins is the same and establishes beyond doubt their common evolutionary origin. The vertebrate rod opsins are highly conserved between species but are less well related to the human cone opsins, whilst the amino-acid sequence of fruit-fly opsin is 36% homologous to bovine rod opsin (O'Tousa et al., 1985),
significantly more similar than could have arisen by chance. Some regions of the molecule consistently show a very much higher degree of conservation than the average for all the opsins studied. These regions include, as might be expected, the 7th transmembrane segment which contains the chromophore binding site. The first two cytoplasmic loops are also highly conserved whilst the 3rd loop is not conserved between insects and vertebrates. The carboxy-terminal tail is conserved only in the sense that it possesses a number of serine and threonine residues that are believed to be sites of in vivo light-dependent phosphorylation. The conservation of the cytoplasmic domains supports the hypothesis that there are significant similarities between the invertebrate and vertebrate in the molecular interactions at this surface.

There is no information pertaining to the molecular interactions, if any, of the extracellular (or intradiscal) portion of the rhodopsin molecule but the sequence studies have revealed a strong homology between the 2nd extracellular loop of all the opsin species studied. This finding underlines the equivalence of the intradiscal and intermicrovillar spaces. Whilst the function of this region is unknown, O'Tousa et al. (1985) pointed out that it could function as a Ca$^{2+}$ binding domain on the basis of limited homology with the EF hand structure of calcium-binding proteins. If this turns out to be true it will be the first case of such a domain being found outside the cytoplasm and implies calcium regulation in both the intradiscal and intermicrovillar spaces.

So far I have emphasised the similarities between vertebrate and invertebrate rhodopsins but a number of functional differences have been established.

The diffusional and rotational mobility of invertebrate rhodopsin is severely restricted (Goldsmith & Wehner, 1977) whereas rhodopsin is free
Figure 1.1; A model to show the major structural features of a generic rhodopsin molecule. Adapted from Hargrave et al. (1983), drawing on the information obtained from sequence studies; see text for details. E1-3, extracellular/intradiscal loops. C1-3, cytoplasmic loops.

- **Conserved segment**
- **Non-conserved segment**
- **Phosphorylated region**
- **Glycosylated region**
- **Chromophore binding-site**
to move in the rod disc membranes (Brown, 1972; Cone, 1972; Liebman & Entine, 1974; Poo & Cone, 1974). This implies that regions of the surface of invertebrate rhodopsin may be specialized for tethering interactions. A microvillar cytoskeleton (Blest et al., 1978; Blest et al., 1982; Saibil, 1982; de Couet et al., 1984) has been suggested to play a part in restricting the mobility of invertebrate rhodopsins as was first proposed by Goldsmith & Wehner (1977) but evidence for direct anchorage of rhodopsin to a cytoskeletal network has not been obtained.

Invertebrate metarhodopsins, unlike those of vertebrates, are generally thermally stable and can be photoregenerated from metarhodopsin to rhodopsin (Hamdorf et al., 1973). This provides a useful experimental tool in those species where the peak absorbance wavelengths of rhodopsin and metarhodopsin differ appreciably because the photoconversions can be manipulated independently.

1.2 PHOTORECEPTOR FUNCTION.

All the photoreceptors discussed here have in common the characteristic of extremely high sensitivity. Most highly developed photoreceptors display discrete measurable membrane events (bumps) in response to individual photons (Yeandle & Fuortes, 1964; Borsellino & Fuortes, 1968; Lillywhite, 1977; Baylor et al., 1979; Doujak, 1984). Such sensitivity entails one or more amplification processes, since the photoisomerization of a single rhodopsin molecule must drive the opening or closing of many membrane ion channels (Cone, 1973). It also implies the modulation of one or more diffusible messenger species within the photoreceptor cell for it is impossible for a single rhodopsin molecule to be in direct macromolecular contact with all the ion channels it influences.
The tasks of phototransduction physiologists are to discover the identities of the second messenger species and also to characterize the macromolecules that modulate and are modulated by those second messengers. They hope thereby to account for the sophisticated electrophysiological responses of photoreceptors to varying illumination.

We know from the work of many investigators that at least five special processes must be integrated for a photoreceptor to function adequately. These are: excitation, inactivation, light-adaptation, dark-adaptation (including bleaching recovery) and the controlled renewal of photoreceptor components. We do not yet have enough information to reconstruct a complete and detailed model of phototransduction even in the rod outer segment and so in the comparative review that follows it is convenient to concentrate on the particular molecular components of photoreceptors that have been most thoroughly investigated. Nevertheless it is important to bear in mind that a particular small diffusible molecule or protein may have more than one role to play in photoreceptor function.

1.3 The cGMP Cascade

The vertebrate pathway of excitation.

When rhodopsin in a dark-adapted vertebrate rod is photoactivated to metarhodopsin it undergoes a conformational change setting in train a series of events which eventually lead to closing of the light-sensitive sodium conductance (Fung & Stryer, 1980; Fung et al., 1981). In brief these are:

1. Transducin-GDP (a three subunit guanine-nucleotide regulatory protein or "G-protein") binds to rhodopsin with a subsequent exchange of GTP for bound GDP and release of an activated form of the transducin.
Metarhodopsin is then free to bind and activate further molecules of transducin.

2. Binding of the activated form of transducin to cGMP-phosphodiesterase enhances the enzyme activity of the phosphodiesterase.

3. Rapid hydrolysis of cGMP within the rod outer segment causes a drop in the intracellular concentration of the second messenger.

4. The lowered intracellular (cGMP) promotes the dissociation of bound cGMP from a population of sodium ion channels, resulting in their closure (Fesenko et al., 1985; Cobbs & Pugh, 1985; Haynes et al., 1986).

These and associated processes are summarised in Figure 1.2.

Vertebrate inactivation processes.

The electrical event caused by a single photon absorption is transient. This implies that each of the activation steps listed is inactivated on a rapid time-scale.

1. Metarhodopsin decays to opsin and all-trans retinal. This is a slow event with a time-scale of several minutes. Other more rapid processes must be involved in inactivation. An important candidate for inactivation, that will be discussed in detail, is phosphorylation of rhodopsin.

2. Hydrolysis of GTP by the transducin catalytic subunit returns transducin to its inactive resting conformation but the precise details of the molecular events remain obscure (Applebury et al., 1986).

3. GTP hydrolysis is a necessary condition for inactivation of the phosphodiesterase (Liebman & Pugh, 1980), but Applebury et al. (1986) point out that measured rates of GTP hydrolysis (Godchaux & Zimmerman, 1979; Baehr et al., 1982) and phosphodiesterase inactivation (Liebman & Pugh, 1980; Goldberg et al., 1983) do not support this hypothesis. The rate measurements may have been confounded by the fact
Figure 1.2: Reproduced, without modification, from Lamb (1986). "A tentative synthesis of the cyclic nucleotide cascade of phototransduction, showing five important biochemical cycles. The rows of dots are used to indicate enzymic catalysis of reactions. An attempt has been made to indicate the location of the reactions: at the cytoplasmic surface of the disk membrane, in the cytoplasm, and at the plasma membrane; note though that $T_\alpha$ is soluble and may diffuse in the cytoplasm. Abbreviations: 11-cis and all-trans, isomers of the chromophore retinal; Rh, rhodopsin, with its photoisomerized (activated) form Rh* (metarhodopsin II); T, G-protein or transducin, with active subunit $T_\alpha$; PDE, phosphodiesterase (inactive); GTP.$T_\alpha$.PDE*, the activated phosphodiesterase; cG, cyclic 3'5'-guanosine monophosphate. Areas of greatest uncertainty concern the sites of action of calcium, and details relating to both the regeneration of native rhodopsin and the activation of PDE. In the latter case, it now seems that the inhibitory $\gamma$-subunit of the PDE actually dissociates from the hydrolytic $\alpha\beta$-unit, but it is not clear to which of these subunits the $T_\alpha$ binds. Thus the activated PDE, indicated as GTP.$T_\alpha$.PDE*, might in fact exist either as $T_\alpha$.($\alpha\beta + \gamma$) or as $\alpha\beta + T_\alpha.\gamma$."

"metarhodopsin II" should be "metarhodopsin II"

The diagram shows:

- Retinal cycle
- Rhodopsin cycle
- G protein cycle
- PDE cycle

The diagram includes reactions involving light, GTP, cGMP, calcium, and sodium ions, among others.
that they were not performed under physiological conditions; for example soluble helper factors may be required for maximum velocity (Sitaramayya, 1986) but the mechanism of phosphodiesterase inactivation is unresolved. Some GTP-requiring mechanism other than GTP hydrolysis by transducin might be responsible for phosphodiesterase inactivation.

4. Intracellular cGMP levels must be allowed to recover. The nature of the guanylate cyclase is unknown but it seems likely that rapid resynthesis of cGMP is involved, because Goldberg et al. (1983) estimated that the entire pool of cGMP within the dark-adapted retina turns over every two seconds. Upon light stimulation the rate of turnover increases dramatically, their calculations placing a lower limit on the turnover time of approximately 20 msecs. A pool of intracellular cGMP that is not directly accessible to the phosphodiesterase is a likely candidate to buffer the intracellular free [cGMP] (Cobbs & Pugh, 1985).

This is the model (in its most basic form) to which invertebrate phototransduction must be compared.

Excitation and inactivation in the invertebrate photoreceptor.

1. The primary event is again photoisomerization of the chromophore that converts rhodopsin to metarhodopsin.

2. A light-activated GTPase was first detected in cephalopod photoreceptors by Calhoon et al. (1980) and more recently a light-activated GTPase has been characterized in insect photoreceptor membranes (Blumenfeld et al., 1985). Initial reports were that the enzyme differed from the vertebrate species in being tightly bound under all conditions of illumination and ionic strength (Calhoon et al., 1980; Vandenberg & Montal, 1984a). However Saibil & Michel-Villaz (1984) reported that most
of the activity was found in the soluble fraction, regardless of treatment, with only a small fraction of the activity tightly bound to the photoreceptor membranes. The implication was that because Calhoon et al. (1980) and Vandenberg & Montal (1984) examined only the membrane fractions they missed the majority of the activity. Strangely, light-activation of the soluble squid GTPase was not observed when it was recombined with the squid photoreceptor membrane but only when it was combined with bovine photoreceptor membranes (Saibil & Michel-Villaz, 1984). Furthermore whilst illumination of squid membranes caused efficient binding and activation of bovine GTPase, the converse was not true.

The presence of only one GTPase that is physiologically light-activated in the cephalopod photoreceptor remains a plausible hypothesis. The soluble GTPase would in that case represent a degraded form of the membrane-bound enzyme or be involved in some entirely distinct function. Indeed it is known that the degree of conservation of guanine-nucleotide regulatory proteins and phosphodiesterase enzymes is such that even when they originate from non-photoreceptor tissues they may be stimulated by light-activated rhodopsin (Bitensky et al., 1982). Given the rhodopsin sequence differences referred to above it is too early to say whether the invertebrate rhodopsin-transducin interface is similar to that of the vertebrate.

The identification of inositol trisphosphate as a light-dependent diffusable messenger in Limulus (Fein et al., 1984; Brown et al., 1984) raises the possibility that a novel G-protein (Merritt et al., 1986) couples rhodopsin photoisomerization to a phospholipase C as is discussed below.

3. A light-activated cGMP-phosphodiesterase has never been identified in any invertebrate system. Ebrey et al. (1980) succeeded in obtaining light-
activation of vertebrate phosphodiesterase in the presence of octopus visual membranes (the source of the transducin in these experiments was not defined) but the interpretation of this experiment is open to the same doubts as for the light-activated GTPase. Saibil (1984) found a light-stimulated increase in cGMP levels in squid photoreceptors but thus far neither a light-inhibited phosphodiesterase nor a light-activated guanylate cyclase has been isolated. Introduction of 10 mM cGMP into *Limulus* photoreceptors by internal dialysis in the dark did not mimic light-stimulation but did decrease the responses to brief dim flashes (Stern & Lisman, 1982). Whilst cGMP metabolism and phototransductive pathways clearly interact in the invertebrate photoreceptor there is no evidence that cGMP is the principal excitatory messenger as is the case in the vertebrate.

4. The finding that inositol trisphosphate mimics the effects of light in its excitation and adaptation of *Limulus* ventral photoreceptors (Fein et al., 1984; Brown et al., 1984) has cast doubt on the role of cGMP as the invertebrate second-messenger.

5. Given the preliminary state of knowledge of the invertebrate photoreceptor's biochemistry little can be said about the inactivation processes that might operate. Although, based on the similarity of invertebrate rhodopsin to its vertebrate counterpart, it has seemed likely that inactivation of invertebrate rhodopsin proceeds via a phosphorylation mechanism. One can also surmise that if inositol polyphosphates are important second messenger substances in rhabdomeric photoreceptors then the appropriate degradative enzymes will be abundantly present.
1.4 The Role of Calcium Ions in Photoreceptors.

Calcium in vertebrate photoreceptors.

There has been a good deal of controversy over the role of calcium since the introduction of the "Calcium Hypothesis" by Yoshikami & Hagins (1973) who proposed that photoisomerization of rhodopsin caused the opening of calcium-permeable channels in the disc or plasma membranes allowing the entry of calcium down its concentration gradient. The increased \([\text{Ca}^{2+}]_i\) was supposed to result in binding of calcium to and closing of the sodium channels. The principle reason for the controversy has been the considerable technical difficulty involved in measuring calcium fluxes in the rod outer segment. There are two main issues. What are the effects of raising or lowering rod outer segment \([\text{Ca}^{2+}]_i\)? What changes in \([\text{Ca}^{2+}]_i\) actually take place on illumination of photoreceptors?

Experimental approaches to answering the first question have employed intracellular injection of calcium (Brown et al., 1977a), raising the extracellular free calcium concentration (Brown & Pinto, 1974; Bastian & Fain, 1979; Bastian & Fain, 1981; McNaughton et al., 1986a) and application of calcium ionophores with normal extracellular calcium (Hagins & Yoshikami, 1974). Generally the effects appear to be a hyperpolarization of the photoreceptor which is transient in the case of calcium injections (Brown et al., 1977a) but slower than light-driven hyperpolarizations (McNaughton et al., 1986b) and which is accompanied by a decrease in the maximum amplitude of the light response.

Attempts to decrease intracellular calcium levels have involved injection of the calcium chelator EGTA (Brown et al., 1977a), internal dialysis with BAPTA or EGTA (Matthews et al., 1985) and decreasing the
extracellular calcium concentration (Yoshikami & Hagins, 1973; Bastian & Fain, 1979; Bastian & Fain, 1981; Yau & Nakatani, 1985). Such treatments seem to cause a further depolarization of the cell and increase the maximum amplitude of the light response. However there is disagreement over the effects on sensitivity. Earlier workers reported a decrease in the photoreceptor sensitivity (i.e. the ratio of amplitude of the response to the light intensity at low amplitudes) whilst Matthews et al. (1985) reported the opposite finding.

For a long time it was believed that light stimulation of rods resulted in an increase in their $[\text{Ca}^{2+}]_i$ due to release from intracellular stores such as the disk lumen. This was on the basis of an observed increase in extracellular calcium on illumination (Yoshikami et al., 1980; Gold & Korenbrot, 1980). However the crucial observation of a light-driven increase in rod outer segment $[\text{Ca}^{2+}]_i$ has never been made, despite evidence that disk preparations can, under certain conditions, accumulate calcium ions and release them upon light stimulation (George & Hagins, 1983); but under the conditions of that study the release from disks was far too slow to be involved in the light response.

More recent evidence has led to a reinterpretation of the observation of an increase in extracellular calcium upon illumination of rods. Yau and Nakatani (1985) showed that in the dark there is a passive flow of calcium ions into the rod that constitutes a significant proportion of the dark current as well as an electrogenic exchange mechanism which pumps out one $\text{Ca}^{2+}$ in exchange for the entry of three $\text{Na}^+$. Light suppresses the passive calcium current and the exchange pump reduces the intracellular calcium concentration below its resting level. There was no evidence for significant release of calcium into the cytoplasm from intracellular stores in the light. The evidence for such a train of events was strengthened by the direct measurement of rod $[\text{Ca}^{2+}]_i$ by
McNaughton et al. (1986a). These workers showed that the normal resting level of calcium ions in the rod outer segment is less than 0.6 µM (possibly explaining the calcium loading of disks achieved by George and Hagins (1983) who used levels of calcium in their artificial cytoplasm between 5 and 50 µM), and that flashes of light always caused a decrease in [Ca\(^{2+}\)].

Almost all of these findings on calcium in the vertebrate rod outer segment are consistent with a model (Bownds, 1980; Yau & Nakatani, 1985; McNaughton et al., 1986b) in which illumination decreases rod outer segment [Ca\(^{2+}\)]i releasing guanylate cyclase from inhibition by calcium (Krishnan et al., 1978; Troyer et al., 1978; Fleischmann & Denisevich, 1979; Gold & Korenbrot, 1981; Lolley & Racz, 1982) so reducing the sensitivity of the photoreceptor and thus contributing to adaptation. This model also helps explain, at least qualitatively, the greatly increased turnover of cGMP that occurs upon illumination (Goldberg et al., 1983).

**Calcium in the invertebrate photoreceptor.**

The role of calcium in invertebrate photoreceptors has been much less controversial. This is in good part due to the relatively large size of *Limulus* ventral photoreceptors which allows calcium sensitive proteins, dyes and chelators such as aequorin, arsenazo III and EGTA to be introduced into intact cells whilst intracellular recordings are maintained. It is generally agreed that:

1. Light causes an increase in [Ca\(^{2+}\)]i (Brown & Blinks, 1974). The response to a brief flash being a rise in [Ca\(^{2+}\)]i which is transient but which persists for longer than the electrical response whilst in the light adapted state [Ca\(^{2+}\)]i is maintained at an elevated level throughout the illumination period (Brown et al., 1977b).
2. Injections of calcium reduce the responsiveness of photoreceptors to light (Lisman & Brown, 1972) and in other respects mimic the effects of light-adapting the photoreceptor (Fein & Charlton, 1977; Fein & Charlton, 1978).

3. There is clear evidence that under physiological conditions the smooth endoplasmic reticulum, which in many invertebrate photoreceptors is highly developed just below the microvilli, accumulates calcium (Perrelet & Bader, 1978; Walz, 1979; Walz, 1982a; Walz, 1982b; Walz & Somlyo, 1984).

4. Calcium is clearly implicated in light-adapting the photoreceptor and this effect is achieved through rises in \([\text{Ca}^{2+}]_i\). The invertebrate photoreceptor seems to behave quite differently from the rod outer segment in this respect.

More recently evidence for a role of calcium in visual excitation has emerged. Payne et al. (1984) detected rapid inward membrane currents when they pressure-injected calcium into \textit{Limulus} ventral photoreceptors. Furthermore when calcium was iontophoresed into photoreceptors that had previously been depleted of calcium the effect was to facilitate the light response (Bolsover & Brown, 1985). The reason why these responses were not seen previously (Brown & Lisman, 1975) may well be that higher concentrations of calcium were attained within the cell by the pressure injection technique than by the earlier iontophoretic applications: it is believed that during light-driven calcium transients \([\text{Ca}^{2+}]_i\) can reach levels of the order of 0.1 mM or more (Brown et al., 1977b). Fein (1986) has also pointed out that it is important that a microscope is used to confirm that injections are delivered specifically to the light-sensitive lobe of the \textit{Limulus} ventral photoreceptor.

The role of calcium in the only invertebrate photoreceptor where it has been investigated in any depth (i.e. \textit{Limulus}) seems quite different from that in vertebrates. In the invertebrate case light-driven calcium changes
are opposite to those in vertebrates and evidence is also emerging that calcium is important in excitation as well as adaptation. The mechanism by which calcium exerts either of these effects in the rhabdom remains unclear. Indeed it is still controversial whether calcium is, under physiological conditions, the principle excitatory transmitter or merely a facilitating factor. This question will be discussed in connection with the role of inositol polyphosphates.

### 1.5 The Role of Inositol Phosphates in Photoreceptors

The similarities between photoreceptor excitation and the activation of cellular metabolism by a hormone or neurotransmitter as well as the obvious importance of calcium in photoreceptor physiology have led to an examination of phosphoinositide metabolism in photoreceptors. Inositol phospholipids have been implicated in cell activation and signalling for many years. Two crucial observations have helped to clarify the role of InsP\textsubscript{3}: stimulation of insect salivary gland with 5-hydroxytryptamine causes a rapid breakdown in phosphatidylinositol 4,5-bisphosphate (PInsP\textsubscript{2}) with concomitant accumulation of inositol 1,4,5-trisphosphate (InsP\textsubscript{3}) and inositol 4,5-bisphosphate (InsP\textsubscript{2}) (Berridge, 1983); and permeabilized pancreatic cells release calcium upon stimulation with InsP\textsubscript{3} (Streb et al., 1983). It is now accepted that InsP\textsubscript{3} may couple the activation of cell-surface receptors to the release of calcium from intracellular stores in many different cell-types. For a recent summary see Sekar & Hokin, (1986). Figure 1.3 summarises the principal biochemical pathways that are involved in phosphoinositide turnover.
Inositol trisphosphate in the invertebrate photoreceptor.

Two groups have independently shown that 1,4,5-D-myo-inositol trisphosphate when injected into *Limulus* ventral photoreceptors first excites and then adapts cells in a manner virtually indistinguishable from light (Fein *et al.*, 1984; Brown *et al.*, 1984). The latter group also demonstrated increased formation of InsP$_3$ upon illumination. Wood, S.F. Reid, M.S. Szuts, E.Z. & Fein, A. (personal communication) have shown a similar light-dependent formation of InsP$_3$ in illuminated squid retinae.

The effects of InsP$_3$ appear to be mediated solely through calcium release (Brown & Rubin, 1984; Corson *et al.*, 1984; Payne *et al.*, 1984; Rubin & Brown, 1985) but whether 1,4,5-inositol trisphosphate is indeed the principal excitatory transmitter remains in doubt because light responsiveness persists after injection of calcium buffers (Payne *et al.*, 1984; Rubin & Brown, 1985; Fein, 1986). Fein (1986) states the dilemma succinctly:

"EGTA may fail to block the light response because a rise in [Ca$^{2+}$]$_i$ is not necessary for excitation although it is sufficient for excitation, as is shown by the calcium injection experiments. Another, as yet unidentified, messenger would then be required for excitation. Conversely, it might be that EGTA fails to block the light response because of spatial differences between exciting the cell by light and by Ins(1,4,5)P$_3$. Ins(1,4,5)P$_3$ is injected into the bulk cytoplasm and its effects are readily buffered by injected EGTA. In contrast, light might preferentially release calcium into the small space between the sub-microvillar endoplasmic reticulum and the microvilli. EGTA might be partially or wholly excluded from this space and/or the calcium released might saturate the buffer in the constricted space. At present we have no experimental means of distinguishing between these possibilities."
Figure 1.3; A generalised model showing the pathways for synthesis, release and breakdown of inositol trisphosphate (InsP$_3$) in response to an extracellular signal. Redrawn from Berridge & Irvine (1984). (a.) - Phosphatidylinositol kinase. (b.) - Phosphatidylinositol-(4)-phosphate kinase. (G.) - G-protein. Inositol trisphosphate and diacylglycerol may have additional second messenger functions.
From the point of view of economy the second hypothesis would be preferred, but this would further degrade the principle of similarity between vertebrate and invertebrate modes of phototransduction. What is certain is that inositol polyphosphate metabolism is crucial to invertebrate photoreception; several alleles of the norp A *Drosophila* visual mutant which does not generate a receptor potential are completely deficient in ocular phosphatidyl inositol phosphodiesterase activity (Yoshioka *et al.*, 1985). This may be the primary genetic defect of the norp A mutant, given that the enzyme is reduced in the head tissue generally as well as the eyes.

A unifying and therefore attractive possibility is that photoexcitation in both invertebrate and vertebrate photoreceptors results in the activation of analogous G-proteins and phosphodiesterase enzymes (Fein, 1986; Merritt *et al.*, 1986). Such a model is consistent with the large body of evidence implicating a G-protein in rhabdom excitation (Fein & Corson, 1979; Bolsover & Brown, 1982; Corson *et al.*, 1983; Minke & Stephenson, 1985; Blumenfeld *et al.*, 1986).

**Inositol trisphosphate in vertebrate photoreceptors.**

Waloga & Anderson (1985) reported that injections of InsP$_3$ hyperpolarize isolated vertebrate rods. There is also evidence for light-stimulated release of inositol trisphosphate in the vertebrate retina (Ghalayini & Anderson, 1984). It is presently difficult to reconcile these data with, on the one hand, the generally accepted notion that inositol trisphosphate exerts its effects through release of calcium into the cytoplasm and, on the other hand, the accumulating evidence that calcium levels decrease inside the rod in response to light (Yau & Nakatani, 1985; Matthews *et al.*, 1985; McNaughton *et al.*, 1986a).
Resolution of this dilemma will require further studies of the role of phosphoinositides in vertebrate photoreceptors.

The other principal product of PInsP$_2$ breakdown, diacylglycerol, has been implicated in the control of a number of cellular processes through its activation of protein kinase C (Berridge, 1986). So far no role has been established for diacylglycerol in either photoreceptor type.

1.6 Light-Dependent Phosphorylation in Photoreceptors.

Vertebrate rhodopsin was the first photoreceptor protein whose phosphorylation state was found to be influenced by light (Kühn & Dreyer, 1972; Bownds et al., 1972). Subsequently light-dependent dephosphorylation of two low molecular proteins with m = 13 kDa and 12 kDa (Polans et al., 1979), a 33 kDa protein (Lee et al., 1984) and light-dependent phosphorylation of two high molecular weight proteins m = 240 kDa and 220 kDa (Szuts, 1985) were also reported in rod outer segments.

Amongst the invertebrates, octopus (Paulsen & Hoppe, 1978); squid (Vandenberg & Montal, 1984b); blowfly (Paulsen & Bentrop, 1984) and probably Drosophila (Matsumoto et al., 1982; Matsumoto & Pak, 1984) rhodopsins are all phosphorylated to higher levels in the light than the dark. Pak's group (op cit.) also reported light-dependent phosphorylations of polypeptides with m = 130 kDa, 80 kDa and 49 kDa besides the 39 kDa band that is presumed to be rhodopsin. In addition a molecule of 2.3 kDa was dephosphorylated by light exposure of the intact animal.
The characteristics of light-dependent rhodopsin phosphorylation.

The crucial trigger for the phosphorylation of rhodopsin is a change in the conformation of the molecule that is caused by illumination (Weller et al., 1975; Kühn, 1978) and which occurs after rhodopsin has decayed to metarhodopsin I (Paulsen & Bentrop, 1983). The conformational change appears to be more subtle than exposure of sites formerly inaccessible to the cytoplasm because the carboxy-terminal sites are available for phosphorylation by another photoreceptor kinase in the dark as well as the light (Kelleher & Johnson, 1986). The new conformation of rhodopsin is phosphorylated by a 68 kDa cGMP- and calcium-independent protein kinase that is found in the rod outer segment (Kühn et al., 1973; Frank & Buzney, 1975; Weller et al., 1975). Experimentally, the β-adrenergic receptor kinase from mammalian lung can also phosphorylate rhodopsin in a light-dependent manner (Benovic et al., 1986) confirming the importance of the state of the protein substrate.

Wilden & Kühn (1982) established that under efficient in vitro labelling conditions an average of seven phosphate groups are incorporated per rhodopsin. Individual rhodopsin molecules were found with phosphorylation numbers between zero and nine. Bovine rhodopsin has seven potential phosphorylation sites located in a group in the carboxy-terminal cytoplasmic tail (Hargrave et al., 1980) whilst the use of selective proteolysis (Miller & Dratz, 1984) confirms that over 80% of light-dependent phosphorylation occurs in the C-terminal region. The other (at least two) phosphorylated residues must be situated elsewhere in the molecule. There have been consistent reports that at very low fractional bleaches very high numbers of phosphate groups are incorporated per bleached rhodopsin:
These results imply some co-operativity between bleached and non-bleached rhodopsins in their activation of rhodopsin kinase (Miller et al., 1977). Alternatively after small bleaches another kinase (Kelleher & Johnson, 1986) could contribute to rhodopsin phosphorylation, in a calcium- and phospholipid-dependent manner.

Invertebrate rhodopsins are also phosphorylated following illumination. Although the highest mean number of phosphates per metarhodopsin that has been observed was only 1.8 in the squid (Vandenberg & Montal, 1984b) the sequence data (O'Tousa et al., 1985; Zuker et al., 1985) indicates that there are six potential phosphorylation sites in the cytoplasmic tail of the rhodopsin of Drosophila.

**Possible functional roles of photoreceptor protein phosphorylation.**

A number of possible functions have been proposed for rhodopsin phosphorylation. A role in excitation has never been seriously entertained in view of the relatively slow ($t_{1/2} = 3-5$ min.) time-course of phosphorylation (Bownds et al., 1972; Kühn & Dreyer, 1972; Frank et al., 1973; Kühn et al., 1973). It now seems that the experimentally observed slow time-courses resulted from the in vitro conditions of high fractional bleaches and kinase-depleted membranes. When these conditions were remedied (Sitaramayya & Liebman, 1983b) phosphorylation rates were accelerated by at least 100-fold. But the degree of amplification inherent in

<table>
<thead>
<tr>
<th>Reference</th>
<th>Maximum no. P_/Rh*</th>
<th>% of Rh bleached</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bownds et al., 1972</td>
<td>50</td>
<td>0.01</td>
</tr>
<tr>
<td>Miller et al., 1977</td>
<td>40</td>
<td>0.002</td>
</tr>
<tr>
<td>Sitaramayya &amp; Liebman, 1983b</td>
<td>20</td>
<td>0.000015</td>
</tr>
</tbody>
</table>
rhodopsin phosphorylation is low and phosphorylation of rhodopsin does not contribute to any known excitatory process.

The phosphorylation of metarhodopsin may perhaps terminate its excited state (i.e. the state capable of activating transducin). The first evidence for such a mechanism was that an ATP or GTP-dependent process with the enzymatic characteristics of rhodopsin phosphorylation quenched the activation of phosphodiesterase by metarhodopsin with a half-time of the order of tens of seconds (Liebman & Pugh, 1980). Subsequently it was confirmed that this process required the presence of either endogenous or added rhodopsin kinase (Sitaramayya & Liebman, 1983a) and that in vitro phosphorylation could be made to occur fast enough to explain the high rate of cGMP phosphodiesterase quenching that is inferred to occur in vivo. (Sitaramayya & Liebman, 1983b; Miller & Dratz, 1984).

It is essential to this interpretation that phosphorylated metarhodopsin be inactive in stimulating transducin and cGMP phosphodiesterase. Shichh et al. (1984) demonstrated that the capacity of regenerated rhodopsin to stimulate GTP-binding to transducin was completely eliminated by the presence of two phosphate groups per rhodopsin after it had been incorporated into artificial phospholipid vesicles. Rod outer segment membranes also partially lost their capacity to promote GTP binding after phosphorylation, the effect being more pronounced after regeneration of rhodopsin.

Miller & Dratz (1984) showed that phosphorylation of rhodopsin inhibits the $V_{\text{max}}$ and shut-off time of phosphodiesterase activity and this effect was progressively reversed by proteolytic trimming of the carboxy-terminal phosphorylation sites. Arshavsky et al. (1985) found a similar effect of rhodopsin phosphorylation on phosphodiesterase activation.
Phosphorylated, regenerated and washed rhodopsin-bearing membranes were 20-50% less effective in stimulating phosphodiesterase than the unphosphorylated control membranes, depending on the fraction bleached.

As Bownds & Brewer have pointed out (1986) these experiments seem to show that phosphorylation of metarhodopsin per se does not completely inhibit phosphodiesterase activation (except in the somewhat unphysiological case where regenerated rhodopsin was incorporated into artificial phospholipid vesicles). However a 48 kDa protein of bovine rod outer segments was shown to require ATP or GTP for it to bind (Pfister et al., 1983) specifically to membranes that were both phosphorylated and illuminated (Kühn et al., 1984). Zuckerman et al. (1984; 1985) demonstrated that antibodies to the 48 kDa protein interfere with phosphodiesterase quench but also contended that ATP + 48 kDa protein has a direct role in the quenching mechanism independent of rhodopsin phosphorylation. This finding contradicts those of Wilden et al. (1986) that whilst the 48 kDa protein can reduce up to 98% of the phosphodiesterase activity induced by rod outer segment membranes if they are phosphorylated, it has no effect on phosphodiesterase activation by non-phosphorylated membranes even in the presence of ATP. However, the discrepancy could be explained if the preparation of Zuckerman et al. (1984; 1985) had been contaminated by rhodopsin kinase. Most recently Sitaramayya has succeeded in obtaining rapid ATP quench of the active state of metarhodopsin using a partially purified preparation of rhodopsin kinase (1986). The extent to which the 48 kDa protein contributes to rhodopsin inactivation therefore remains controversial.

Dissenting views remain concerning this simple model of metarhodopsin inactivation. Whilst the capacity of metarhodopsin
phosphorylation and binding of 48 kDa protein to terminate the stimulation of phosphodiesterase in vitro can not seriously be disputed it can be argued that these processes are not relevant to the physiologically observed termination of excitation. Hermolin et al. (1982) reported that phosphorylation of rod outer segment membranes could be reduced by more than threefold under conditions (up to 10 mM cGMP or 5'-GMP) which did not perturb the normal quenching of phosphodiesterase by ATP. Donner & Hemilä (1985) reported that 2 mM adenosine, an inhibitor of rhodopsin kinase, reduced rhodopsin phosphorylation by 58 to 88% whilst the recovery of the dark current from low fractional bleaches was slowed only 30-40% by this treatment. The problem with interpreting these results is that some light-stimulated phosphorylation persisted in these experiments whilst incorporation of two, or possibly even one, phosphate groups per photobleached rhodopsin may significantly curtail the active state (Miller & Dratz, 1984) and quench the activation of phosphodiesterase (Shichi et al., 1984). This prediction follows from a model in which multiple phosphorylations are necessary to prevent the rate of non-enzymic dephosphorylation in a completely bleached photoreceptor from generating a large spurious signal (Lisman, 1985). The model is discussed in more detail below.

More recently Bownds and Brewer (1986) compared the time-course of phosphorylation after a flash with the time-courses for the recovery of the dark current and recovery of sensitivity in a suspension of physiologically intact rods. Under conditions of low [Ca^{2+}]e the half-times for phosphorylation and recovery of the dark current were comparable but the current was observed to have fully recovered 2 minutes after the flash, when phosphorylation was only approximately 85% complete. Loss of sensitivity occurred too fast to be observed on this experimental time scale.
but regain of sensitivity under physiological conditions was also slower than phosphorylation. These results indicate that phosphorylation may occur fast enough to account for the decline in light response but that other factors, in this case the $[\text{Ca}^{2+}]_e$ also affect this process.

A novel approach to this problem has been the genetic manipulation of a *Drosophila* rhodopsin gene so as to eliminate the C-terminal region of the molecule, followed by the reinsertion of the altered gene into the germ line (unsourced abstract of Zuker, C. Ozaki, K. Rubin, G.M. & Pak, W.L. "Physiological effects of deleting potential phosphorylation sites from *nina E*, R1-6 opsin gene.") The transformants were reported to be indistinguishable from a control which had been constructed with an intact rhodopsin gene, in their electrophysiological response to light. They had significantly reduced photopigment phosphorylation.

It would be surprising if the role of metarhodopsin phosphorylation were very different amongst different species. Further investigations will be necessary in this area to reconcile the apparently conflicting experimental evidence.

Although it is not directly relevant to deciding the physiological roles of rhodopsin phosphorylation there is a large body of evidence implicating a leaky multiple step process in the termination of the active state of metarhodopsin in both invertebrates and vertebrate photoreceptors. The prolonged depolarizing afterpotential (PDA) was first observed in the *Limulus* median ocellus (Nolte *et al.*, 1968) but has now been observed in the photoreceptors of a variety of invertebrates:

"In these photoreceptors, an intense coloured stimulus, which shifts a substantial amount of visual pigment – rhodopsin – to its thermally stable photoproduct – metarhodopsin – induces a prolonged depolarizing afterpotential (PDA), which saturates the voltage response of the cell for several seconds to many minutes after the stimulus has
been turned off. Once a maximal PDA has been induced, no further depolarizing
response can be obtained from the cell even with the strongest available stimulus until (a)
the PDA decays or (b) metarhodopsin regenerates to rhodopsin, either thermally or
photically. Of these, only photoregeneration of rhodopsin reactivates the cell rapidly." (Minke et al., 1975)

Minke (1986) has summarised the similarities between the PDA and
the prolonged dark excitation in vertebrate rods, i.e that: they are both
related to the amount of metarhodopsin present and can be terminated by
regeneration to rhodopsin; that they appear to be composed of and decay
into discrete bump-like events; that they are accompanied by a drastically
reduced sensitivity to light and that they are localized events that depend
on the local concentration of metarhodopsin. These electrophysiologically
observed phenomena appear to be the basis of the "equivalent background
brightness" (Stiles & Crawford, 1932), a subjective phenomenon that
resembles a steady background light to human observers who are placed
in darkness following a high bleach.

Non-specific phosphatase inhibitors, including fluoride, as well as non-
hydrolyzable GTP analogues have been used to activate photon-like noise
and prolong light-evoked stimulation in Limulus (Fein & Corson, 1979;
Corson & Fein, 1983; Corson et al., 1983) and Musca photoreceptors
(Minke & Stephenson, 1985). Whilst these experiments undoubtedly
provide good evidence for the involvement of a G-protein the interpretation
of the role of the phosphatase inhibitors is confused. Fluoride has been
suggested to have two relevant effects:
1. Activation of transducin (Corson & Fein, 1983)
2. Inhibition of oxidative phosphorylation and high-energy phosphate
metabolism generally (Payne, 1982).
Fluoride is also a well-known inhibitor of a number of cellular phosphatase enzymes. The other "phosphatase inhibitors" used have a similar range of biochemical effects.

Other treatments that reduce intracellular ATP levels (Stern et al., 1985) and/or promote phosphatase action at the expense of kinases (Stern & Lisman, 1982a,b) also increased the spontaneous bump rate. Stern et al. (1985) therefore proposed that these treatments have a common action in limiting rhodopsin phosphorylation.

A model that explains these phenomena in terms of multiple-step leaky inactivation of metarhodopsin was proposed by Lamb (1981). The model was extended by Paulsen & Bentrop (1984) and Lisman and associates (Stern et al., 1985; Lisman, 1985) who showed that metarhodopsin phosphorylation has the required characteristics of the inactivation process. The most important features of the model are:

1. Metarhodopsin is the preferred substrate of the kinase whilst phosphorylated rhodopsin, at least in invertebrates, is the preferred substrate of the phosphatase (Paulsen & Bentrop, 1984).
2. More than one but not all the phosphorylations are required to inactivate metarhodopsin whilst multiple phosphorylations serve to limit the number of spontaneous reversions to the active state (Lisman, 1985). Individual phosphorylations may have distinct characteristic effects on quenching (Miller & Dratz, 1984).
3. The PDA and its vertebrate homologue are due either to the local limitation of a component necessary for rhodopsin phosphorylation, such as ATP or rhodopsin kinase, or to the formation of inaccessible rhodopsin conformations at high bleaches (Paulsen & Bentrop, 1984; Lisman, 1985).

Another possible role for rhodopsin phosphorylation is in photoreceptor light-adaptation. A number of workers have defined two components of
light-adaptation (Kühn et al., 1977) in the rod outer segment. One, where the sensitivity is proportional to the amount of bleached rhodopsin in the photoreceptor has been termed "pigment adaptation". The second is not simply dependent on the fraction of inactivated rhodopsin and will be referred to as "fast adaptation" (Dowling, 1963). Dark adaptation represents recovery from (i.e. reversal of) these two components. In the first case recovery is slow and strictly limited by regeneration of rhodopsin. In the second case the speed of recovery is variable and after low bleaches may be quite rapid.

A role for metarhodopsin phosphorylation in photoreceptor light-adaptation was first suggested on the basis of its time course in vivo (Bownds et al., 1972; Kühn et al., 1973; Kühn, 1974). Miller et al. (1975) obtained the first experimental evidence correlating decreased phosphorylation of rhodopsin with increased sensitivity of rod outer segments. Kühn et al. (1977) suggested that metarhodopsin phosphorylation might participate in fast adaptation but excluded the possibility of involvement in pigment adaptation on the dubious grounds that they had demonstrated that dephosphorylation of phosphorylated rhodopsin in the dark was independent of pigment regeneration. Whilst recovery of active pigment (a component of dark adaptation) is limited by the rate of resynthesis from opsin and 11-cis -retinal this fact is irrelevant to the signal for the initial light-adaptation.

Hermolin et al. (1982) found that increasing calcium concentrations in a suspension of permeabilized rod outer segments inhibited metarhodopsin phosphorylation at all levels of bleaching and suggested that a light-dependent decrease in rod outer segment [Ca$^{2+}]_i$ occurring after a flash would depress sensitivity. This model was proposed at a time when it conflicted with the generally held view of calcium fluxes in the
photoreceptor and must have been strengthened by the recent findings discussed in section 1.4.

If metarhodopsin phosphorylation is involved in light-adaptation the effects of simple inactivation of rhodopsin will be negligible except at very high light levels. In the vertebrate retina background light that causes a barely measurable bleach ($< 0.0005$) can adapt the retina, reducing the sensitivity of the threshold by a factor of $10^4$ (Lamb, 1981). Clearly, even improbably optimistic estimates of the number of pigment molecules that could be inactivated by phosphorylation per photon (50) are inadequate. An amplification process driven by phosphorylation of rhodopsin and involving diffusible messengers would have to be involved.

**Dephosphorylation and the phosphatase.**

Whilst the phosphorylation of rhodopsin and other proteins has been extensively studied, at least in the rod outer segment, much less is known about the process of rhodopsin dephosphorylation. Indeed, progress on phosphoprotein phosphatases generally lags behind that on protein kinases (Krebs & Beavo, 1979; Ingebritsen & Cohen, 1983).

In the frog retina dephosphorylation of rhodopsin appears to be a slow process with a half-time of 13 minutes. The phosphatase has proven elusive. A number of investigators report that loss of dephosphorylation capacity accompanies any treatment that might damage the rod outer segment (Kühn et al., 1973; Miller & Paulsen, 1975; Weller et al., 1975) and dephosphorylation has not been reported to occur in isolated photoreceptor membranes. It has recently been reported that increased phosphatase activity is responsible for some of the abnormalities found in the retinae of dogs with an inherited retinal dystrophy (Takemoto et al., 1986). It is not
yet clear whether a perturbed phosphoprotein phosphatase is the principle genetic defect in this disorder.

In contrast Paulsen & Bentrop (1984) have shown that dephosphorylation of rhodopsin in blowfly photoreceptors occurs with a half-time of 20 seconds. There is a plausible physiological explanation for this difference. When an invertebrate rhodopsin molecule has followed the pathway of: photoisomerization, decay to metarhodopsin, and phosphorylation it may be photoregenerated to phosphorylated rhodopsin immediately. The speed with which the phosphate groups are removed may limit the rate at which rhodopsin again becomes a viable photon receptor. The speed of the phosphatase reaction also makes it a potential target for regulation of photoreceptor sensitivity at high light levels. Regeneration of functional rhodopsin in the vertebrate retina is almost two orders of magnitude slower; half-time = 30 minutes (Dowling, 1963). Neither hydrolysis of metarhodopsin nor its regeneration from 11-cis-retinal seem to be affected by the phosphorylation state of rhodopsin (Kühn et al., 1973). There would therefore be no advantage in a high rate of dephosphorylation nor would the phosphatase enzyme be an effective control point.

These considerations not only suggest that the dephosphorylation of rhodopsin would best be studied in an invertebrate system but that it might also prove to be a more interesting and significant process in the rhabdom than in the rod outer segment. However prior to this study there has been only one published investigation of a potential phosphoprotein phosphatase in any invertebrate photoreceptor (Romero-Saravia & Hamdorf, 1983). That study identified an "alkaline phosphatase" associated with the rhabdomeric microvilli of an octopus and suggested that it might have a role either in rhodopsin dephosphorylation or cation
exchange across the microvillar membrane. Saibil & Michel-Villaz (1984) mentioned a similar "alkaline phosphatase" activity contaminating their preparations in an investigation of squid GTPase activation but found the activity exclusively in the supernatant fraction.
2. CYTOCHEMICAL DISTRIBUTION AND BIOCHEMISTRY OF A NOVEL PHOSPHATASE IN THE PHOTORECEPTIVE MICROVILLI OF A CRAB

2.1 SUMMARY

4-nitrophenylphosphatase (4-NPPase) activity is concomitant with a number of phosphatase enzymes and may be used to demonstrate them biochemically or cytochemically under appropriate conditions. In the present study two major 4-NPPase activities were identified in retinal photoreceptors of the crab *Leptograpsus variegatus*.

1. One of these shows maximal activity below pH 5.5, does not require magnesium and cannot be observed cytochemically at pH 9.0. This is presumed to represent the lysosomal acid phosphatase previously localized and characterized (Blest *et al.*, 1980; de Couet & Blest, 1982).

2. The other activity requires magnesium, being maximal at pH 7.5 with magnesium concentrations above 10 mM; it is markedly inhibited by calcium in the millimolar range, whereas the magnesium-independent form is not. The magnesium-dependent enzyme occurs principally within the photoreceptive microvilli and within the palisade bridges. Staining within these regions is heavy, indicating a significant concentration of the enzyme.

3. The conditions required for the cytochemical demonstration of the rhabdomeral enzyme *in situ* are the same as those that support the magnesium-dependent enzyme *in vitro*; they rule out Na⁺/K⁺ adenosine triphosphatase (Na⁺/K⁺ ATPase, E.C.3.6.1.3), alkaline phosphatase
E.C.3.6.1.3), alkaline phosphatase (E.C.3.1.3.1) and acid phosphatase or nucleotidase (E.C.3.1.3.2 and E.C.3.1.3.31 respectively) as sources of the activity.

4. Despite its circumscribed ultrastructural distribution, the magnesium-dependent enzyme is soluble under all conditions that were tested.

2.2 Introduction

In contrast to the wealth of information (Miki et al., 1973; Pannbacker et al., 1972; Robinson & Hagins, 1977; Wheeler & Bitensky, 1977; Stryer et al., 1981) which has been gleaned from vertebrate preparations, we have little evidence as to how phototransduction is accomplished by invertebrate (rhabdomeric) photoreceptors. The paucity of biochemical data from arthropods contrasts with the fact that elegant electrophysiological experiments are possible in these systems. The reasons for the lack of biochemical data are simple and cogent. Even the larger arthropod eyes contain too little material to allow even moderately pure preparations of the photoreceptive segments to be made in reasonable quantity.

An alternative approach, adopted here, is to combine biochemistry performed on crude preparations with ultrastructural cytochemistry. The crab *Leptograpsus variegatus* is suitable for this type of study because the anatomy of the photoreceptors in this species is well characterized (Blest et al., 1980; Stowe, 1980a; Stowe, 1980b) as is the daily turnover of photoreceptive membrane (Blest et al., 1980; Stowe, 1980b; Stowe, 1983; de Couet & Blest, 1982). We also know that in this species discrete transient current events (bumps) can be observed in response to single photons (Doujak, 1984).
Here it is reported that during a survey of Na+/K+ ATPase distribution in the photoreceptor cell membrane using the potassium-stimulated 4-NPPase method pioneered by Ernst (1972a; 1972b), it became apparent that the rhabdom itself contained high 4-NPPase activity which was not attributable to Na+/K+ ATPase. The source of this activity is neither artefactual nor a non-specific acid or alkaline phosphatase. It is proposed that the high concentration of the rhabdomeral enzyme indicates a major role in phototransduction.

2.3 MATERIALS AND METHODS

Crabs.

Crabs (Leptograpsus variegatus, Fabricius 1793) were collected from a rocky location on the New South Wales coast and kept in a tank at 16 °C under a light / dark cycle of 14:10 hours in summer and 12:12 hours in winter. Dissections for both cytochemical and biochemical experiments were started several hours after 'dawn' and finished at least three hours before 'dusk'.

Materials.

Ouabain and guanosine triphosphate (GTP, dilithium salt) were obtained from Boehringer (Mannheim); 4-nitrophenyl phosphate (disodium salt, 4-NPP), adenosine monophosphoric acid (5'-AMP), β-glycerophosphate (disodium salt), adenosine triphosphate (ATP, disodium salt), 'phosphorus' standard solution (potassium dihydrogen orthophosphate), para-bromotetramisole oxalate (pBT, (+) and (-) forms, R30 402), and Malachite Green hydrochloride were from Sigma (St. Louis): L-cysteine and Brij 35™ were from Fluka (Buchs). Other reagents...
were of analytical grade or e.m. grade, except for Araldite™ which was of industrial grade from Ciba-Geigy (Australia). All solutions were made up in quartz-distilled, deionized water.

Cytochemistry.

Retinae were fixed in 0.1 M piperazine ethane sulphonyl acid (PIPES), 0.14 M sucrose, 3% (w/v) paraformaldehyde, 0.25% (w/v) glutaraldehyde, adjusted to pH 7.4 with potassium or sodium hydroxide as appropriate. They were cut into blocks having linear dimensions less than 0.5 mm and under standard conditions were allowed to remain under fixative for 15 minutes from the start of the dissection. At the end of this time they were washed three times with 0.1 M tris(hydroxymethylamine) aminomethane hydrochloride (Tris HCl), 0.16 M sucrose, pH 7.4; each wash was for five minutes at 4 °C. All subsequent steps were performed at room temperature.

A number of vials were set up containing approximately 5 ml of either the following basic medium or a modification thereof: 0.1 M Tris HCl, 0.105 M sucrose, 20 mM L-cysteine, 20 mM 4-NPP, 10 mM SrCl₂, pH 9.0 at 25 °C. Either 10 mM KCl or 10 mM NaCl was also present and other modifications were made to the medium as noted in the results section. All modifications to the medium were osmotically compensated with sucrose. Fragments, all derived from a single retina, were separated into groups that were incubated for two hours on a reciprocal shaker. Following this incubation the tissues were given three five-minute washes with 0.13 M Tris HCl, 0.19 M sucrose, pH 9.0; followed by two five minute washes with 2% (w/v) lead nitrate. Excess lead nitrate was removed by two five-minute washes with 0.34 M sucrose; three five-minute washes with 0.1 M Tris HCl, 0.16 M sucrose; and three further washes with 0.1 M
Cytochemistry of Microvillar 4-NPase.

PIPES, 0.14 M sucrose, pH 7.4. The fragments were postfixed in 1% (w/v) osmic acid in the same PIPES buffer for one hour; rinsed briefly with distilled water and dehydrated through a standard ethanol series. Infiltration was performed by treating with propylene oxide for 10 minutes, 50:50 propylene oxide/Araldite™ overnight, followed by extensive resin infiltration prior to embedding and polymerization at 60 °C. All the steps were performed under normal laboratory fluorescent lighting.

Electron microscopy

In order to screen various conditions for their effects on phosphate deposition gold to silver sections were cut from two or more separate blocks under each experimental condition. They were examined unstained in Jeol 100c or Hitachi H500 electron microscopes using accelerating voltages of 75 and 50/75 kV respectively.

For observations at high resolution, silver to grey sections were examined in a Hitachi H600 microscope with an accelerating voltage of 75 kV. Some of these sections were stained with uranyl acetate alone or in combination with lead citrate.

Biochemical fractionation of crab retina

Retinae from a number of crabs were dissected under a buffer containing 20 mM Tris HCl, 0.3 M sucrose, 0.1 mM dithiothreitol, pH 7.4 at 4 °C. Care was taken to peel axonal material away from the basement membrane. Retinae were homogenized in the cold with ten strokes of a Potter-type teflon-in-glass pestle spinning at approximately 1,000 r.p.m. using a ratio of two retinae per 1 ml of buffer. The homogenate was centrifuged for 2 min. at 12,000g (approximately) in an Eppendorf minicentrifuge. The supernatant (S12) was aspirated off the pellet (P12)
and spun for 90 minutes at 120,000g maximum at 4 °C in the ultracentrifuge to give supernatant (S120) and pelleted (P120) fractions.

**Enzymatic assays**

Assays were performed in a total volume of 0.75 ml in capped 1.5 ml polypropylene tubes with each determination performed in quadruplicate. Final concentrations in the basic assay buffer (to which changes were made as noted) were: 0.1 M Tris HCl, 0.1 M sucrose, 20 mM L-cysteine, 5 mM ouabain, 20 mM 4–NPP, both in the presence and absence of 10 mM MgCl₂, pH 7.4 at 25 °C. Assays were started by adding 75 µl of the appropriate retinal fraction, mixing and standing at room temperature for 5 min. followed by the addition of 75 µl of a ten times concentrated stock of 4–NPP to bring the total volume to 0.75 ml. The tubes were vortexed immediately and incubated for up to 60 min. at 25 °C. Under these conditions the reaction is linear with time. Assays were stopped by the addition of 0.25 ml of 13.3% (w/v) sodium dodecyl sulphate (95% grade), 0.12 M diaminoethanetetra-acetic acid (EDTA, disodium sulphate), pH 7.0 (White, 1982). Blanks were constructed both with and without magnesium by adding stop solution at the beginning of the assay. Immediately before reading absorbance at 405 nm, 100µl of NaOH was added to each tube to make the contents alkaline. Where necessary this procedure was scaled down to smaller volumes.

For phosphoester substrates other than 4-nitrophenyl derivatives inorganic phosphate release was determined by one of two different methods according to the concentration range involved. Where sufficient phosphate was present the assay was performed and stopped exactly as described above with the substitution of alternative substrates for the
4-NPP. Colorimetric determination of the phosphate was by the method of Taussky & Shorr (1953) as described by White (1982).

For determination of inorganic phosphate release down to 1 nmol per assay tube the Malachite Green-phosphomolybdate method was used essentially as described by Lanzetta et al., (1979). Assays were performed in quadruplicate in polypropylene tubes in the assay buffer described previously. The final assay volume was 0.50 ml including 50 μl of retinal fraction and 50 μl of substrate solution. The reaction was stopped by the addition of 0.80 ml of colour reagent. Departures from the published procedure were that 2% (w/v) Brij 35™ was substituted for Sterox™, and that a fixed amount of phosphate was added to the assay tubes immediately before addition of colour reagent to bring the blank absorbance at 660 nm above 0.12 versus water. The reason was that the assay was not linear with phosphate concentration below this absorbance level.

Protein determination

Protein concentration was estimated by Markwell et al.'s (1978) modification of the Lowry procedure (Lowry et al., 1951).

2.4 Results

Distribution and nature of cytochemical deposits

Tissue preservation was good at the light microscope level but left much to be desired when viewed in the electron microscope. In an experiment in which fixation was allowed to continue for 60 min. (not shown) structural preservation was good, demonstrating that poor fixation is a consequence of the brevity of the fixation and the hostility of
the cytochemical treatments. Despite generally poor preservation the rhabdom itself, including the microvillar membranes, showed excellent preservation wherever reaction product (r.p.) was not present. Reaction product could be easily discerned, in both stained and unstained sections, as amorphous grains with dimensions of 50 nm or less, which in some cases were observed to have coalesced into continuous 'sausages' extending for 1 micron or more (Fig. 2.2a).

Experiments in which either sodium was present in isolation or in which 10 mM potassium was present in addition to sodium (introduced with the substrate), showed indistinguishable r.p. distribution and intensity. The overwhelming majority of the reaction product was situated within the rhabdom and palisade bridges (Fig. 2.1a), but r.p. was also consistently found associated with the outer cell membrane of the photoreceptor cell (Fig. 2.2a). Apart from these locations, deposits were occasionally seen associated with the nuclear membrane, and as originally reported for other tissues (Ernst, 1972b), over condensed chromatin and within the mitochondria. In most regions the cytoplasm of the photoreceptor cells was clear of r.p., but in cases of especially heavy rhabdomeral deposits r.p. was present, at a lower level, in the cytoplasm. Outside the photoreceptor cells deposits were absent with the occasional exception of the cytoplasm of distal pigment cells when these were in a particularly poor state of preservation.

Cytochemical experiments under the standard conditions were repeated as controls for all perturbation experiments and were always positive. It was a frequent finding that within an individual rhabdom the microvillar bundles from some photoreceptor cells were heavily labelled whilst other cells within the same rhabdom had little or no r.p. within their microvilli. This phenomenon gives rise to the zebra pattern
**Figure 2.1:** Unstained transmission electron micrographs to show the ultrastructure of photoreceptor cells and the distribution of reaction product (r.p.) within them under standard cytochemical conditions. – **a.** Longitudinal view of rhabdom (Rh) showing r.p. deposited in the absence of potassium. The palisade (p), multilamellar bodies (MLB) and surrounding reflecting pigment cells (RPC) are free of r.p. Retinula cell pigment granules (G) are the large dense spherical bodies not to be confused with r.p. granules. – **b.** Transverse section through rhabdom showing r.p. deposited in the presence of 10 mM KCl. – **a, b.** Bar 2 µm.

**Figure 2.2a:** Unstained oblique section through a rhabdom with r.p. deposited under standard conditions in the absence of potassium. – Note the r.p. on retinula cell plasma membranes (*arrowheads*) and the zebra pattern formed by r.p. within the rhabdom. – **b.** Detail of (**a**) showing longitudinal and transverse sections through microvilli that are filled with r.p. – **a.** Bar 2 µm. – **b.** Bar 0.5 µm.
seen in Figure 2.3c. In view of the counter examples (Fig. 2.14c), the implication is that some essential condition for lipoprotein synthesis is not present in the unlabelled cells rather than that the enzyme is absent in these cells.

experiments were carried out in the presence of ketoenolase which is an inhibitor of alkaline phosphatases from organisms as diverse as dog and

E. coli at 0.1 mM levels (Van Bella, 1975).
seen in Figure 2.2a. In view of the counter examples (Fig. 2.1a), the implication is that some essential conditions for r.p. deposition are not present in the unlabelled cells rather than that the enzyme is absent in vivo.

Unfortunately, deposition of r.p. was incompatible with preservation of the fine structural detail of the microvillar membrane (Fig. 2.2b) and staining did not alter this picture (Fig. 2.6). Despite this limitation one can conclude from views such as that of Figure 2.2b that r.p. is deposited within the microvillar membrane which must have retained sufficient integrity at the time of the deposition to limit the spread of the reaction. The conditions necessary for the cytochemical reaction were investigated by ultrastructural screening and are summarized in Table 2.1. These experiments indicate a heat- and fixative-sensitive enzyme that actively liberates phosphate from 4-NPP in the presence of 10 mM strontium (Fig. 2.4) acting as capture ion. The reaction does not proceed in the presence of 10 mM calcium and requires added magnesium (Fig. 2.3). Neither 10 mM ouabain nor removal of potassium inhibited r.p. production (Fig. 2.1b & 2.5). These data support the conclusion that the microvillar enzyme is not a manifestation of the Na⁺/K⁺ ATPase. They suggest that the enzyme is not an acid phosphatase because of the high pH at which the experiment was carried out, the lack of r.p. within lysosomes, the absence of reaction with β-glycerophosphate and 5'-AMP and the absence of microvillar reactions in the acid phosphatase study of de Couet & Blest (1982). Likewise there seems good reason to rule out a non-specific alkaline phosphatase, since alternative substrates were not utilized, and experiments were carried out in the presence of L-cysteine which is an inhibitor of alkaline phosphatases from organisms as diverse as dog and E. coli at 0.1 mM levels (Van Belle, 1972).
Figure 2.3; Sections stained with lead citrate and uranyl acetate showing rhabdoms incubated in the absence of magnesium. – a. Longitudinal section. – b. Transverse section. – a. Bar 2 µm. – b. Bar 0.5 µm.

Figure 2.4; Unstained transverse section of a rhabdom demonstrating that r.p. is deposited in the presence of 10 mM ouabain (10 mM KCl present). – Bar 1 µm.

Figure 2.5; Unstained transverse section through a rhabdom demonstrating that r.p. is not deposited when strontium is omitted from the standard cytochemical incubation. – Bar 1 µm.

Figure 2.6; A rhabdom double-stained with lead citrate and uranyl acetate showing that staining has little effect whether r.p. is present or not. Cytochemistry was performed under standard conditions in the absence of potassium. – Bar 0.5 µm.
<table>
<thead>
<tr>
<th>Fixation</th>
<th>Deletions</th>
<th>Replacements</th>
<th>Additions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard procedure...</td>
<td>(+) Strontium...</td>
<td>(-) Sr(^{2+}) with 10 mM Ca(^{2+})...</td>
<td>(-) 10 mM ouabain (+)</td>
</tr>
<tr>
<td>2.5% glutaraldehyde...</td>
<td>(-) Magnesium...</td>
<td>(-) Sr(^{2+}) with 2.5 mM Ca(^{2+}) &amp; 7.5 mM Sr(^{2+})...</td>
<td>(+) 1 mM EGTA (+)</td>
</tr>
<tr>
<td>Standard fixation for more than 30 min...</td>
<td>(-) 4-NPP...</td>
<td>(-) 4-NPP with 10 mM 5'-AMP...</td>
<td>(-) 4-NPP with saturated inorganic phosphate... (-)</td>
</tr>
<tr>
<td>Heat to 60 °C for 30 min. after fixation...</td>
<td>(-)</td>
<td>(-) 4-NPP with 10 mM (\beta)-glycerophosphate...</td>
<td>(-)</td>
</tr>
<tr>
<td>4-NPP...</td>
<td></td>
<td>(-)</td>
<td></td>
</tr>
<tr>
<td>K(^{+}) with 10 mM Na(^{+})...</td>
<td>(+)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Wash steps: 5mM EGTA in first two post-incubation washes (-).

Table 2.1: Summary of variations to the standard conditions used in cytochemical processing and the resultant effects on r.p.deposition: (+) r.p. present in equal strength to control. — (-) No r.p. whatsoever within rhabdom or on plasma membranes.
In isolation, the cytochemical evidence can be challenged on the grounds that enzymes may show anomalous properties with respect to substrates and inhibitors as a result of fixation and the biochemically unnatural conditions (e.g. pH 9.0, 10 mM SrCl₂).

There are magnesium-dependent and -independent 4-NPPase activities in crab retinal homogenates.

Phosphatase activity was found in a crude homogenate of crab retina incubated under buffer conditions identical to those of the cytochemical incubation medium. Only 29% of this 4-NPPase activity could be inhibited by the combined addition of 10 mM ouabain and deletion of potassium from the medium. Deletion of strontium marginally inhibited the total activity.

The reason for performing cytochemistry at pH 9.0 is to ensure precipitation of strontium phosphate. Under the more physiological biochemical assay conditions described in "Materials and Methods", Figure 2.7 shows that the upper limit for the contribution of the Na⁺/K⁺ ATPase to the total was 24%. Of the remaining activity measured under standard conditions, approximately one quarter required no added magnesium.

4-NPPase activity does not sediment with the membrane fractions.

When the experiment was performed in the standard homogenization buffer, i.e. no magnesium and low salt (20 mM Tris HCl) the magnesium-dependent activity unequivocally remained in the S12 and subsequently the S120 fractions (Table 2.2).

This indicates that the activity is not due to an integral membrane protein. The results of experiments in which the homogenization was
Figure 2.7: An experiment in which total 4-NPPase activity was assayed in a crude homogenate of retina at pH 7.4 with increasing concentrations of ouabain present. Standard biochemical conditions were observed with the following modifications: (□) 10 mM KCl, 10 mM MgCl$_2$ — (■) no KCl, 10 mM MgCl$_2$ — (〇) no KCl, no MgCl$_2$. Error bars represent the standard deviations of quadruplicate determinations.
Table 2.2; Fractionation of magnesium-dependent 4-NPPase activity by differential centrifugation in a low salt (20 mM Tris HCl), zero magnesium, standard homogenization medium. Errors are standard deviations of quadruplicate determinations.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Magnesium-dependent activity at pH 7.4 (nanomoles Pi liberated / min / mg of protein)</th>
<th>% yield relative to homogenate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>2.3 ± 0.3</td>
<td>100</td>
</tr>
<tr>
<td>S12</td>
<td>5.2 ± 0.4</td>
<td>97</td>
</tr>
<tr>
<td>P12</td>
<td>0.4 ± 0.1</td>
<td>7</td>
</tr>
<tr>
<td>S120</td>
<td>5.7 ± 0.5</td>
<td>95</td>
</tr>
<tr>
<td>P120</td>
<td>0 ± 0.7</td>
<td>0</td>
</tr>
</tbody>
</table>

Figure 2.8 shows that the magnesium-dependent activity has a broad pH optimum around 7.5 to 7.8, whilst magnesium-independent activity is maximal at pH 5.5, or below. This and the evidence of differential inhibitor sensitivities led to the conclusion that the two activities arise from separate magnesium-activated enzymes, which if not observed at all pH's may be present, but are only activated when magnesium is added.
performed in high salt (20 mM Tris HCl, up to 150 mM NaCl) and high magnesium (up to 25 mM) were more difficult to interpret quantitatively, because of the introduction of significant levels of magnesium into the assay buffer so that magnesium-dependent and -independent activities could not be assigned. However, examination of the total activity distribution in these experiments allows an assertion that on no occasion was greater than 10% of the total activity found in the sedimented fractions and that the specific activity of these fractions was roughly tenfold less than in the supernatant. Thus, conditions have not yet been discovered which change the soluble nature of the activity. This does not rule out the possibility that the 4-NPPase is a peripherally bound membrane protein which would be consistent with the highly localized distribution of the activity under cytochemical conditions. Alternatively the enzyme could be trapped by weak associations with the microvillar cytoskeleton, which is known to be highly labile in other decapod crustaceans (de Couet et al., 1984). Because the increase in specific activity in going from S12 to S120 fractions was small the former was used as the source of the enzyme for the experiments described in the rest of this chapter.

The magnesium-dependent and -independent activities differ in their pH optima.

Figure 2.8 shows that the magnesium-dependent activity has a broad pH optimum around 7.0 to 7.5, whilst magnesium-independent activity is maximal at pH 5.5, or below. This and the evidence of differential inhibitor sensitivities lead to the conclusion that the two activities arise from separate enzymes. The low pH species (which is not observed at all at pH 9.0) corresponds to the acid phosphatase previously reported in this
Figure 2.8: Variation with pH of magnesium-dependent (open symbols) and magnesium-independent (filled symbols) 4-NPPase activities in S12 fraction. — Squares: assays performed in 0.1 M Tris HCl buffer. — Triangles: assays performed in 0.1 M 2-(N-morpholino)ethane-sulphonic acid-Tris buffer. Other conditions were those described as standard in the Materials and Methods section. Error bars represent the mean and standard deviations of quadruplicate determinations.
tissue (de Couet & Blest, 1982). It is evident that the failure in that study to see clear-cut changes in acid-phosphatase levels during a daily cycle may well have been due to contamination by the 'acid tail' of the magnesium-dependent enzyme.

The effects of divalent cations

The major portion of the 4-NPPase activity observed under standard buffer condition required magnesium. Consequently, the effects of varying both magnesium concentration and those of the divalent cations strontium, calcium and cerium were tested, all as solutions of their chlorides (Fig.s 2.9 & 2.10). Figure 2.9 shows the 4-NPPase is stimulated measurably at magnesium concentrations as low as 10 µM and that saturation is approached at 10 mM magnesium. For the purposes of this study the magnesium-dependent activity is defined as the difference in activity between that measured with no added magnesium and that with 10 mM magnesium. The possibility that some stimulation of activity occurs due to magnesium carried over from the tissue cannot be excluded, but the degree of dilution involved (of the order of 100 x ) suggests that this effect will be quantitatively small. The definition used avoids the problems of interpreting the data obtained in the presence of EDTA, which may remove magnesium tightly bound to the enzyme and appears in this case to have effects other than on magnesium (Fig. 2.9).

As predicted from the cytochemistry, calcium strongly inhibited the 4-NPPase, an effect possibly of physiological significance since Schröder et al., (1980) have reported calcium levels between 3 and 10 mM in crayfish rhabdom using the technique of laser microprobe mass spectroscopy. When used after glutaraldehyde fixation, antimonate cytochemistry does not confirm this result. Background staining only is
Figure 2.9: Magnesium stimulation (○) of total 4-NPPase in S12 fraction at pH 7.4. — Inset shows (●) the effect of increasing EDTA concentration in the absence of magnesium; the ordinate scales are the same. Standard buffer conditions were used in other respects. Error bars represent the standard deviations of quadruplicate determinations.
Figure 2.10: Inhibition of magnesium-dependent 4-NPPase activity in S12 fraction at pH 7.4 by three cations: (□) CaCl$_2$, — (○) SrCl$_2$, — (■) CeCl$_2$, under standard buffer conditions. Error bars represent the standard deviations of quadruplicate determinations.
seen in the photoreceptive microvilli of crab (own unpublished observations), the honeybee (Perrelet & Bader, 1978) and the leech (Walz, 1979). Walz resolved this dilemma when he showed that, by omitting the glutaraldehyde fixation, he visualized dense deposits, almost certainly of calcium, on the inner surface of the leech's microvillar membranes (Walz, 1979).

Strontium, the capture ion used in this study, proved fortuitously to have no inhibitory effect even when present at 10 mM, whilst cerium which has recently been reported to be suitable for very high resolution cytochemical localization of a number of phosphatases (Robinson & Karnovsky, 1983) completely inhibited magnesium-specific activity at levels as low as 2 mM.

**Effects of phosphatase inhibitors and alternative substrates.**

Neither magnesium-dependent nor -independent activities responded to (-)-p-bromotetramisole oxalate at 0.1 mM which is a potent alkaline phosphatase inhibitor (Van Belle *et al.*, 1977). The magnesium-dependent activity was significantly more sensitive to both fluoride and (+)-tartaric acid (Fig. 2.11) than was the magnesium-independent enzyme. Typically both of these inhibitors are very effective against lysosomal acid phosphatase, but in this tissue it has been reported (de Couet & Blest, 1982) that 4-NPPase at pH 5.0 is relatively weakly inhibited. Neither β-glycerophosphate nor 5'-AMP gave measurable magnesium-dependent activity, confirming the findings of the cytochemical approach in this respect and excluding both general lysosomal acid phosphatase and acid nucleotidase as sources of the activity. Significant ATPase and GTPase activities were detected in the S12 fraction (Table 2.3).
Figure 2.11; Comparison of the effects of three phosphatase inhibitors on magnesium-dependent (■) and -independent (□) 4-NPPase activities measured at pH 7.4 in the S12 fraction under standard buffer conditions. — a. 0.1 mM (-) para-bromotetramisole oxalate. — b. 5 mM sodium fluoride. — c. 5 mM (+)-tartaric acid. Error bars represent the standard deviations of quadruplicate determinations.
Table 2.3: Phosphate ester hydrolysis by S12 fraction of crab retina with a number of different substrates at pH 7.4. Specific activity values are expressed as nanomoles of phosphate liberated per minute per mg of protein. Errors represent the standard deviation of quadruplicate determinations.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Concentration (mM)</th>
<th>Magnesium-independent activity</th>
<th>Magnesium-dependent activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-NPP</td>
<td>20&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.7 ± 0.6</td>
<td>5.4 ± 0.4</td>
</tr>
<tr>
<td>ATP</td>
<td>2</td>
<td>0 ± 0.9</td>
<td>3.4 ± 1.2</td>
</tr>
<tr>
<td>GTP</td>
<td>2</td>
<td>1.2 ± 0.6</td>
<td>11.9 ± 1.6</td>
</tr>
<tr>
<td>β-glycerophosphate</td>
<td>2</td>
<td>0.3 ± 0.25</td>
<td>0.9 ± 0.25</td>
</tr>
<tr>
<td>5'-AMP</td>
<td>2</td>
<td>0.3 ± 0.2</td>
<td>1.3 ± 1.6</td>
</tr>
</tbody>
</table>

<sup>a</sup> There is no increase in specific activity between 2 and 20 mM 4-NPP.
2.5 Discussion

The specificity of 4-NPPase cytochemistry.

The adoption by Ernst (1972a & 1972b) of the potassium-stimulated 4-NPPase half-reaction to visualize the Na+/K+ ATPase arose from the realization at the end of the '60s that lead capture cytochemistry with ATP as substrate could not reliably demonstrate that enzyme (Ernst & Hootman, 1981). Specificity is assured by the special nature of the conditions required to demonstrate the enzyme of interest, i.e. the presence of potassium and the absence of the specific inhibitor ouabain. Thus, the method depends absolutely on the proper observance of controls (preferably performed on biochemical preparations as well as cytochemically). In this respect the present observations simply reinforce the caveats voiced by Ernst, who nevertheless reasoned (Ernst, 1972b) that the background cytochemical staining that he observed in the absence of potassium was probably not due to a separate enzyme. In part his grounds were that r.p. was situated on the cytoplasmic side of the plasma membrane and could not therefore arise from Mg^{2+} ATPase. Whilst this conclusion may be valid for the avian salt gland the argument is not. Acid phosphatase (Fersht, 1977), alkaline phosphatase (Fersht, 1977), and Na+/K+ ATPase (Ernst, 1972a) all hydrolyze 4-NPP and are known to operate through a phosphorylated protein intermediate, as do, by definition, phosphoprotein phosphatases. Thus, the common factor shared by the enzymes reported to hydrolyse 4-NPP may be protein dephosphorylation and is certainly not the handling of nucleoside triphosphates. The origins of the rhabdomeral enzyme could therefore be a phosphoprotein phosphatase, a nucleotidase hydrolyzing 4-NPP, or a new class of phosphohydrolase which has not previously been reported to
have this property. It is conceivable that the 4-NPPase arises from two or more enzymes having different natural substrates. The r.p. observed on plasma membranes seems to have similar characteristics to the rhabdomeral enzyme. Thus, if the rhabdomeral enzyme is a true membrane protein, the plasma membrane staining could arise from the same enzyme. This might imply no more than the high concentration of membrane with respect to cytoplasm that the microvillar arrangement represents.

What is the physiological role of the 4-NPPase?

Some inferences about this can be made because it is highly concentrated in a specialized subcellular compartment – the rhabdom – which does two remarkable things. It traps light and performs some or all of the steps of phototransduction. In *Leptograpsus* it also turns over in a massive diurnal cycle of shedding and resynthesis (Blest *et al.*, 1980; Stowe, 1980b; Stowe, 1983). The enzyme seems to be present in larger amounts than would be expected if it had a control or trigger role in the turnover cycle. It can be distinguished from the lysosomal enzymes already known to participate in the degradation phase of membrane turnover (Blest *et al.*, 1980; de Couet & Blest, 1982). The utility of a degradative enzyme, active at neutral pH, outside secondary lysosomes and present in massive amounts in a biologically active region at a time when major degradation is not occurring is questionable.

Alternatively, the enzyme's role may be in phototransduction. One test of whether an enzyme participates in this process is to look for stimulation or inhibition by light. Some important enzymes may be concerned with resetting the system to zero, and would not necessarily respond to illumination. At present it is not possible directly to test the
light dependency of the 4–NPPase in a valid way because it is impossible to obtain the enzyme in close association with the photoreceptive membrane.
3. INOSITOL TRISPHOSPHATASE AND INOSITOL BISPHOSPHATASE ACTIVITIES OF CRAB RETINA

3.1 SUMMARY

1. Crab retinal extracts promoted rapid magnesium-dependent release of inorganic phosphate from 1,4,5-inositol trisphosphate.
2. Experiments using 1,4,5-inositol trisphosphate labelled with $^{32}$P in the 4' and 5' positions indicated the presence of both inositol trisphosphatase and inositol bisphosphatase activities.
3. These activities were not inhibited by 10 mM 2,3-bisphosphoglycerate but 10 mM CaCl$_2$ was markedly inhibitory.
4. The inositol trisphosphatase activity was overwhelmingly cytosolic and was separated from the main 4–NPPase activity by DEAE-cellulose column chromatography.
5. The ability of the breakdown enzymes to discriminate amongst the three available phosphate groups was not tested. However, during the course of the inositol trisphosphate breakdown at least one of the pair of vicinal phosphates was removed; this is believed to be sufficient to inactivate the molecule.

3.2 INTRODUCTION

When inositol trisphosphate (InsP$_3$) is pressure injected into the ventral photoreceptors of *Limulus* it excites them in a manner indistinguishable from light (Fein *et al.*, 1984; Brown *et al.*, 1984). Whether InsP$_3$ release is an element of the principle excitatory pathway of
visual transduction in rhabdomeral photoreceptors remains unresolved (Fein, 1986). If InsP$_3$ is part of this pathway then the synthetic and degradative pathways of the phosphoinositide cycle (see Figure 1.3) should be especially well-developed in rhabdomeric photoreceptors. In particular good temporal resolution by the visual system requires that the effective concentration of the diffusible messenger can be reset to the resting level very rapidly after a transient stimulus. There are several ways in which this could be achieved, but one of the simplest would involve hydrolysis of one or more of the phosphoester bonds of InsP$_3$. This mechanism is attractive because it is known that the 1,4 inositol bisphosphate is inactive in the *Limulus* ventral photoreceptor whilst the 4,5 bisphosphate retains considerable potency (Fein *et al*., 1984) and because removal of the 5' phosphate of InsP$_3$ is a well-established breakdown route in other systems (Downes *et al*., 1982; Berridge *et al*., 1983; Storey *et al*., 1984; Connolly *et al*., 1985). If this is the relevant pathway one would predict higher levels of the enzyme inositol trisphosphatase in the photoreceptor than in other tissues given the radically different time-courses of responses to visual and hormonal stimuli.

This study was undertaken to identify the InsP$_3$ degrading activities in crab retina. This should not only contribute to our understanding of the role of InsP$_3$ in invertebrate visual transduction but would also aid in determining the physiological function of the microvillar 4–NPPase. 4–NPP hydrolysis by an inositol trisphosphatase has never been reported. However, the cytochemical distribution of 4–NPPase activity that was described in Chapter 2 is consistent with the pattern that might be predicted for an inositol trisphosphatase which is involved in visual transduction.
3.3 MATERIALS AND METHODS

Crabs.

*Leptograpsus variegatus* were obtained and kept as described in Chapter 2.

Materials.

At the outset of these experiments 1,4,5-D-myo-inositol trisphosphate was not commercially available and was either obtained from Dr. R. Irvine or prepared locally as described below. Subsequently inositol trisphosphate was obtained from Sigma. (4,5) $^{32}$P-inositol trisphosphate was the generous gift of Dr. R. Irvine. Dowex-1x8-400, N-2-hydroxyethylpiperazine-N’-2-ethanesulphonic acid (HEPES), inosine, sodium pyruvate and 2,3-bisphosphoglyceric acid were obtained from Sigma; adenine was obtained from Fluka.

Other materials were obtained from the sources noted in Chapter 2.

Preparation of inositol trisphosphate.

(Based on the method of Downes *et al.*, (1982) as modified by R. Irvine.)

Human blood, less than two weeks old, was obtained from the Red Cross Blood Bank, Woden Valley Hospital. Approximately 500 ml of blood was diluted with 1.5 litres of an ice-cold buffer containing: 130 mM KCl, 10 mM NaCl, 10 mM HEPES, pH 7.0 and the cell suspension was centrifuged at 4 °C for 20 minutes at 1500 x g. The supernatant and buffy layer were aspirated off and discarded and the red blood cells were given a further three washes. The buffer for the second pair of washes was 65 mM tri-potassium citrate, 5 mM trisodium citrate, 10 mM HEPES buffered to pH 7.0 with citric acid.
40 ml of 50 mM sodium pyruvate, 50 mM inosine, 100 mM glucose, 50 mM disodium hydrogen orthophosphate, 5 mM adenine in the same citrate/HEPES buffer was added to the cells. The suspension was incubated for 2 hours at 37 °C and then poured into one litre of ice-cold 10 mM Tris HCl, 1 mM EDTA to lyse the cells. Lysis was allowed to proceed for 15 minutes. The erythrocyte ghosts were pelleted at 15-20,000 x g for 30 minutes and the supernatant was discarded. The pellet was resuspended in an excess of the Tris/EDTA buffer and the ghosts were again collected by centrifugation. The washing and collection of the ghosts was repeated several more times until the supernatant was completely colourless.

1 M CaCl₂ was added to the pelleted ghosts to give a final concentration of 2 mM and they were stirred and incubated at 37 °C for 45 minutes. The reaction was quenched by pouring the ghosts into one litre of ice-cold water which were pelleted as before. The supernatant was passed over a 1 cm column of Dowex-1 in the formate form. The column was eluted with 10 ml of 0.4 M ammonium formate, 0.1 M formic acid followed by 10 ml of 1 M ammonium formate, 0.1 formic acid. The eluate from the last step was diluted to 50 ml and passed over a 0.5 cm column of Dowex in the chloride form. Inositol trisphosphate was eluted with 2.5 ml of 1 M LiCl and the sample was dried *in vacuo* at 45 °C. The pellet was washed several times with ethanol to remove the LiCl and air dried.

**Specificity of inositol trisphosphate hydrolysis.**

Assays were performed in duplicate in the standard 4-NPPase assay buffer and in a final volume of 250 µl. Assays were performed in the presence and absence of 10 mM MgCl₂. The substrate was 0.1 mM inositol trisphosphate which contained approximately 8,000 decays per minute per assay tube of ³²P-labelled InsP₃; labelled predominantly in the
5' phosphate with none in the 1' phosphate (Downes et al., 1982). The assay was started by the addition of an S12 fraction of crab retina and allowed to proceed for 15 or 30 minutes. At the end of this time the assay was stopped by the addition of 250 µl of 12% (w/v) trichloracetic acid. Samples were incubated on ice for 30 minutes before the precipitated protein was pelleted at approximately 12,000 x g. The samples were diluted to 2.5 ml with distilled water and neutralized with a small volume of 0.1 M NaOH. Each sample was run onto a 0.5 cm Dowex-formate column in a pasteur pipette and the columns were eluted according to the following regime (Downes & Michell, 1981):

1. Distilled water..........................................................1.0 ml
2. 0.1 M formic acid, 0.2 M ammonium formate............7.5 ml
3. 0.1 M formic acid, 0.4 M ammonium formate............7.5 ml
4. 0.1 M formic acid, 1.0 M ammonium formate............7.5 ml

The distilled water wash was combined with the load fraction. These samples and the ammonium formate eluates were collected in scintillation vials and the distribution of radioactivity amongst the fractions was determined by Cerenkov counting.

Determination of total phosphate in retinal extracts.

Duplicate samples of up to 40 µl were pipetted into acid-washed pyrex tubes and dried down under vacuum. The samples were digested in the presence of 5 M sulphuric acid and 60% perchloric acid according to Hess & Derr (1975). 125 µl of distilled water was added to the digested samples followed by 650 µl of the modified ammonium molybdate-Malachite Green-Brij 35 reagent of Lanzetta et al., (1979), prepared as described in section 2.3. The samples were allowed to stand for 10 minutes before having their Absorbance at 660 nm measured. The total phosphate concentration was
calculated by reference to inorganic phosphate standards that had been processed in the same way.

Column chromatography procedures are described in section 4.3. Other methods were as described in section 2.3.

### 3.4 Results

When 100 µM 1,4,5-inositol trisphosphate and 10 mM magnesium chloride were added to an S12 fraction from crab retina under the standard buffer conditions used for 4-NPPase assays (section 2.3), there was a rapid and dramatic release of inorganic phosphate. In order to ascertain the source of this phosphate the enzymatic release of phosphate was compared with the total amount of phosphate present in the sample. In a typical experiment an S12 supernatant from crab retina containing 36 µg of protein released approximately 1100 nmoles of inorganic phosphate in the presence of 100 µM InsP3 over 30 minutes at 25 °C. This is compared with 198 ± 20 nmoles of inorganic phosphate released from 200 µM 4-NPP under the same assay conditions. The total phosphate of tissue origin in these assays was determined to be 120 nmoles by total acid hydrolysis of the S12 fraction. Inositol trisphosphate was completely stable for up to one hour at room temperature in the standard assay buffer whether magnesium ions were present or not.

These findings suggest that there is very high InsP3 hydrolyzing activity in the retina of *Leptograpsus*.

**Hydrolysis of 32P-labelled 1,4,5 inositol trisphosphate.**

S12-mediated hydrolysis of 32P-labelled InsP3 was investigated in order to confirm the source of the inorganic phosphate release and to determine the extent and specificity of the InsP3 breakdown. The breakdown
products were separated by Dowex-formate column chromatography into three fractions which comprised: inositol trisphosphate, inositol bisphosphates and a fraction including inositol monophosphates and inorganic phosphates (Downes & Michell, 1981; Berridge et al., 1983).

InsP$_3$ hydrolysis was not observed in control samples that were precipitated with trichloroacetic acid prior to InsP$_3$ addition (not shown.) Figure 3.1 shows that there was negligible InsP$_3$ breakdown during a 15 minute incubation of the S12 fraction with 100 µM InsP$_3$ when magnesium was omitted from the assay. The addition of 10 mM MgCl$_2$ during the incubation markedly changed this picture. After a 15 minute incubation in the presence of magnesium approximately two-thirds of the InsP$_3$ had been degraded. The breakdown products after this time included inositol bisphosphates, inorganic phosphate and possibly inositol monophosphates. After 30 minutes of incubation virtually all the exogenous InsP$_3$ and all the inositol bisphosphates that were formed from its breakdown had been hydrolyzed. The $^{32}$P label was in the form of inorganic phosphate and inositol bisphosphate. Figure 3.1 also shows that over a 30 minute period inositol trisphosphate breakdown occurred in the absence of added magnesium. This hydrolysis was less extensive than in the presence of 10 mM magnesium and was preceded by a significant lag time when no breakdown occurred. It could represent a truly magnesium-independent inositol trisphosphatase activity or simply be a product of endogenous magnesium, the long incubation time and the extremely high activity in the S12 extract.

Clearly there are both inositol trisphosphatase and inositol bisphosphatase activities in the S12 fraction. Whether there is also an inositol monophosphatase cannot be decided using this data because inositol monophosphates cannot be separated from inorganic phosphate by Dowex-formate chromatography. Nor is it possible to distinguish
Figure 3.1; Fractionation of the breakdown products of inositol 1,4,5-trisphosphate. An S12 fraction of crab retina was incubated with 100 µM inositol trisphosphate, labelled in the 4' and 5' positions with $^{32}$P, for the times indicated. Standard assay buffer conditions (section 2.3) were used at pH 7.4 and 25 °C. The breakdown products were separated into four fractions by chromatography on 0.5 cm columns of Dowex-formate in pasteur pipettes. The fractions were obtained by the following elution scheme. "Flow through"; standard assay buffer & distilled water wash. "0.2 M"; 0.2 M ammonium formate/0.1 M formic acid. "0.4 M"; 0.4 M ammonium formate/0.1 M formic acid. "1.0 M"; 1.0 M ammonium formate/0.1 M formic acid. Error bars represent the standard deviations of duplicate determinations. See section 2.3 for further details of the method. Note, the radioactive material appearing in the flow through fraction after 30 min. incubation may be due to saturation of the column capacity.
15 minute incubation with no added magnesium

15 minute incubation with 10 mM magnesium chloride

30 minute incubation with no added magnesium

30 minute incubation with 10 mM magnesium chloride

Counts per minute

Flow through

Fraction

Flow through

Fraction

0.2 M

0.4 M

1.0 M

0.2 M

0.4 M

1.0 M
between hydrolysis of the 4’ and 5’ phosphates using this technique. However at least one of this pair of vicinal phosphates was removed which would be expected to terminate the biological activity of the molecule (Downes et al., 1982).

**Inhibitor studies.**

2,3-bisphosphoglycerate also bears a pair of vicinal phosphates and competitively inhibits the inositol trisphosphatase of a mammalian system (Downes et al., 1982). In the crab system, the release of phosphate from 50 µM InsP₃ was unaffected by up to 10 mM 2,3-bisphosphoglycerate. In fact, whilst the total phosphate release was unaffected by bisphosphoglycerate, it elevated the fraction of the activity that did not require exogenous magnesium for expression. This is interpreted as being due to contamination of the commercial bisphosphoglycerate preparation with magnesium or another divalent cation. The vicinal phosphate groups form a particularly strong binding site for divalent cations and such contamination would be difficult to avoid. Bisphosphoglycerate itself was not hydrolysed by the S12 fraction which indicates that InsP₃ breakdown enzymes in crab retina are more specific than the enzyme from human erythrocyte membranes (Downes et al., 1982).

Calcium has also been reported to inhibit the soluble inositol trisphosphatase of human platelets (Connolly et al., 1985). In this system 10 mM CaCl₂ inhibited the magnesium-dependent rate of phosphate release from InsP₃ by >60% but under the same conditions 10 mM SrCl₂ had no observable effect. This discrimination between millimolar concentrations of calcium and strontium ions is an additional feature that the inositol trisphosphatase shares with the microvillar 4–NPPase.
Fractionation of inositol trisphosphatase activity of crab retina

Both membrane bound and cytosolic inositol trisphosphatases have been described and both forms are present together in various mammalian preparations (Downes et al., 1982; Storey et al., 1984; Joseph & Williams 1985). The S12 fraction, used as the source of the inositol trisphosphatase in the experiments described up to this point, contains both membrane and cytosolic components and cannot be used to provide information regarding the distribution of inositol trisphosphatase.

Figure 3.2 shows the time-courses of magnesium-independent and magnesium-stimulated InsP$_3$ hydrolysis in authentic membrane and cytosolic fractions prepared from a crude homogenate of crab retina. After high-speed centrifugation the inositol trisphosphatase is recovered overwhelmingly in the S120 fraction. There was minimal loss of activity following glass microfibre filtration to remove the red screening lipid (Figure 3.2, "GF/C").

In order to test the hypothesis that the 4–NPPase and inositol trisphosphatase activities reside on the same protein a GF/C filtered cytosolic fraction similar to that shown in Figure 3.2 was subjected to DEAE-cellulose column chromatography. The results are shown in Figures 3.3 and 3.4. The distribution of the main 4–NPPase activity in the column eluates was quite different from that of the inositol trisphosphatase. The main peak of the 4–NPPase activity eluted in a broad peak around 90 mM NaCl whilst significant inositol trisphosphatase activity was only eluted in the high salt (1.5 M NaCl) wash. The assays used to construct the column profile shown in Figure 3.3 were single time-point assays without duplicates. Figure 3.4 shows a more rigorous determination of the inositol trisphosphatase activity in the sample that was loaded and the eluted fractions with the highest inositol trisphosphatase and 4–NPPase activities respectively. The ratios of the
Figure 3.2: Time course of inorganic phosphate release from 1,4,5-inositol trisphosphate by various fractions derived from a homogenate of crab retina. A crude homogenate of crab retina was centrifuged at 120,000 x g for 90 min. to give S120 and pelleted fractions. The pellet was resuspended in the original volume of homogenizing buffer to give a P120 fraction, and the S120 fraction was repeatedly filtered through glass microfibre filters to give the GF/C fraction. 20 µl samples of these fractions were incubated with 45 µM inositol 1,4,5-trisphosphate in the presence (●) or absence (○) of 10 mM MgCl₂ in a total volume of 250 µl. Standard assay buffer conditions (section 2.3) were employed at pH 7.4 and 25 °C. Assays were stopped at 5 min. intervals with an equal volume of 12% (w/v) trichloroacetic acid and the amount of phosphate released was assayed using Lanzetta et al.'s (1979) method as described in section 2.3.
Absorbance at 660 nm

Homogenate

S120 fraction

P120 fraction

GF/C fraction

Absorbance at 660 nm

0 5 10 15 20

time (minutes)

0 5 10 15 20

time (minutes)
Figure 3.3: Separation of inositol trisphosphatase activity from 4-NPPase by DEAE-cellulose column chromatography. Approximately 6 mls of a GF/C fraction of crab retina, prepared as described in the legend to figure 3.2, was applied to a 10 ml DEAE-cellulose column (1). The column was washed with 20 mM TrisHCl, 100 mM sucrose, 0.5 mM DTT, pH 7.4 (2) and developed with a linear gradient of 0-100 mM NaCl in the same buffer (3). In (4) the column was washed with 1.5 M NaCl in the same Tris buffer. Fractions of approximately 2 mls were collected and samples assayed for: (•) 4-NPPase activity using the standard assay procedure; (▲) and phosphate release from 45 µM inositol trisphosphate using the method of Lanzetta et al. (1979). Both assays were performed in the presence of 10 mM magnesium chloride. See section 3.3 for details of the assays. N.b. In this experiment colour development in the inorganic phosphate assay was measured at 630 nm, rather than the usual 660 nm, in an E.L.I.S.A. plate reader. The original GF/C material and the fractions marked * were saved for further assay.
Figure 3.4: Phosphate release from inositol trisphosphate by the column purified fractions of Figure 3.3. 20 µl samples of the stated fractions were assayed for magnesium-dependent (●) and magnesium-independent (○) release of phosphate from 45 µM inositol trisphosphate as described in the legend to Figure 3.2.
rates of the magnesium-dependent phosphate liberation (determined over the first 5 minutes) from InsP\textsubscript{3} and 4-NPP in the three fractions were:

- Column load (GF/C fraction) ........................................6.5
- Peak 4-NPPase fraction (No. 20) ................................. 0
- Peak inositol trisphosphatase fraction (No. 28) ........... 17.6

It is clear that the inositol trisphosphatase is distinct from the main portion of the 4-NPPase activity present in the retina and that conversely only minor 4-NPPase activity could be associated with the inositol trisphosphatase.

### DISCUSSION

The discovery of significant inositol trisphosphatase and bisphosphatase activities in crab retina confirms the importance of InsP\textsubscript{3} in such arthropod tissue. The cellular origin of the enzymes and their subcellular distribution remains unknown. Localization of the enzyme within the retina would represent an important advance in our understanding of the role of inositol phosphates in phototransduction. As was shown here this cannot be achieved by the use of the non-specific phosphatase substrate 4-NPP because there is little or no 4-NPPase activity associated with inositol trisphosphatase activity. InsP\textsubscript{3} itself is an unappealing substrate for cytochemical work due partly to its scarcity and cost and partly to the fact that the millimolar concentrations required for cytochemical work precipitate in the presence of phosphate capture ions such as lead and strontium (R. Irvine personal communication.) Probably the best route to localizing the InsP\textsubscript{3} breakdown enzymes would be an immunocytochemical one.

In the preliminary experiments reported here, the positional specificity and kinetics of the trisphosphatase and bisphosphatase
enzymes were not examined. Based on observations of the enzymes of mammalian (Downes et al., 1982; Storey et al., 1984; Connolly et al., 1985) and insect (Berridge, 1983) origins the inositol trisphosphatase would be expected to selectively remove the 5' phosphate with subsequent removal of the 4' phosphate by the bisphosphatase enzyme. Confirmation of this pathway for the crab enzymes would probably only be worthwhile if the enzymes can be shown to have a microvillar or, at least, a photoreceptor origin because considerable expense and effort is required to prepare $^{32}$P-labelled InsP$_3$, which is not commercially available.
4. PARTIAL PURIFICATION FROM SQUID RETINA OF A 4-NPPASE PREVIOUSLY LOCALIZED TO THE PHOTORECEPTIVE MICROVILLI OF A CRAB.

4.1 SUMMARY

1. The microvillar 4-NPPase of crab that was described in Chapter 2 was further characterized with respect to its divalent cation requirements and substrate specificity.
2. The crab enzyme is shown to require µM levels of calcium, in the presence of millimolar levels of magnesium, for optimal activity. Manganese and cobalt but not calcium, nickel or zinc will substitute for magnesium in stimulating the 4-NPPase.
3. The activity is unstable unless millimolar concentrations of an appropriate divalent cation - magnesium, calcium or manganese - are maintained during fractionation steps.
4. The activity is a pure phosphomonoesterase and is distinct from the ATPase and GTPase activities that can also be identified in the crab retinal homogenate.
5. A similar enzyme was identified in the local squid species Sepioteuthis australis. This activity closely resembles the crab enzyme in a number of physical characteristics and was purified approximately 100-fold. The only polypeptide associated with the activity was a 35 kDa subunit. A 35 kDa polypeptide also copurifies with the 4-NPPase activity of crab.
6. On the basis of apparent native molecular masses of 70 ± 5 kDa for squid and 80 ± 5 kDa for crab (estimated by gel filtration on calibrated
columns of Sephadex) a homodimeric structure is proposed for the enzyme.

4.2 INTRODUCTION

One feature peculiar to the rhodopsin-bearing membrane of invertebrates is the abundance of a 4-NPPase (Romero-Saravia & Hamdorf, 1983; Trowell, 1985). Whilst a 4-NPPase has been described in the retinae of vertebrates (Ueno et al., 1980; Ueno et al., 1981; Ueno et al., 1984b) it was attributed to the Na⁺/K⁺-ATPase and no activity was found in association with the rhodopsin-bearing membrane. The invertebrate activity was initially described in octopus as an "alkaline phosphatase" (Romero-Saravia & Hamdorf, 1983) but the data presented in that study made it clear that the enzyme differed markedly from alkaline phosphatases isolated from several mammalian tissues. The 4-NPPase that was subsequently described in a crab (Trowell, 1985) resembled the octopus enzyme in its close association with rhodopsin-bearing membrane, its high specific activity in crude homogenates and its requirement for magnesium. It seems that the two activities derive from an enzyme performing the same function in both tissues and, on the basis of its biochemical properties, that the enzyme is not a classical alkaline phosphatase (E.C.3.1.3.1.)

The high specific activity of the 4-NPPase and its association with the phototransductive membrane point to a role in phototransduction. Its properties led to previous suggestions that it functions as a phosphoprotein phosphatase (Romero-Saravia & Hamdorf, 1983; Trowell, 1985) and it is an excellent candidate for the rhodopsin phosphatase. This possibility is particularly attractive because a number of phosphoprotein phosphatases do exhibit such a divalent cation-dependent activity (Pallen
The study described here was undertaken in order partially to purify the 4-NPPase from crab and squid and to further characterize them biochemically. This work is a prerequisite for reconstitution experiments in which the ability of the 4-NPPase to dephosphorylate rhodopsin is tested directly and is also a preliminary to the generation of monoclonal antibodies against the 4-NPPase.

4.3 MATERIALS AND METHODS

Crabs.

Leptograpsus variegatus were obtained and kept as described in Chapter 2.

Squid.

Squid (Sepioteuthis australis) were caught in local waters and transported to the laboratory on ice. The eyeballs were excised and opened within 12 hours of capture. After being allowed to drain the eyeballs were frozen and stored at -70 °C until use. Squid eyeballs were thawed and disrupted by vigorous stirring in buffer at a ratio of 5 ml per retina. The suspension was filtered through a 1 mm plastic mesh, which retains most of the tissue but allows the passage of almost all retinal fragments. The resulting suspension was homogenized thoroughly in a teflon-on-glass homogeniser spinning at approximately 1,000 r.p.m.
Materials.

Protein molecular weight markers for gel filtration, DEAE-cellulose, phospho-amino acids, phosvitin, 4-nitrophenylphosphate, 4-nitrophenyl phosphoryl choline, bis-(4-nitrophenyl) phosphate, phenylmethylsulphonyl fluoride (PMSF), calmodulin (bovine brain - P2277), phosphate standard solution, 4-nitrophenol standard solution, Tris base, EDTA, EGTA, ATP, AMP, D-myo-inositol-(1,4,5)-trisphosphate (InsP3), β-glycerophosphate, β-mercaptoethanol, dithiothreitol (DTT), N,N,N',N'-tetramethylethylene-diamine (TEMED), Brilliant Blue R250, ammonium persulphate and 95% sodium lauryl sulphate were from Sigma. Sephadex gel filtration media and phenyl-Sepharose were from Pharmacia. Acrylamide, N,N'-methylene bis-acrylamide, sodium dodecyl sulphate (SDS) and protein molecular weight markers for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) were from Bio-rad.

The cysteine proteinase inhibitor L-trans-epoxysuccinylleucylamido-(3-methyl)-butane (Ep-475) (Barrett et al., 1982) was the kind gift of Dr. K. Hanada (Taisho Pharmaceutical Company, Tokyo) to Dr. A.D. Blest.

Other reagents were obtained from the sources indicated in sections 2.3 and 3.3.

Column chromatography procedures.

The following buffers were used:

A.......20 mM TrisHCl, 2 mM MgCl₂, 0.5 mM DTT, pH 7.0
B.......20 mM TrisHCl, 2 mM MgCl₂, 0.5 mM DTT, 0.5 mM PMSF, 0.2 mM Ep-475, pH 7.0
C.......0.65 M ammonium sulphate, 20 mM TrisHCl, 2 mM MgCl₂, 0.5 mM DTT, pH 7.0
D........100 mM sucrose, 20 mM TrisHCl, 2 mM MgCl₂, 0.5 mM DTT, pH 7.5
E........100 mM sucrose, 20 mM NaCl, 20 mM TrisHCl, 2 mM MgCl₂, 0.5 mM DTT, pH 7.5
F........100 mM sucrose, 100 mM NaCl, 20 mM TrisHCl, 2 mM MgCl₂, 0.5 mM DTT, pH 7.5
G........1 M NaCl, 100 mM sucrose, 20 mM TrisHCl, 2 mM MgCl₂, 0.5 mM DTT, pH 7.5

Squid or crab retinae were homogenised in buffer B and the homogenates were centrifuged at 12,000 x g for 10 minutes. The S₁₂ supernatant from this step was centrifuged at 120,000 x g for 90-120 min. The S₁₂₀ supernatant from this step was filtered several times through Whatman GF/C glass microfibre discs to remove suspended lipid and the filtrate was used as the starting material for further biochemical purification.

Dialysis membrane was boiled in 0.1 M NaHCO₃, 10 mM EDTA and washed extensively in distilled deionised water prior to its use.

The general scheme for the column purification of 4-NPPase from squid was as follows. (See the Figure legends for details of column procedures.) The filtered S₁₂₀ extract was concentrated from approximately 200ml to 40 ml by ultrafiltration through an Amicon YM30 membrane and applied to a 98 x 2.5 cm Sephadex G-100 column. The column was developed with buffer A at a flow rate of approximately 20 ml hr⁻¹. Fractions eluting from the column with significant 4-NPPase activity were pooled, made 0.65 M in ammonium sulphate by slow addition of the solid salt and the solution was applied to a phenyl-Sepharose column. Active fractions eluting around 0.5 M ammonium
sulphate were pooled, concentrated by ultrafiltration to approximately 9 ml and refractionated on a Sephadex column. The fractions that eluted in the single peak of activity were pooled and applied to a DEAE-cellulose column. A broad region of activity eluting between 20 and 80 mM NaCl was collected, concentrated to approximately 3 ml by ultrafiltration and dialysed versus buffer A.

For the rapid assay of 4–NPPase in column fractions 50 µl samples were transferred to a 96 well cluster plate. 50 µl of a pH 9.0 buffer containing 0.2 M Tris HCl, 0.2 M sucrose, 0.04 M L-cysteine, 0.02 M MgCl₂ and 4 mM 4–NPP was added rapidly to each well with a multichannel pipette and the plate was incubated with vigorous shaking until significant colour development was observed (5-60 minutes). The assay was stopped by the rapid addition of 50 µl of 6.7% sodium lauryl sulphate, 0.12 M EDTA adjusted to pH 9.0 with NaOH. Absorbance in the wells was determined at 405 nm using a Titertek "Multiskan" ELISA plate-reader.

Gel slices were washed into an Eppendorf tube with 675 µl of assay buffer containing 0.1 M sucrose, 0.1 M TrisHCl, 20 mM cysteine, 10 mM MgCl₂, pH 9.0. The assay was started by adding 75 µl of 0.1 M 4–NPP and continued at 25 °C for 30 minutes with shaking. It was stopped by the addition of 250 µl of a solution containing 6.7% (w/v) sodium lauryl sulphate, 0.12 M EDTA, pH 9.0. The assay tubes were allowed to settle and the release of 4-nitrophenolate into the supernatant was measured spectrophotometrically at 405 nm.

Protein in column fractions was estimated by measuring the Absorbance at 280 nm versus a water blank.

Non-denaturing polyacrylamide gel electrophoresis.

Samples were loaded onto a 1.5 mm thick 5% vertical slab polyacrylamide gel prepared in a non-denaturing continuous buffer
Partial Purification of 4-NPPase. 66

system (30% acrylamide, 0.8% bisacrylamide, 0.375 M Tris HCl pH 8.9). Electrophoresis was carried out at 20 mA per slab constant current until the tracking dye approached the bottom of the gel. Two longitudinal tracks 1 cm wide were cut from the body of the slab gel which was then frozen. One of the tracks was cut into 2 mm slices which were assayed for 4-NPPase activity as described above. The other track was frozen in Laemmli's sample buffer (Laemmli, 1970) modified by the addition of 5 mM EDTA.

Cylindrical gels of diameter = 5 mm were similarly prepared and were electrophoresed at 3 mA per tube. Duplicates were either sliced for assay or frozen in sample buffer.

SDS-polyacrylamide gel electrophoresis.

Electrophoresis was carried out at room temperature in a vertical slab system comprising a 3% stacking gel and a 7.5-15% acrylamide gradient separating gel according to the procedure of Laemmli (1970). Gels were fixed and stained with Brilliant blue R250 and/or silver-stained according to the method of Wray et al., (1981). The molecular weights of retinal proteins were estimated by reference to a standard curve obtained by plotting the log of the molecular weight versus the relative mobility of protein standards.

Molecular weight estimation by gel permeation chromatography.

Columns (Pharmacia, 98cm x 1.6 cm) were packed with Sephadex G-100 and G-150 according to the manufacturer's instructions. All samples and standards were prepared in buffer D. Samples were run into the column using a sample applicator cup, in a volume of 2 ml. The column was developed in buffer D at a constant flow rate of approximately 10 ml per hour. 2 ml fractions were collected and assayed for protein at 280 nm.
4-NPPase activity, where present, was assayed in 96 well cluster plates as described above.

Other methods were as described in sections 2.3 and 3.3.

4.4 RESULTS

Initially, experiments were performed on relatively crude preparations from crab retina in order to eliminate some obvious possible natural functions for the 4-NPPase.

Activity of 4-NPPase towards alternative phosphoester substrates.

A crude homogenate of retina was screened for activity towards a number of substrates. Those supporting significant phosphatase activity were further tested on 4-NPPase positive fractions purified by gel filtration or ion-exchange chromatography. No activity was detected towards bis-(4-nitrophenyl)phosphate nor to 4-nitrophenylphosphoryl-choline (Kurioka & Matsuda, 1976). This suggests that the 4-NPPase is a pure phosphomonoesterase and is not derived from a phosphodiesterase. ATP, GTP (Trowell, 1985) and InsP₃ (Chapter 3) were previously shown to be hydrolyzed by soluble extracts of the retina. The DEAE-cellulose column fraction that contained peak 4-NPPase caused no hydrolysis of these substrates (Table 4.1 and Chapter 3).

Divalent cation requirement for enzyme stability.

During early attempts to purify the 4-NPPase it became apparent that column procedures resulted in almost total loss of enzyme activity. This loss of activity was not observed in a portion of the sample that was stored under identical physical conditions for the duration of the chromatography run and this was interpreted as indicating separation of
Table 4.1; Crab retinal 4-NPPase does not copurify with GTPase or ATPase.

Magnesium-stimulated phosphohydrolyase activity in an S120 extract was measured against various phosphoester substrates. Values are means ± standard deviations of 4 determinations. See "Materials and Methods" for details of assays.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Specific activity in the filtered S120 extract (nmol Pi / min. / mg of protein)</th>
<th>Specific activity in the peak 4-NPPase fraction eluted from a DEAE-cellulose column (nmol Pi / min. / mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>500 µM 4-nitrophenyl phosphate</td>
<td>10 ± 0.7</td>
<td>150 ± 6</td>
</tr>
<tr>
<td>500 µM GTP</td>
<td>3.9 ± 0.2</td>
<td>none detectable</td>
</tr>
<tr>
<td>500 µM ATP</td>
<td>0.3 ± 0.1</td>
<td>none detectable</td>
</tr>
</tbody>
</table>
the 4–NPPase from a stabilizing factor. Inclusion of 2 mM Mg\(^{2+}\) and 0.1 mM Ca\(^{2+}\) in all the buffers (Fig. 4.1) resulted in a tenfold increase in the activity recovered from the column.

The effect was investigated by dialysing samples of an S120 fraction of the 4–NPPase for 36 hours versus buffers containing various added divalent cations (Table 4.2). The requirement for millimolar concentrations of divalent cations was confirmed with the order of efficacy being Ca\(^{2+}\) ≥ Mg\(^{2+}\) > Mn\(^{2+}\). Chelation of residual calcium with EGTA in the presence of 2 mM Mg\(^{2+}\) had no effect on the stability of the activity.

Consequently, 2 mM MgCl\(_2\) was routinely added to all buffers in order to stabilise the activity. Whilst Ca\(^{2+}\) would have been marginally more effective it was avoided in order to prevent possible stimulation of calcium-dependent proteinases that are believed to operate in arthropod retinal tissues (Blest et al., 1984).

**Effects of transition metal ions.**

The requirement for a divalent cation to stabilize the enzyme suggested that other divalent cations besides magnesium might be capable of supporting the activity (as is the case for several other phosphoprotein-phosphatase-associated 4–NPPase activities of mammalian origin (Pallen & Wang, 1983; Clari et al., 1975; Ullman & Perlman, 1975; Roberts & Bazer, 1976; Li et al., 1979; Rose & Heald, 1961; Li et al., 1978). 1 mM manganese, cobalt, nickel and zinc ions (as solutions of their chlorides) were tested for their ability to stimulate the 4–NPPase activity in S120 extracts. If the total activity measured in the presence of 1 mM magnesium is taken as 100% then the corresponding value for manganese was 105% whilst the values for cobalt, nickel and no addition
Table 4.2: Effect of the inclusion of divalent cations in the dialysis buffer on the stability of crab 4-NPPase activity after dialysis for 36 hours.

The dialysis buffer was 100 mM sucrose, 20 mM TrisHCl, 0.5 mM dithiothreitol, pH 7.5 with the stated additions. Total 4-NPPase activity was measured in the presence of 10 mM magnesium chloride, 0.3 mM CaCl$_2$, and 0.2 mM EGTA (final concentrations.) Values are mean ± standard deviation of 4 determinations.

<table>
<thead>
<tr>
<th>Dialysis Buffer</th>
<th>Total 4-NPPase as % of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>23 ± 4</td>
</tr>
<tr>
<td>2 mM MgCl$_2$</td>
<td>100 ± 8</td>
</tr>
<tr>
<td>2 mM CaCl$_2$</td>
<td>115 ± 8</td>
</tr>
<tr>
<td>2 mM MnCl$_2$</td>
<td>73 ± 8</td>
</tr>
<tr>
<td>2 mM MgCl$_2$ + 0.1 mM EGTA</td>
<td>115 ± 8</td>
</tr>
</tbody>
</table>
Figure 4.1: Loss of 4-NPPase during gel filtration in the absence of added divalent cations. 2 ml samples were applied to a 98 x 1.6 cm column packed with Sephadex G-100 and eluted at approximately 10 ml/hour. 2 ml samples were collected and assayed for their ability to hydrolyse 4-NPP in the presence of 10 mM MgCl₂ as described in "Materials and Methods". (●) – Sample buffer and elution buffer were 100 mM Tris HCl, 20 mm Tris HCl, 0.5 mM DTT. (○) - Sample and elution buffer were modified by the addition of 2 mM MgCl₂, 0.1 mM CaCl₂. Activity is expressed as a percentage of the total activity present in the original sample.
were 92%, 57% and 44% respectively (Table 4.3). 1 mM zinc ions completely inhibited all 4-NPPase activity.

**Effect of calcium ions on 4-NPPase activity.**

The 4-NPPase was initially described as requiring millimolar magnesium for maximal activity (Romero-Saravia & Hamdorf, 1983; Trowell, 1985). Millimolar concentrations of calcium inhibited the magnesium-dependent activity (Trowell, 1985). Romero-Saravia and Hamdorf (1983) reported that neither Ca\(^{2+}\) nor Zn\(^{2+}\) in the 0.1-1.0 mM range substitute for Mg\(^{2+}\) in stimulating the enzyme. However buffers prepared by normal laboratory procedures are known to contain micromolar concentrations of Ca\(^{2+}\) ions and one would also expect tissue Ca\(^{2+}\) released during homogenization to contribute to this background. Consequently the calcium concentration was manipulated in the micromolar range by including the calcium-specific chelator EGTA.

Inclusion of 10 µM Ca\(^{2+}\) beyond that present in the control assay increased the magnesium-dependent activity at the expense of the magnesium-independent (Table 4.4). Addition of 0.2 mM EGTA had the opposite effect. EGTA also largely reversed the effect of added calcium when the two additions were combined.

These results suggest that a quantitative analysis of the calcium-dependency of the phosphatase should be performed in order to determine whether calcium is indeed switching the 4-NPPase between magnesium-requiring and magnesium-independent forms. It is also important to establish to what extent the phosphatase could be under calcium regulation *in vivo.*
Table 4.3: Effectiveness of divalent cations in substituting for magnesium in the stimulation of 4-NPPase activity of an S120 extract prepared from crab retina.

The divalent cations were all used as 1 mM solutions of their chlorides in the standard assay (see "Materials and Methods"), modified by the omission of L-cysteine. Values are the means and standard deviations of 4 determinations.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% of activity with MgCl₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>No addition</td>
<td>45 ± 4</td>
</tr>
<tr>
<td>1 mM MgCl₂</td>
<td>100 ± 7</td>
</tr>
<tr>
<td>1 mM MnCl₂</td>
<td>105 ± 4</td>
</tr>
<tr>
<td>1 mM NiCl₂</td>
<td>57 ± 3</td>
</tr>
<tr>
<td>1 mM CoCl₂</td>
<td>92 ± 5</td>
</tr>
<tr>
<td>1 mM ZnCl₂</td>
<td>none detectible</td>
</tr>
<tr>
<td>1 mM MgCl₂ + 20 mM L-cysteine</td>
<td>90 ± 7</td>
</tr>
</tbody>
</table>
Table 4.4: Experiment to test the effect of adding 10 µM calcium, alone or in combination with calmodulin, on the 4-NPPase activity of a filtered S120 extract of crab retina.

75 µl samples were incubated for 15 min. at 25 °C with 12 µg / ml of pig brain calmodulin in a pH 7.5 buffer containing 100 mM sucrose, 20 mM TrisHCl, 0.5 mM dithiothreitol with the stated additions. 675 µl of standard assay buffer containing appropriate levels of magnesium, calcium and EGTA were added and the assay was started by the addition of 75 µl of 20 mM 4-NPP. (For assay details see "Materials and Methods"). Values are means ± standard deviations of 4 determinations of the relative rates of 4-NPP hydrolysis.

<table>
<thead>
<tr>
<th>Treatment (final concentrations in assay)</th>
<th>Activity in the absence of added magnesium</th>
<th>Additional activity in the presence of 10 mM MgCl₂</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>No addition</td>
<td>0.22 ± 0.01</td>
<td>0.38 ± 0.02</td>
<td>0.60 ± 0.01</td>
</tr>
<tr>
<td>10 µM CaCl₂</td>
<td>0.17 ± 0.01</td>
<td>0.43 ± 0.01</td>
<td>0.60 ± 0.01</td>
</tr>
<tr>
<td>10 µM CaCl₂ + 0.2 mM EGTA</td>
<td>0.42 ± 0.02</td>
<td>0.19 ± 0.02</td>
<td>0.61 ± 0.01</td>
</tr>
<tr>
<td>10 µM CaCl₂ + 1.2 µg / ml calmodulin</td>
<td>0.19 ± 0.01</td>
<td>0.58 ± 0.02</td>
<td>0.77 ± 0.02</td>
</tr>
</tbody>
</table>
Stimulatory effects of calmodulin.

The phosphatase activity reported here resembles mammalian protein phosphatase 2B or 'calcineurin' in respect of its activity towards 4-nitrophenyl phosphate (Pallen & Wang, 1983); activation by magnesium (Li, 1984), although other investigators have reported little or no stimulation by magnesium; and requirement for μM calcium ions (Li, 1984; Pallen & Wang, 1984). The possibility arises that the microvillar 4-NPPase activity resides in a molecule that functionally resembles mammalian calcineurin. If so one might predict that calmodulin should stimulate the 4-NPPase activity (Li, 1984; Pallen & Wang, 1984). Not only do we know that calcium plays an important role in excitation and adaptation in the rhabdom, but de Couet et al., (1986) have recently shown that the microvilli of crab and squid are strongly immunopositive for calmodulin. Table 4.4 shows that calmodulin does stimulate magnesium-dependent p-NNPase activity in an S120 fraction of crab retina in a calcium-dependent fashion.

Unequivocal confirmation of the in vivo role of the 4-NPPase will require purified preparations of the enzyme. The following series of experiments was conducted to achieve this goal.

Retinal 4-NPPase from crab and squid have very similar properties.

*Leptograpsus* is a convenient species for cytochemistry because it is easily maintained in the laboratory. The ultrastructure and daily turnover cycle of photoreceptor membrane have been thoroughly investigated (Blest et al., 1980; Stowe, 1980a & 1980b; Stowe, 1983) and the biochemical characterization of the 4-NPPase was previously directly related to an unequivocal localization of the enzyme on photoreceptive membrane (Trowell, 1985). This is an important advantage because the evidence for intracellular distribution of cephalopod retinal 4-NPPase is
indirect. However the cephalopod retina is the preferred starting material for purification of the enzyme because insufficient retinal material is obtainable from the number of crabs that can reasonably be collected. In order to exclude the possibility that the 4-NPPase purified from squid is one that performs a different function from the crab enzyme, each step of the purification scheme was worked out in parallel on small samples of both the crab and squid enzyme. Thus the two are shown to have very similar physical properties besides having similar tissue distribution and biochemical properties (Romero-Saravia & Hamdorf, 1983; Trowell, 1985). This approach has the added advantage that it minimises the chances of selectively purifying a minor contaminating 4-NPPase activity through successive column fractionations. Magnesium-stimulated 4-NPPase was also found in the crab lamina. However, the specific activity in an S120 fraction prepared from the lamina was only 45% of that in the equivalent retinal fraction. It was concluded that even if contamination of the retinal with small fragments of lamina tissue were to occur it would not present a significant problem for purification procedures.

Ammonium sulphate precipitation.

In both species ammonium sulphate precipitation of the magnesium-dependent 4-NPPase from the filtered S120 extracts caused an almost complete loss of activity in the redissolved precipitate. This procedure was therefore avoided and for the multi-step purification of the 4-NPPase from squid retina a preliminary, low resolution, separation by gel filtration on Sephadex G-100 was used to reduce the protein loaded onto subsequent column steps as shown in Figure 4.6. Concentration of samples was achieved by ultrafiltration.
Hydrophobic interaction chromatography on phenyl sepharose.

Only one class of 4–NPPase activity was detected in the crab retinal S120 extract after fractionation on a phenyl-Sepharose column (Fig. 4.2a). The activity bound at 0.6 M ammonium sulphate and eluted in a broad peak around 0.2 M ammonium sulphate.

Under the same conditions two classes of 4–NPPase activity were detected in the S120 extract of squid retina (Fig. 4.2b). The first passed unretarded through the column in buffer C; this activity was found to bind at higher ammonium sulphate concentrations in batch test experiments. The second class of 4–NPPase activity bound in buffer C and was eluted between 0.5 and 0.55 M ammonium sulphate. The latter activity behaved identically to crab 4–NPPase during DEAE-cellulose column chromatography. It was established in batch test experiments that no further 4–NPPase activities could be eluted from the phenyl-Sepharose below an ammonium sulphate concentration of 0.2 M.

Native molecular weight estimation by gel filtration.

S 120 fractions were prepared from crab and squid retinae. Chromatography of this material on calibrated Sephadex columns (G-150 and G-100) yielded apparent molecular masses of 80 ± 5 kDa for the major crab 4–NPPase and 70 ± 5 kDa for the squid enzyme (Fig. 4.3).

Ion-exchange chromatography on DEAE-cellulose.

Both crab 4–NPPase and the more hydrophobic of the squid 4–NPPase activities bound to DEAE-cellulose only under relatively favourable conditions, i.e. low salt (20 mM NaCl, 20 mM Tris HCl, pH 7.5), and were eluted at salt concentrations between 20 and 80 mM NaCl. Step gradients gave a single skewed peak (Fig 4.4a.). Linear gradient elutions revealed a broader distribution of activity (Fig.s 4.4b & 4.8). The squid 4–NPPase of
Figure 4.2; Phenyl Sepharose chromatography of filtered S120 extracts prepared from the retinas of crab (a.) and squid (b.). 1. 10 ml samples, in buffer A, were made 0.6 M in ammonium sulphate by addition of the solid salt and were applied to a 10 x 1.6 cm column of phenyl Sepharose equilibrated in the same buffer containing 0.6 M ammonium sulphate. 2. The column was eluted with a linear gradient of 0.6 M to 0 M ammonium sulphate in the same buffer. 6 ml fractions were collected and assayed for 4-NPPase as described in "Materials and Methods". In the case of the squid no activity bound to, or eluted from, phenyl Sepharose below 0.2 M ammonium sulphate - not shown. (O) - 4-NPPase activity. (---) - salt concentration.
Figure 4.3: Estimation of molecular mass of the major retinal 4-NPPase activities of crab (o) and squid (Δ) by gel filtration on a calibrated column of Sephadex G-100. 2 ml samples of filtered S120 fractions were applied to a 98 x 1.6 cm column packed with Sephadex G-100 and eluted at approximately 10 ml/hour. 2 ml fractions were collected and assayed for enzyme activity and protein as described in "Materials and Methods". Standards were: bovine immunoglobulin G (m = 153 kDa); yeast alcohol dehydrogenase (m = 141 kDa); bovine transferrin (m = 76 kDa); bovine serum albumin (m = 69 kDa); human haemoglobin (m = 64.5 kDa); carbonic anhydrase ( m = 29 kDa); bovine alpha-chymotrypsinogen (m = 25 kDa); cytochrome C (m = 12.4 kDa). Ve = elution volume of the peak activity or protein fraction. Vo = void volume determined with dextran blue 2,000,000.
Figure 4.4: Behaviour of 4-NPPase of crab and squid during DEAE-cellulose chromatography. 10 ml of DEAE-cellulose was packed into a 20 ml plastic syringe and equilibrated with buffer D. 2 ml fractions were collected and assayed as described in the "Materials and Methods" section.

a.) Chromatography of a filtered S120 fraction of squid. 6 ml of a squid retinal extract was applied to the column in buffer D (1.) and the column was washed with 10 ml of the same buffer (2.). It was developed with 20 ml of buffer E containing 20 mM NaCl (3.) followed by 20 ml of buffer F containing 100 mM NaCl (4.) and finally 20 ml of buffer G containing 1M NaCl. It was established in separate experiments (not shown) that the peak of activity eluting in region 4 corresponds to the activity binding to phenyl-Sepharose in buffers that contain 0.6 M ammonium sulphate. Conversely the second peak of activity would not bind to phenyl Sepharose under these conditions.

b.) Chromatography of a filtered S120 fraction of crab. 10 ml of a crab retinal extract was applied to the column in buffer D (1.) and the column was washed with 20 ml of the same buffer (2.). The column was developed with 20 ml of a linear gradient of 20 mM to 120 mM NaCl in buffer D (3.). The final wash was buffer G containing 1M NaCl.
lower hydrophobicity bound more tightly to the DEAE-cellulose column and was eluted in the 1 M NaCl wash; its properties were not investigated further.

Native polyacrylamide gel electrophoresis.

Squid (purified through 4 column steps as described below) and crab 4-NPPase (purified by gel filtration and DEAE-cellulose chromatography) had relative mobilities of 0.51 ± 0.03 and 0.60 ± 0.03 (Fig.s 4.5a & b) in the non-denaturing PAGE system described in 'Materials and Methods'. In both cases these were the only regions of activity present on the gels. When crab S120 extract that had not been further purified was electrophoresed in the same system the major peak of activity migrated with a relative mobility of 0.63 ± 0.03 (Fig. 4.5c). The buffer conditions for assaying gel slices are similar to those that were originally used to demonstrate the microvillar enzyme cytochemically (Trowell, 1985). The electrophoretic profiles of the crab retinal extracts show that the purified 4-NPPase activity is the major activity originally present in the retina and, by inference, that it is the microvillar enzyme. The particular squid 4-NPPase species that was purified by the column chromatography steps migrated with a similar, though not identical, mobility to the crab enzyme in this system. This reinforces the conclusion that the two activities are similar in their physical properties.

Purification of the retinal 4-NPPase from squid.

Approximately half the activity in the crude extract was lost during the centrifugation steps (Table 4.5). There was no change in specific activity of the total 4-NPPase through these stages. This contrasts with almost total release of crab 4-NPPase into the supernatant and may indicate incomplete release of the rhabdomeric 4-NPPase from cephalopod
Table 4.5: Summary of the purification of a retinal 4-NPPase from squid.

<table>
<thead>
<tr>
<th>Purification stage</th>
<th>Specific activity (nmol Pi / min. / mg of protein.)</th>
<th>Purification factor</th>
<th>% yield</th>
<th>% of the total activity requiring magnesium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenisation</td>
<td>1.4</td>
<td>1</td>
<td>100</td>
<td>70</td>
</tr>
<tr>
<td>120,000 x g centrifugation</td>
<td>1.4</td>
<td>1</td>
<td>46</td>
<td>83</td>
</tr>
<tr>
<td>Preliminary Sephadex G-100 gel filtration</td>
<td>4.8</td>
<td>3</td>
<td>61</td>
<td>95</td>
</tr>
<tr>
<td>Phenyl Sepharose chromatography</td>
<td>8.9</td>
<td>6</td>
<td>11</td>
<td>98</td>
</tr>
<tr>
<td>2nd Sephadex G-100 gel filtration</td>
<td>2.8</td>
<td>20</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>DEAE-cellulose chromatography</td>
<td>11.2</td>
<td>80</td>
<td>3</td>
<td>20</td>
</tr>
</tbody>
</table>
Figure 4.5: Recovery of 4-NPPase from slices of non-denaturing polyacrylamide gels. Assays were performed at pH 9.0 in the presence of 10 mM MgCl$_2$ as described under "Materials and Methods".

a.) Electrophoresis profile of squid 4-NPPase after purification through four column steps as described in "Results".

b.) Electrophoresis profile of crab 4-NPPase after purification by gel filtration and DEAE-cellulose.

c.) Electrophoresis profile of crab 4-NPPase from a filtered S120 extract that was electrophoresed without further purification.
Partial Purification of 4-NPPase.

microvilli (Romero-Saravia & Hamdorf, 1983) or the presence of a large amount of a distinct 4-NPPase activity that is tightly associated with non-photoreceptive membrane.

No 4-NPPase activity was detected in the ultrafiltrate passing through an Amicon YM30 membrane, which places a lower limit on the molecular mass of all species with 4-NPPase activity of approximately 30 kDa.

Paradoxically, after preliminary purification by gel filtration (Fig. 4.6) there was an increase in yield relative to the sample loaded (Table 4.5). This was a reproducible observation associated with the first gel filtration step during purification. It suggests that the S120 fraction contains an inhibitory factor with a molecular mass distinct from the 4-NPPase although the low level of inhibition that was revealed may be caused by non-specific interactions.

The broad region of activity that eluted from the phenyl-Sepharose column at approximately 0.5 M ammonium sulphate (Fig. 4.7) was saved, concentrated to 9 ml and rechromatographed on Sephadex G-100 (Fig. 4.8). A single peak of activity was resolved from a major protein peak having a slightly lower molecular mass.

When the active fractions were pooled and fractionated on DEAE-cellulose (Fig. 4.9) the activity was eluted as a broad band of activity probably comprising two discrete major bands. Both of the major bands were eluted between 20 and 50 mM NaCl at constant pH. No significant further activity was eluted by a step change to 1M NaCl.

All the 4-NPPase activity eluting from DEAE-cellulose between 20 and 50 mM NaCl was combined and concentrated to approximately 3ml. After extensive dialysis versus buffer A this material displayed a single sharp peak of activity in a non-denaturing polyacrylamide gel electrophoresis
Figure 4.6; Purification of 4-NPPase from squid retinae. Sephadex G-100 gel filtration of the concentrated S120 extract of squid retinae. The sample was applied to the top of a 95 x 2.6 cm column of Sephadex G-100 in a volume of 40 ml. The column was eluted with buffer A at a flow rate of approximately 20 ml/hr. Fractions of approximately 6 ml were collected and assayed for protein (□) and 4-NPPase activity (●) as described in "Materials and Methods". The fractions indicated by the horizontal bar were pooled, made 0.65 M in ammonium sulphate and applied to a phenyl Sepharose column (Figure 4.7).
Figure 4.7; Purification of 4-NPPase from squid retinae. Phenyl Sepharose chromatography of the pooled active fractions recovered from a preliminary gel filtration step (Fig. 4.6). The sample, in buffer A, was made 0.65 M in ammonium sulphate and applied to a column of phenyl Sepharose (12 x 3.1 cm) pre-equilibrated in the equivalent buffer C (1.). The column was washed with 100 ml of buffer C (2.), followed by 400 ml of a linear gradient running from 0.65 M (buffer C) to 0 M (buffer A) ammonium sulphate (3.). 8 ml fractions were collected and assayed for protein (__) and 4-NPPase activity (o) as described in the "Materials and Methods" section. The fractions indicated by the horizontal bar were retained for further purification.
Figure 4.8: Purification of 4-NPPase from squid retina. Sephadex G-100 gel filtration of the pooled and concentrated fractions that were obtained from the phenyl Sepharose column of Figure 4.7. The sample was applied to the top of a 95 x 2.6 cm column of Sephadex G-100 in a volume of 9 ml. The column was eluted with buffer D at a flow rate of approximately 20 ml/hour. Fractions of approximately 6 ml were collected and assayed for protein (■) and 4-NPPase activity (○) as described under "Materials and Methods". The fractions indicated by the horizontal bar were used in the DEAE-cellulose column step.
Figure 4.9: Purification of 4-NPPase from squid retina. DEAE-cellulose column chromatography of the active fractions eluted from the second gel filtration column (Figure 4.8). The sample (34 ml) was applied to an 8 x 3.1 cm column of DEAE-cellulose that had been equilibrated with buffer D (1.). The column was washed with 85 ml of buffer E which contains 20 mM NaCl (2.) followed by 180 ml of a linear gradient of 20 mM NaCl (buffer E) to 100 mM NaCl (buffer F) (3.). Fractions of approximately 6 ml were collected and assayed for protein (___) and 4-NPPase activity (○) as described under "Materials and Methods". The fractions indicated by the horizontal bar were pooled and concentrated for non-denaturing gel electrophoresis as shown in Figure 4.5a.
system (Fig. 4.5a). The peak of activity had a mobility relative to bromophenol blue of $0.51 \pm 0.03$ with some tailing towards the origin.

SDS-PAGE (Fig. 4.10), in the second dimension, of a track cut from the non-denaturing gel resulted in 4 regions of protein staining (Brilliant Blue R250). Two minor bands with $m = 69$ kDa and 54 kDa were just visible to the naked eye whilst there was a major band at 35 kDa and a complex group of four major bands between $m = 26$ and 30 kDa. This pattern was observed reproducibly in material from two separate purifications and no additional staining was observed when the gel was destained and restained with an ultrasensitive silver stain (Wray et al., 1981). Of the four protein groups only the peak of the 35 kDa band corresponded to the region of 4-NPPase activity identified in the adjacent track. The tail of activity towards the origin was also compatible with the distribution of protein within the 35 kDa band. The 35 kDa band extended in the non-denaturing dimension to form a second spot connected to the anodic side of the activity. This is interpreted as being an inactive form of the same enzyme that exhibits a slightly higher mobility in the non-denaturing system.

The overall purification factor for squid 4-NPPase (Table 4.5) was approximately 100 fold in two independent runs. This must be read as an order of magnitude estimate only, because of possible factors such as the assay being unable to discriminate between three major forms of 4-NPPase activity only one of which is present in the final sample, or the removal of inhibitory components during purification. The values presented relate to total 4-NPPase activity measured in the presence of 10 mM magnesium chloride.

The value of the overall purification factor indicates that the squid 4-NPPase activity is associated with a major retinal protein. Consequently one might be able to identify the homologous polypeptide in
Figure 4.10; SDS-PAGE in the second dimension of purified squid retinal 4-NPP hydrolysing activity that had been separated by non-denaturing PAGE in the first dimension. The track adjacent to that shown in Figure 4.5a. was used in this experiment. Electrophoresis was performed in the buffer system of Laemmli (1970) using a 3% stacking gel and a 7.5-15% separating gel. The gel is stained for protein with Brilliant Blue R250.
crab by electrophoresis of the active fractions generated by a single column chromatography step. Figure 4.11 shows the electrophoretic profile of the region of activity eluted from a phenyl-Sepharose column. A polypeptide of 35 kDa was the only single observable band whose distribution matched that of the activity. An alternative but more complex possibility is that the crab 4–NPPase activity is associated with a polypeptide doublet comprising bands of slightly more than 45 kDa but that the activity is not exhibited by either polypeptide on its own. In either case a dimeric structure for the crab 4–NPPase seems likely.

Taken with the native molecular weight determinations this evidence supports a dimeric structure for the 4–NPPase in both crab and squid.

The 4–NPPase that was originally identified in crab and octopus required magnesium ions for maximum activity. The soluble 4–NPPase present in the squid S120 extract also behaves and purifies as a magnesium-requiring enzyme (Table 4.5). After elution from phenyl-Sepharose 98% of the 4–NPPase activity was magnesium-dependent but the activity recovered from the subsequent step had been converted to a form exhibiting complete magnesium independence. In this case 10 mM magnesium chloride actually inhibited the activity slightly (to 92% of the non-magnesium activity). There was slight recovery of magnesium stimulation during the ion-exchange step. This phenomenon was not due to the selective purification of an unrepresentative subset of the 4–NPPase because the activity was almost completely (98%) magnesium-dependent before this step; the loss in activity was low, as would indeed be expected from such a mild treatment as gel filtration; and the activity had already been fractionated once by gel filtration. The only possible conclusion is that transient exposure to 0.65 M ammonium sulphate in the presence of magnesium changes the conformation of the 4–NPPase so as to eliminate the requirement for added magnesium.
Figure 4.11: Comparison of protein band distribution with the 4-NPPase activity profile in fractions of a crab retinal extract purified on phenyl Sepharose. a.) Shows the distribution of activity in the peak fractions whilst b.) is an SDS polyacrylamide gel electrophoretogram of the same fractions. Column chromatography was performed and the fractions were assayed as previously described. Fractions eluting around the peak of activity were dialysed extensively against distilled water, lyophilised, taken up in sample buffer and subject to electrophoresis according to Laemmli (1970). One half of the total protein present in each fraction was loaded onto the gel. The gel was silver stained by Wray et al.'s method (1981).
4.0 Discussion

Divalent cation-dependent 4-NPase activity is a characteristic of alkaline phosphatase (2.4.1) but it is also intimately associated with RTase and ATPase activities. Ultimately, a definitive assignment requires that the enzyme be obtained in a purified form and used to reconstitute the catalytic function. The present study describes a procedure by which this can be achieved.
4.5 Discussion

Divalent cation dependent 4-NPPase activity is a characteristic of alkaline phosphatases (E.C. 3.1.3.1) but it is also intimately associated with a number of vertebrate phosphoprotein phosphatases. The initial description of a rhabdomeric 4-NPPase was in terms of an "alkaline phosphatase" (Romero-Saravia & Hamdorf, 1983). In the broader sense of this term its use is indisputable. Both in octopus and crab the enzymes show an optimum pH above neutrality. Conventional "alkaline phosphatases" (Fernley, 1971) are usually characterized by their close association with microvillar membranes specialized for transport, such as those of intestinal brush border and placenta, or tissues that are involved in calcification, notably bone and periodontal tissue. In this case the microvillar analogy may be misleading because there is no evidence that rhabdomeral microvilli are involved in transport. In fact the data of Romero-Saravia and Hamdorf (1983) does not support their conclusion that the enzyme is one of a "tightly defined class of alkaline phosphatases". Far from 4-NPP being a specific substrate, conventional alkaline phosphatases show no selectivity for 4-NPP over β-glycerophosphate or 5'-AMP (Fernley, 1971). Despite these quibbles Romero-Saravia & Hamdorf (1983) suggested that the function of the 4-NPPase might well be the dephosphorylation of rhodopsin.

It is easy enough to eliminate potential functions of the rhabdomeral 4-NPPase in an ad hoc way, as is demonstrated here for GTPase and ATPase activities. Ultimately a definitive assignment requires that the enzyme be obtained in a purified form and used to reconstitute the putative function. The present study describes a procedure by which this can be achieved.
The several similarities between the retinal 4-NPPase activities of crab and squid suggests that they may well be derived from an enzyme with a common function in both species. The arguments rehearsed previously (Romero-Saravia & Hamdorf, 1983; Trowell, 1985) would suggest that this function may be the dephosphorylation of rhodopsin following its light-dependent phosphorylation by rhodopsin kinase. It will be possible to test this hypothesis using the enriched preparation of the squid 4-NPPase now available.

The properties of the 4-NPPase in some respects resemble those of the 4-NPPase associated with the vertebrate phosphoprotein phosphatase 2B or "calcineurin". The similarities extend to its stimulation by transition metal ions, micromolar levels of calcium and calmodulin. The phenomenon of the divalent cation requirement of 4-NPPase activity being switched from one stable state to another has also been observed with calcineurin, in that case following exposure to transition metal ions (Gupta et al., 1986), and may perhaps underlie conflicting observations as to the magnesium-dependency of that enzyme (Li, 1984).

Despite these similarities the polypeptide composition of the molecule is quite distinct from that of calcineurin. The calcium and calmodulin-dependency is particularly intriguing because calcium is believed to play an important role in photoreceptor excitation and adaptation yet the pathways by which calcium exerts its effects remain largely unknown (Kelleher & Johnson, 1986). Calmodulin, which is capable of linking control of 4-NPPase activity to light-driven changes in photoreceptor calcium concentration, has been demonstrated in abundance in the rhabdoms of the crab and squid species used in the present study (de Couet et al., 1986). However it is not yet possible to say what levels of calcium are required or if this pathway is important in the intact photoreceptor.
Neither the 4–NPPase from squid nor the crab enzyme (in small scale tests) is purified as a single sharp peak in the phenyl-sepharose or DEAE-cellulose column steps. Because this was a reproducible observation for both species and on columns of different scales it is unlikely to have been the result of poor column performance and rather represents true heterogeneity of the 4–NPPase. That such heterogeneity is liable to occur is borne out by two observations: the presence of an enzymically inactive form of the 35 kDa polypeptide revealed in the electrophoresis steps, and the switch from a magnesium-requiring form to one that does not require magnesium during the second gel filtration step. As a matter of routine during purification of the squid 4–NPPase, the whole of the broad region of activity was retained for further purification in order to minimise the possibility of purifying an unrepresentative subsample of the activity originally present. This policy was vindicated by the reproducible observation of only a single band of activity after the native PAGE step and the association of only a single polypeptide species with this activity as shown by SDS-PAGE. Whether the heterogeneity revealed is present in vivo or is merely a manifestation of the instability of the 4–NPPase is unknown.

The initial cytochemical identification of the crab 4–NPPase indicated some form of membrane association but the activity was released into the supernatant on homogenization of the retina. Romero-Saravia and Hamdorf (1983) also concluded that the 4–NPPase of octopus is membrane-bound but because they used a vesicle preparation it is not clear just how tight this association is. Saibil & Michel-Villaz (1984) reported finding 4–NPPase almost exclusively in the supernatant after sedimentation of squid photoreceptor membranes. Here it is shown that, in another species of squid, retinal 4–NPPase activity may be at least partly released into the supernatant by homogenization. Whether these
are species differences or are caused by different treatments cannot be
determined because the octopus species used in the initial study was not
available locally.
5. **LIGHT-DEPENDENT PHOSPHORYLATION OF SOME RETINAL PROTEINS OF CRAB AND SQUID**

5.1 **SUMMARY**

1. Crab retinal membranes were phosphorylated by an *in situ* labelling procedure. The phospholipid region and a 48 kDa protein were labelled in the dark. Incorporation into both regions was markedly enhanced by illumination.

2. Phosphorylation of a broad band of molecular weight 33-39 kDa, believed to represent opsin, was only achieved when labelling conditions were optimised. The incorporation into this region was almost entirely light-dependent.

3. An enriched photoreceptor membrane fraction from squid was prepared after *in vitro* labelling of the membranes using *γ*-32P-ATP. The pattern of protein phosphorylation in this preparation was complex and very similar in the light and dark.

4. Only the major protein band in these membranes displayed light-dependent incorporation of radioactivity. The molecular weight range of this protein, 47-51 kDa, is characteristic of squid opsin.

5. Soluble protein fractions derived from crab retina were ineffective in promoting dephosphorylation of the phosphoproteins of squid photoreceptive membrane.

6. The 48 kDa phosphoprotein of crab retina was partially dephosphorylated in the presence of a crude soluble extract of crab retina. This dephosphorylation was markedly stimulated by 10 mM MgCl₂.
5.2 Introduction

The hypothesis that rhodopsin is dephosphorylated in invertebrate photoreceptors by the enzyme that exhibits 4–NPPase activity seems a useful one. The dephosphorylation of rhodopsin can occur rapidly in the photoreceptors of at least one invertebrate species (Paulsen & Bentrop, 1984). Two invertebrate phyla include species which have a high level of 4–NPPase activity in close association with their photoreceptive membrane (Romero-Saravia & Hamdorf, 1983; Trowell, 1985) and it is a well established fact that phosphoprotein phosphatases from many sources are associated with divalent cation-stimulated 4–NPPase activities; as was discussed in Chapters 3 and 4.

The most valid way to test this hypothesis is to attempt the in vitro dephosphorylation of phosphorylated rhodopsin using a purified extract of the 4–NPPase. The use of substrates such as phosvitin or phosphorylated histones would be a poor test of the hypothesis; it is known that rhodopsin kinase is unusually specific for its substrate and will not phosphorylate these artificial substrates (Weller et al., 1975; Shichi & Somers, 1978). Whilst phosphoprotein phosphatases generally exhibit less substrate specificity than the corresponding kinases (Krebs & Beavo, 1979), the rhodopsin phosphatase of blowfly seems to discriminate precisely between phosphorylated rhodopsin and metarhodopsin (Paulsen & Bentrop, 1984). Only the former is significantly dephosphorylated in vivo.

The partial purifications of the retinal 4–NPPases of crab and squid were detailed in Chapter 4. The other prerequisite for performing reconstitution experiments is the availability of $^{32}$P–phosphorylated rhodopsin in sufficient quantity. Two routes were taken to achieve this goal:
1. Retinal proteins were phosphorylated in situ by incubating isolated crab retinae with $^{32}$Pi following the protocol of Paulsen & Bentrop (1984).

2. A crude homogenate of squid retina was incubated with $\gamma^{32}$P-ATP in vitro along the lines followed by Vandenberg & Montal (1984b).

Each approach has its advantages and disadvantages; the in situ method probably reflects the in vivo phosphorylation pattern more closely but it proved unsuitable for the preparation of substrate quantities of $^{32}$P-rhodopsin. Live squid retina was not available.

The S120 fraction of crab retina was tested for its ability to dephosphorylate squid rhodopsin as well as some other retinal phosphopeptides of crab. A preliminary attempt has been made to obtain copurification of rhodopsin phosphatase with 4-NPPase during column chromatography of crab S120 fraction.

### 5.3 Materials and Methods

**Crabs and Squid.**

*Leptograpsus variegatus* were obtained and kept as described in Chapter 2. *Sepioteuthis australis* eyeballs were prepared and frozen as described in Chapter 4.

**Materials.**

Carrier-free $^{32}$P orthophosphate and adenosine 5'-$[\gamma^{32}$P]triphosphate, triethylammonium salt ($\gamma^{32}$P-ATP, specific activity = 3,000 Ci/mmol), were obtained from Amersham. Other chemicals were obtained from the sources stated in sections 2.3, 3.3, and 4.3.
In situ phosphorylation of crab retina

Based on the protocol of Paulsen & Bentrop (1984).

Crabs were dark-adapted for a minimum of two hours. Subsequent procedures were performed in the dark or under dim red illumination (λ > 630 nm). Retinae were dissected under dim red light at room temperature in a bath of the following medium: 115 mM NaCl, 50 mM HEPES, 5 mM glucose, 2 mM KCl, 2 mM MgCl₂, 2 mM CaCl₂, pH 6.8, saturated with carbygen (95% O₂/5% CO₂). The eyecups were bisected and groups of half retinae were distributed amongst several vials. 50 µl per retina of the same medium plus carrier free ³²P-orthophosphate, at an activity concentration of 250 µCi ml⁻¹, was added to each vial. The retinae were incubated in the dark at room temperature for 15 minutes. After this they were illuminated using a 50 W fluorescent lamp at a distance of 50 cm. In some cases the light from the lamp was filtered through blue (λ_max = 400 nm, half-height bandwidth = 60 nm) or orange (Schott OG 570) absorbance filters. Control vials were protected from the light with aluminium foil.

At the end of this time the retinal samples were rapidly washed three times with ice-cold distilled water. The retina was separated from the cornea and where applicable the lamina was teased away from the retina. The retinal samples were either disrupted immediately in SDS-sample buffer (Laemmli, 1970) and stored frozen until electrophoresis or they were homogenized in ice-cold 20 mM Tris HCl, 0.5 mM DTT, pH 7.5 in order to investigate the phosphorylation of the proteins specifically of the particulate fraction. The homogenate was centrifuged for 60 minutes at 120,000 x g at 4 °C. The pellet was saved and resuspended in ice-cold distilled water and centrifuged again. The washing and centrifugation was repeated. The pellets were saved and either stored frozen at −20 °C prior to their use in dephosphorylation experiments or they were
resuspended in SDS sample buffer. Samples containing rhodopsin were never heated prior to electrophoresis. The same route was used to prepare lamina tissue for electrophoresis and autoradiography in experiments which compared the phosphorylation of proteins in the lamina with those in the retina.

In an attempt to increase the level of $^{32}$Pi incorporation into retinal polypeptides the procedure was modified in the following respects. The dissection and incubation buffer was changed to: 0.42 M NaCl, 50 mM HEPES, 47 mM MgCl$_2$, 35 mM CaCl$_2$, 12 mM KCl, 5 mM glucose, pH 6.8 to make it more physiological (Wiersma, 1961). The activity concentration remained unchanged at 250 µCi ml$^{-1}$ but the volume of incubation buffer per retina was increased to 200 µl. Incubation in the dark was extended to 30 minutes. The intensity of the illumination was increased by replacing the 50 W fluorescent bulb with a 150 W Phi lips Comptalux photographic lamp filtered through 10 cm of water (as a heat filter). The initial homogenization of tissue was performed in ice-cold 50 mM sodium fluoride, 10 mM EGTA, 10 mM EDTA but ice-cold distilled water was retained for the subsequent washes.

**Dephosphorylation of the 48 kDa protein of crab**

Phosphorylated crab retinal membranes were prepared according to the first protocol. The phosphorylated 120,000 x g pellet was resuspended in 150 µl of double strength 4–NPPase assay buffer and divided into three 50 µl aliquots which were adjusted by the addition of 2 µl of the appropriate stock solution to contain the following: (a) no addition, (b) 5 mM EDTA, (c) 10 mM MgCl$_2$. To start the reaction 50 µl of an S120 extract of crab retina in standard homogenization buffer was added to each of the membrane aliquots and the mixture was incubated at 25 °C for 60 minutes. The reaction was terminated by the addition of 6.5 ml of ice-
cold 5 mM EDTA and the membranes were collected by spinning at 100,000 x g for one hour. The pellets were subjected to SDS-PAGE and the gel was dried down and autoradiographed. The density of labelling in the 48 kDa band was estimated by laser-densitometry of a lightly-exposed autoradiograph. Three separate scans were performed on each track; each scan was traced and photographically enlarged onto Ilfospeed paper. The areas under the 48 kDa peaks were compared by cutting out and weighing the peaks.

*In vitro* phosphorylation of squid retinal membranes.

Based on the protocol of Vandenberg & Montal (1984b).

The following procedures were all performed in the dark or under dim red illumination except where it is stated otherwise. Eyeballs that had been prepared under normal illumination and frozen at -70 °C, as described in section 4.3, were thawed and disrupted in the following ice-cold buffer: 56 mM TrisHCl, 11 mM EGTA, 2.2 mM DTT, 0.11 mM ouabain, 0.11 mM PMSF, 0.11 mM Ep 475, pH 8.0 at a ratio of 2-3 eyeballs per ml. The retinal suspension was filtered through a 1 mm plastic mesh and the filtrate was homogenized thoroughly using a teflon-on-glass pestle spinning at approximately 1,000 r.p.m. The homogenate was centrifuged at approximately 1,000 x g for 5 minutes at room temperature. The milky supernatant was collected, made 20 mM in MgCl₂ and divided into two portions. Each portion was made 4 mM in γ-³²P-ATP (approximately 25 mCi mmol⁻¹) by the addition of one tenth volume of a 10 x stock solution. Final concentrations in the incubation medium were: 50 mM Tris HCl, 18 mM MgCl₂, 10 mM EGTA, 4 mM γ-³²P-ATP, 2 mM DTT, 0.1 mM ouabain, 0.1 mM PMSF, 0.1 mM Ep 475, pH 8.0. The medium also contained 5% ethanol which originated from the γ-³²P-ATP stock solution.
The tubes were illuminated with a 150 W Philips Comptalux photographic lamp for 30 minutes at 25 °C using the same heat filter as for the crab phosphorylation. A control sample was protected from the light using aluminium foil. The light was turned off and one fifth volume of 1 M sodium fluoride, followed by 5 ml of ice-cold distilled water, was added to each sample. The samples were loaded onto 0.5 ml sucrose cushions and centrifuged for 60 minutes at 120,000 x g. The material at the sucrose interface was aspirated off and loaded onto 20-50% (w/v) continuous sucrose density gradients (Luthe, 1983) constructed in 50 mM Tris HCl buffer pH 7.5. Gradients were spun at 120,000 x g for 90 minutes. The diffuse pink band at approximately 35% sucrose was aspirated off, diluted in distilled water and sedimented at 120,000 x g for 60 minutes. The bright cherry-red pellets were prepared for electrophoresis or stored for use in dephosphorylation experiments as was outlined in the previous section.

**Attempted dephosphorylation of squid photoreceptor membrane.**

$^{32}$P-labelled squid photoreceptor membranes, that had been prepared as in the previous section and stored frozen, were homogenized in a small volume of distilled water. 50 µl aliquots of this membrane suspension were placed into airfuge tubes and the divalent cation composition was adjusted by the addition of 2 µl of the appropriate stock solution. 150 µl of one of the following was added to start the reaction:

1. Crab S120 fraction in standard dissection buffer with no divalent cations.
2. Buffer only.
3. One of the 4-NPPase containing fractions generated by phenyl-Sepharose column chromatography of the crab S120 fraction. These
fractions were made 50 mM in Tris HCl, pH 7.5 prior to their use in this experiment.

The samples were incubated with shaking at 25 °C for 30 minutes. The incubation was terminated by rapidly centrifuging the suspension at approximately 100,000 x g in a Beckman airfuge. The membrane pellets were twice resuspended in distilled water and sedimented before being prepared for SDS-PAGE.

**Gel autoradiography.**

After staining and photography, polyacrylamide gels were impregnated with a solution containing 40% (v/v) methanol, 10% (v/v) acetic acid and 3% (v/v) glycerol for at least 3 hours. They were dried onto 3 MM paper in a Biorad gel drier at 80 °C. The dried-down gel was covered with Cling Wrap™ and was subjected to autoradiography for up to one week at −70 °C using Kodak X-OMAT RP-5 film with a Cronex Lightning-plus CH intensifying screen.

Other methods have been described in the relevant sections of Chapters 2, 3 and 4.

**5.4 Results**

The pattern of protein phosphorylation revealed by *in situ* labelling of crab retinae.

The pattern of phosphorylation induced by illumination of 32Pi-loaded crab retinae was examined by autoradiography of total retinal proteins after SDS-PAGE (Fig. 5.1):

1. In dark-incubated control retinae there was a single weakly phosphorylated band with an apparent molecular weight of 48 kDa, as determined by reference to molecular weight markers.
Figure 5.1: The effect of illumination on retinal protein phosphorylation; SDS-PAGE and autoradiography of whole and particulate fractions of crab retina and of whole crab lamina.

Crab half-eye-cups were incubated in the presence of $^{32}\text{Pi}$ for 15 min. in the dark. The tissues were either maintained in the dark for a further 15 min. or illuminated (50 W fluorescent tube, 50 cm) for 15 min. The tissues were washed and the retinas and laminae were dissected apart and processed for SDS-PAGE, either immediately or following preparation of a particulate fraction. In this experiment only, 2 mg/ml bovine serum albumin (BSA) was included in the first centrifugation step during preparation of the particulate fraction. See section 5.3 for further details.

a. Proteins as revealed by Coomassie Brilliant Blue staining of the gel.

b. Autoradiograph of the gel tracks shown in a.

Molecular weight values are expressed in kDa.

Track no.

1. Particulate fraction of retina. DARK
2. Particulate fraction of retina. LIGHT
3. Whole retina. DARK
4. Whole lamina. DARK
5. Whole retina. LIGHT
6. Whole lamina. LIGHT

Note that more protein is loaded onto those tracks which are derived from dark-incubated tissues.
a. BSA

b.
2. Light-incubated retinae showed strongly enhanced phosphorylation of the 48 kDa band as well as a highly phosphorylated diffuse band migrating ahead of the dye front in the region where phospholipids are found (Vandenberg & Montal, 1984b). Using the original phosphate incorporation protocol no other phosphorylated bands were visible in autoradiograms of whole retina.

Autoradiography of the particulate and membrane fraction of crab retina revealed a similar pattern of phosphorylations:
1. In the dark the 48 kDa band was barely visible whilst a band at the top of the gel representing material that did not enter the gel was also visible.
2. Illumination caused significant labelling of the phospholipid region and greatly increased phosphorylation of the 48 kDa band. A weakly radioactive band was also visible in the region between 14 kDa and the dye front.

Clearly, under these experimental conditions, inorganic phosphate enters the cells and is incorporated into nucleotide triphosphates. Early events of the phototransductive process must also have been preserved since the phosphorylation of protein and phospholipid was light-stimulated. In order to eliminate the possibility that some of the phosphorylations observed were due to the contamination of the retina with neural material from the lamina the pattern of phosphorylation in this tissue was also investigated. As shown in Figure 5.1 both the 48 kDa protein and the phospholipid region are light-dependently phosphorylated in the lamina but at a level not significantly greater, and probably lower, than in the retina. The 48 kDa protein is present in both retinal and other neural tissue and it may be homologous to the 49 kDa phosphoprotein identified by Pak's group in *Drosophila* (Matsumoto & Pak, 1984) since, contrary to the initial report, this protein is found in the brain as well as the retina of *Drosophila* (T. Tanimura personal communication). The
48 kDa protein was easily resolved from actin by autoradiography of a nitrocellulose blot that had been probed with a commercial anti-actin antibody (unpublished results of H.G. de Couet & S.C. Trowell.)

The nature of the retinal protein phosphorylations induced by either blue or orange light were identical (not shown) and indistinguishable from that caused by white light. This result is expected given the coincidence of the peak absorption wavelengths of the rhodopsin and metarhodopsin in *Leptograpsus* (Briggs, 1961).

Throughout a number of experiments, using the original labelling conditions, no phosphorylated band with the characteristics of rhodopsin was ever observed. This may have been due to the experimental conditions employed; neither the total retinal protein nor the membrane and particulate fraction of retina contained enough rhodopsin for a Coomassie-stained rhodopsin band to be visible after SDS-PAGE (Fig. 5.1). The specific activity of $^{32}$P-nucleotide triphosphates within the photoreceptor could have been too low; or the level of illumination used may have been insufficient to allow rhodopsin phosphorylation to reach a detectable level.

Conceivably the activity of rhodopsin phosphatase may be sufficiently high to dephosphorylate rhodopsin as rapidly as it is phosphorylated under these experimental conditions. Without a specific inhibitor of the phosphatase it will not be possible to test this possibility because of the coincidence of the absorption maxima of *Leptograpsus* rhodopsin and metarhodopsin (Briggs, 1961). It is also possible that rhodopsin phosphorylation does not occur at all in this species; which would make *Leptograpsus* unique amongst those organisms in which photoreceptor protein phosphorylation has been investigated. It was important to attempt to eliminate this explanation because if it were true the 4-NPPase could not possibly represent a rhodopsin phosphatase.
The phosphorylation of membrane and particulate fraction proteins was re-examined using the modified procedure described in the Methods section. Autoradiography of the dried-down gel for 18 hours showed light-stimulated phosphorylation of both the 48 kDa band and the phospholipid region. Other bands were barely distinguishable above background and their labelling was not influenced by light. After autoradiography for 5 days (Fig. 5.2) a large number of radioactive bands became apparent. Light did not influence the phosphorylation of any distinct bands, other than the 48 kDa protein, but a diffuse region lying between 33 and 39 kDa was more heavily labelled in the illuminated sample than in the dark control. The presence of 166 mM sodium fluoride (free concentration = 2 mM) during the period of illumination reduced the overall extent of phosphorylation in a non-selective way. These results do not prove that rhodopsin is phosphorylated in *Leptograpsus* because the identity of the 33-39 kDa band could not be independently determined. However it seems highly probable that the diffuse band represents phosphorylated rhodopsin because it has the following characteristics of that molecule: the band migrates as a diffuse region during SDS PAGE, it is phosphorylated in a light-dependent manner and its molecular weight falls into the range that has been determined for a number of other arthropod rhodopsins (reviewed in Hillman *et al.*, 1983; *Calliphora*... Paulsen & Bentrop, 1984; *Cherax* ... de Couet & Sigmund, 1985).

Given the low level of rhodopsin labelling achieved and the problems inherent in the purification of elongated, fused rhabdoms of the *Leptograpsus* type, an alternative source of phosphorylated rhodopsin was required. Frozen squid retinae were a readily available source of rhodopsin-bearing membrane. A membrane fraction that was significantly enriched in rhodopsin was obtained using the simple sucrose density gradient purification scheme that is described in section
Figure 5.2: Enhanced phosphorylation of retinal proteins; SDS-PAGE and autoradiography of proteins of the particulate fraction of crab retina.

Crab half-retinae were incubated in the presence of $^{32}$Pi for 30 min. in the dark according to the modified procedure described in section 5.3. They were illuminated (150 W Contralux lamp, 10 cm) for 15 min. Control half-retinae were maintained in the dark throughout this period. The particulate fraction was subjected to SDS-PAGE. See section 5.3 for further details.

a. Proteins as revealed by Coomassie Brilliant Blue staining of the gel.

b. Autoradiograph of the gel tracks shown in a.

Molecular weight values are expressed in kDa.

Track no.

1. Particulate fraction of crab retina. DARK
2. Particulate fraction of crab retina. LIGHT
5.2. The major protein in these preparations was a broad band between 47 and 61 kDa. The major band could be seen on the basis of the charge and the nature of the band; the endogenous rhodopsin band is identical to that of the rodopsin and the fact that it was phosphorylated in a molecular weight of 50 kDa. The preparation was believed to be different before and after phosphorylation of the membranes using the details of the separation bands (Fig. 5.3) but only after separation by SDS-PAGE and fibroblasts (Fig. 5.3) and that were phosphorylated and then phosphorylation. In accordance with the extent of the protein that appeared to be selected prior to the purification of the rhodopsin.

Effect of the 4-NPPase assay on rhodopsin.

An 6120 fraction of squid photoreceptor was incubated with a crab 6120 fraction, in the presence of magnesium and calcium, and found that of control membranes, which were incubated in the absence of crab 4-NPPase activity under the same buffer conditions. The 4-NPPase
5.3. The major protein in these preparations forms a broad band between 47 and 51 kDa (Figure 5.3). This protein was assigned as opsin on the basis of the cherry-red colour of the membranes; the diffuse nature of the band; the molecular weight range of the protein which is identical to that of the rhodopsins of squid from other genera (Nashima et al., 1979); and the fact that it was the only protein in these preparations that could be phosphorylated in a light-dependent manner. A second major band with a molecular weight just below 100 kDa was sometimes present and is believed to be due to rhodopsin dimerization. Phosphorylation of the membranes using endogenous kinases and γ-32P-ATP was attempted both before and after membrane purification using the protocol detailed in section 5.3. Autoradiography of the membrane proteins after separation by SDS-PAGE revealed a number of phosphorylated protein bands (Fig. 5.3) but only the 47-51 kDa protein exhibited light-dependent phosphorylation. The phospholipid region was also very heavily labelled.

In accordance with Vandenberg & Montal’s results (1984b) the extent of opsin phosphorylation was relatively low in membranes that were phosphorylated after they had been gradient-purified (Fig. 5.3) and appeared to be selectively enhanced when phosphorylation was performed prior to the purification (Fig. 5.4).

Effect of the 4–NPPase on phosphorylated retinal membranes of squid and crab.

An S120 fraction from crab was tested for its ability to dephosphorylate squid photoreceptor membranes (Fig. 5.4). No difference could be detected between the phosphorylation profile of membranes that were incubated with a crab S120 fraction, in the presence of magnesium and calcium, and that of control membranes which were incubated in the absence of crab 4–NPPase activity under the same buffer conditions. The 4–NPPase-
Figure 5.3: Effect of illumination on the phosphorylation of squid photoreceptor membranes; SDS-PAGE and autoradiography of proteins of squid rhodopsin-bearing membrane.

Squid rhodopsin-enriched membranes were prepared according to the protocol described in section 5.3 prior to incubation with 4 mM $^{32}$P-$\gamma$-ATP (25 Ci/mol) for 30 min. in the light (150 W Contralux lamp, 10 cm) or dark. The membranes were washed and subjected to SDS-PAGE. See section 5.3 for further details.

a. Proteins as revealed by Coomassie Brilliant Blue staining of the gel.

b. Autoradiograph of the gel tracks shown in a.

Molecular weight values are expressed in kDa.

Track no.

1...............................Squid photoreceptor membranes. DARK
2...............................Squid photoreceptor membranes. LIGHT
Figure 5.4: Attempted dephosphorylation of squid photoreceptor-membrane phosphoproteins; SDS-PAGE and autoradiography of the proteins of squid rhodopsin-bearing membrane.

Rhodopsin-bearing membranes were prepared following light-driven $^{32}$P-phosphorylation of a crude homogenate of squid retina. The protocol was exactly as described in section 5.3.

The washed, phosphorylated membranes were incubated for 30 min. with soluble extracts prepared from crab retina under normal laboratory illumination.

a. Proteins as revealed by Coomassie Brilliant Blue staining of the gel.

b. Autoradiograph of the gel tracks shown in a.

Molecular weight values are expressed in kDa.

All tracks were loaded with the same amount of $^{32}$P-labelled squid photoreceptor membranes. The incubation conditions for each track were 100 mM sucrose, 20 mM TrisHCl, 0.5 mM DTT, pH 7.5 with the following modifications:

1. 10 mM MgCl$_2$, 0.1 mM CaCl$_2$.

2. An S120 fraction of crab retina in the presence of 5 mM EDTA, 5mM EGTA.

3. An S120 fraction of crab retina in the presence of 10 mM MgCl$_2$, 0.1 mM CaCl$_2$.

4.-6. Three fractions which eluted from a phenyl-Sepharose column in the 4–NPPase peak. 10 mM MgCl$_2$, 0.1 mM CaCl$_2$. 
Light-Dependent Phosphorylation.

containing fractions that eluted from a phenyl-Sepharose column were also unable to dephosphorylate squid photoreceptor membrane proteins.

These results lead to one of the following conclusions:

1. The 4-NPPase activity is not a phosphoprotein phosphatase.
2. There are sufficient species differences between the photoreceptor proteins and in particular the rhodopsins of squid and crab to prevent cross-reactivity of the putative phosphatase.
3. The procedures used to enrich the photoreceptive membrane and 4-NPPase fractions result in the loss of the conditions necessary for expression of the phosphoprotein phosphatase activity.

A number of additional experiments will be required to distinguish amongst these possibilities. As a preliminary to this work the ability of the crab S120 fraction to dephosphorylate the 48 kDa phosphoprotein of crab retina was investigated.

After incubation of the membrane and particulate fraction of crab retina with the crab S120 fraction (Fig. 5.5) $^{32}$P-incorporation into the 48 kDa protein was reduced by 14% with respect to the level found in control membranes. Inclusion of 10 mM magnesium with the S120 fraction caused a total reduction of 52% in the labelling with respect to the control membranes. These results confirm that there is a magnesium-stimulated phosphoprotein phosphatase in the crab S120 fraction even though the same fraction showed no phosphoprotein phosphatase activity against squid photoreceptor membranes. They say nothing about whether the phosphoprotein phosphatase is the same as the 4-NPPase, nor do they bear on the in vivo substrate specificity of the phosphoprotein phosphatase.
Figure 5.5: Partial dephosphorylation of the 48 kDa phosphoprotein of crab retina using an S120 extract of crab retina; Brilliant Blue-stained SDS-PAGE of the retinal particulate fraction prior to autoradiography.

Crab retinae were $^{32}$P-phosphorylated in the light as described in Figure 5.1. The particulate fraction was isolated and three aliquots were incubated in a 50 mM Tris HCl, pH 7.5 buffer for 1 hr at 25 °C under the following conditions:

1. No addition.
2. S120 fraction prepared from crab retina + 5 mM EDTA
3. S120 fraction prepared from crab retina + 10 mM MgCl$_2$.

The membranes were washed with 5 mM EDTA and prepared for electrophoresis. See section 5.3 for further details. Percentage figures indicate the relative density of labelling in the 48 kDa phosphoprotein as determined by laser densitometry.

Molecular weight values are expressed in kDa.
The results presented in this chapter strongly suggest that the rhodopsin photoreceptor absorbs light into the underlying phosphorescent material. The red light found these two proteins in the rhodopsin phosphorescent and was shown to reduce the protein by 1%.

The similar peak absorbance wavelength of Lentigo puncta rhodopsin and metarhodopsin precedes experimental separation of the phosphorylation and dephosphorylation process. It is possible to examine the underlying neural interface.

Calphor (Paulson & Bentrop, 1984). It has also been impossible to
5.5 Discussion

The results presented in this chapter strongly suggest that the rhodopsins of *Leptograpsus variegatus* and *Sepioteuthis australis* fall into the same range as other members of the decapod crustacea (Winterhager & Stieve, 1982; de Couet *et al.*, 1984) and the cephalopod molluscs (Nashima *et al.*, 1979; Saibil, 1982) respectively.

The 48 kDa protein of crab retina is labelled strongly under conditions which leave other retinal proteins unlabelled. Matsumoto & Pak (1984) found that the 49 kDa phosphoprotein of *Drosophila* retina was phosphorylated very rapidly *in vivo* following a 1 msec strobe flash. The two proteins may be homologous. Matsumoto & Pak (op. cit.) claimed that the protein was located exclusively in the photoreceptor layer and was absent from *Drosophila* lamina on the basis of Coomassie staining of two dimensional SDS-PAGE, but they could easily have missed small amounts of highly phosphorylated 49 kDa protein. Their conclusion was that the 49 kDa was: "probably part of the machinery underlying photoreceptor mechanisms." Since a 48 kDa protein was also the most prominent light-dependently phosphorylated band in crab lamina (which is easily obtained virtually uncontaminated by retinal tissue) it seems that the protein is more likely to have a role in neural activity generally, rather than specifically in phototransduction. It would be instructive to generate monoclonal antibodies directed against the 48 kDa protein in order to examine its subcellular distribution within the retina as well as in other neural tissues.

The similar peak absorbance wavelengths of *Leptograpsus* rhodopsin and metarhodopsin preclude experimental separation of the phosphorylation and dephosphorylation processes as is possible in *Calliphora* (Paulsen & Bentrop, 1984). It has also been impossible to
obtain highly purified preparations of rhabdoms or photoreceptive membranes from this crab; presumably because of the elongated shape of the rhabdom and the absence of a large volume of extracellular matrix which has assisted in the purification of blowfly rhabdoms (Paulsen & Schwemer, 1983; Paulsen, 1984). Given these constraints it is unsurprising that phosphorylation of an opsin band was only observed with difficulty. There is no reason to suppose that light-dependent rhodopsin phosphorylation in this species is less developed than in any other invertebrate.

The attempt to dephosphorylate squid rhodopsin using crude and partially purified 4–NPPase-containing fractions of crab retina was unsuccessful. The hypothesis presented in the introduction to this chapter, i.e. that the 4–NPPase is a rhodopsin phosphatase, may have to be abandoned. The 4–NPPase may be responsible for the dephosphorylation of the 48 kDa phosphoprotein. However, rhodopsin phosphatase is known to be elusive and labile. For example, Paulsen & Bentrop (1984) found that for dephosphorylation of *Calliphora* rhodopsin to proceed, extracellular calcium is required during the phosphorylation as well as the dephosphorylation step. The reconstituted system employed here may have been deficient in some unidentified factor required for phosphatase activity. It is also possible that a species difference between the visual pigments of crab and squid blocks cross-reactivity of the phosphatase. Apart from the difference in molecular weights between crab and squid opsins it seems that invertebrate opsins are immunologically diverse; de Couet & Sigmund (1985) found that *Leptograpsus* rhodopsin was not recognised at all by a battery of monoclonal antibodies that had been generated against the rhodopsin of another decapod crustacean, *Cherax destructor*. 
In order to test these possibilities further *in vitro* phosphorylation of crab retinal membranes using $\gamma^{32}$P-ATP may have to be employed. This was avoided up to now because of the unavailability of a pure photoreceptor membrane fraction; the limited amount of material which is available would make the screening of column-purified fractions for phosphatase activity very costly. It may be possible to avoid these disadvantages using *in vitro* phosphorylation of *Cherax* photoreceptor membrane which, because of the ovoid shape of the rhabdom (de Couet *et al.*, 1984), can be obtained highly purified although in much smaller quantity than from squid.
6. GENERAL DISCUSSION

Details of the cytochemical demonstration, biochemical characterization and biochemical fractionation of a microvillar 4-NPPase activity were discussed in Chapter 2. The discovery and preliminary characterization of inositol trisphosphatase and bisphosphatase from crab retina was discussed in Chapter 3. Following the partial purification and further biochemical characterization of the 4-NPPase that is described in Chapter 4 the similarities amongst the 4-NPPases of arthropod and cephalopod retinae and those found in mammalian tissues were discussed. The pattern of light-stimulated protein phosphorylations in crab and squid retinal membranes and preliminary attempts to reverse these phosphorylations were discussed in Chapter 5. The remainder of this Chapter will be confined to points that have not so far been discussed in detail and suggestions for additional experiments.

The study presented in this thesis is an attempt to overcome some of the technical problems that have slowed research on the phototransductive physiology of arthropod rhabdoms, by using cytochemical and biochemical methods in parallel. The attraction of a cytochemical screening procedure is that enzymes can be localized with an authority and ease that cannot be matched by the best available techniques for the isolation and purification of subcellular organelles. My starting point was to map the distribution of Na+/K+ ATPase in crab retina. This limited aim was chosen because the cytochemical techniques for the demonstration of Na+/K+ ATPase are extremely well characterized; knowledge of the sodium pump distribution should be a pointer to the membrane sites of large ionic fluxes.
Significant retinal phosphatase activity was visualized using a 4-NPPase cytochemical procedure that has been used to demonstrate Na+/K+ ATPase in tissues where it is highly concentrated (e.g. Ernst, 1972b). However, the 4-NPPase activity of crab retina differed in many respects from the potassium-stimulated activity that characterizes the sodium pump. Its concentration in the photoreceptor microvilli suggested a more direct involvement in phototransduction than that of an ion pump. With hindsight it appears that this was the second identification of such an activity in an invertebrate photoreceptor. Romero-Saravia & Hamdorf (1983) had discovered a very similar magnesium-stimulated activity in a retinal membrane preparation from octopus. Because the activity appeared to them to be membrane-bound they seriously considered the possibility that the activity might reside in the rhodopsin molecule itself. Whilst the crab activity is closely associated with the rhodopsin-bearing membrane it is not an integral membrane protein. It is unlikely that the 4-NPPase is an integral membrane protein in one phylum and not in the other because Saibil & Michel-Villaz (1984) found an abundant soluble 4-NPPase in the photoreceptors of squid. It seems fair to rule out the possibility that the 4-NPPase resides in the rhodopsin.

The question arises as to whether the 4-NPPase is specifically a retinula cell enzyme or not. Clearly its distribution within the retina is highly circumscribed but in Chapter 4 an experiment was performed to show that the crab lamina also possesses a magnesium-stimulated 4-NPPase with a specific activity approximately half that in retinal homogenates. The purpose of the experiment was to exclude the possibility that the enzyme present in retinal extracts could be a contaminating activity of extra-retinal origin; consequently the lamina 4-NPPase was not characterized further. Since seven of the eight
retinula cells that comprise each rhabdom terminate in the lamina and the other one extends processes in this region (Stowe, 1977) it is possible that the activity in the lamina is derived from retinula cell processes. However, since it is not established whether the lamina 4–NPPase activity is the same as the retinal one it is impossible to decide whether photoreceptors have made special use of an enzyme that is distributed throughout the nervous system or whether the retinal 4–NPPase is unique.

Whilst it is clear that millimolar concentrations of calcium inhibit the magnesium-dependent 4–NPPase (Chapter 2) and cannot replace magnesium in stimulating the activity (Romero-Saravia & Hamdorf, 1983) such concentrations are non-physiological. 100 µM calcium on the other hand was shown to be stimulatory (Chapter 5), but precisely what range of calcium concentrations is effective in stimulating the 4–NPPase is unknown. Nevertheless, calcium transients are known to reach at least 100 µM in *Limulus* ventral photoreceptors (Brown et al., 1977b) so it is certainly possible that calcium stimulation of the 4–NPPase represents a physiological modulation. Some of the evidence that calcium is involved in both excitation and adaptation of invertebrate photoreceptors was rehearsed in Chapter 1.

The crucial question is: what is the physiological function of the 4–NPPase? In Chapters 2, 3 and 4 a number of small phosphoester molecules were eliminated as substrates for the 4–NPPase molecule. The evidence linking divalent-cation-stimulated 4–NPPase activity with phosphoprotein phosphatases was discussed in Chapter 3 and in more detail in Chapter 5. The similarities between the microvillar activity and those that copurify with mammalian phosphoprotein phosphatases, and particularly with the calcineurin 4–NPPase, led to the development of the
hypothesis that the function of the enzyme in the intact photoreceptor is to dephosphorylate rhodopsin.

Evidence was presented to show that the rhodopsin of both the species used in this study is phosphorylated upon the illumination of their photoreceptive membranes. Therefore these organisms must possess a rhodopsin phosphatase yet cross-specific dephosphorylation of squid rhodopsin by the 4-NPPase of crab was not observed. The most obvious explanation is that rhodopsin dephosphorylation will not proceed in the system as presently reconstituted, otherwise one must accept that neither the cytosolic fraction of crab retina nor the photoreceptor membranes of squid harbour rhodopsin phosphatase activity. The identity of the rhodopsin dephosphorylating activity is left open.

In this thesis I have presented no definitive evidence to show that the function of the 4-NPPase is the dephosphorylation of rhodopsin. The possibility remains that this is not the true function of the microvillar phosphatase. For example, its true function could be the dephosphorylation of the 48 kDa phosphoprotein. It may even be unrelated to the dephosphorylation of phosphoprotein substrates. If this is the case the 4-NPP probably mimics a phospho-enzyme intermediate during the dephosphorylation step as was discussed in Chapter 2. What the substrate of such an enzyme might be is presently unclear. In any event the enzyme would seem to be important for the function of the rhabdom.

Suggestive evidence has been presented that the function of the 4-NPPase may be rhodopsin dephosphorylation. Phosphoprotein phosphatases have proven more difficult to characterize than the corresponding protein kinases (Krebs & Beavo, 1979; Ingebritsen & Cohen, 1983). Rhodopsin phosphatases from both vertebrates and invertebrates have been especially difficult experimental targets as was discussed in
Chapter 1. Dephosphorylation of phosphorylated rhodopsin has never, to my knowledge, been observed in an isolated photoreceptor membrane preparation. Failure to observe the dephosphorylation step after photoreceptor disruption could arise from one of three sources:

1. A conformational change in rhodopsin that makes it unavailable for dephosphorylation.
2. Deterioration of the phosphatase enzyme so that it no longer handles phospho-rhodopsin.
3. Loss of a cofactor necessary for the dephosphorylation.

Whatever the cause of the loss of phosphatase activity may be, it occurs very readily and most preparations of rod outer segments that are described in the literature have already undergone this change. A clue to one of the factors that may be important is the observation that extracellular calcium is required during the phosphorylation step (Paulsen & Bentrop, 1984) if the dephosphorylating capacity of photoreceptor cells is to be preserved. Whether the action of calcium is intracellular or extracellular is unknown. Calcium also inhibits the extent to which rhodopsin is phosphorylated in an unpurified, freshly dissected retinal suspension from squid (Vandenberg & Montal 1984b). This effect could be mediated by preservation or activation of the rhodopsin phosphatase activity as an alternative to inhibition of the kinase.

Further investigation of the role of calcium in promoting rhodopsin dephosphorylation may be productive. It is not yet known whether the rhabdomeral calmodulin that is abundant in the rhabdoms of Leptograpsus, and some other arthropods (de Couet et al., 1986), is relevant to this process or has a cytoskeletal function.

The phosphorylation and dephosphorylation of proteins is an integral part of the control of many cellular processes. Our understanding of these processes can only be enhanced by unravelling the action of the
relevant phosphatases. The rhodopsin case is a challenging one, yet the indications are that it should be amenable to further study, at least in the invertebrate system. Just one possible approach would be to use purified, exogenous alkaline and phosphoprotein phosphatases to determine whether rhodopsin is inherently resistant to dephosphorylation or if the lability lies elsewhere in the system.
REFERENCES


Bastian, B.L. & Fain, G.L. (1979) Light adaptation in toad rods: requirement for an internal messenger which is not calcium. *J. Physiol.* 297 493-520


References.


References.


Kühn, H & Dreyer, W.J. (1972) Light dependent phosphorylation of rhodopsin by ATP. *FEBS Letters* 20 1-6


Li, H.-C. Hsiao, K.-J. & Sampathkumar, S. (1979) Characterization of a novel alkaline phosphatase activity which co-purifies with a phosphorylase (phosphoprotein) phosphatase of Mr = 35,000 cardiac muscle. J. Biol. Chem. 254 3368-3374


Walz, B. (1982b) Ca\(^{2+}\)-sequestering smooth endoplasmic reticulum in invertebrate photoreceptor. I. Intracellular topography as revealed by OsFeCN staining and in situ Ca\(^{2+}\) accumulation. *J. Cell Biol.* **93** 839-848


ADDENDUM