EXCITATION AND REGENERATION IN VISION

BIOCHEMICAL AND BIOPHYSICAL ASPECTS

J. J. H. H. M. DE PONT

EXCITATION AND REGENERATION IN VISION

Biochemical and Biophysical Aspects

PROMOTOR: PROF. DR. S. L. BONTING

This investigation was carried out in the Department of Biochemistry, University of Nijmegen, Nijmegen (The Netherlands) and the Zoological Station, Naples (Italy) under the joint supervision of Prof. Dr. S. L. Bonting and Dr. F. J. M. Daemen.

EXCITATION AND REGENERATION IN VISION

BIOCHEMICAL AND BIOPHYSICAL ASPECTS

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN
DOCTOR IN DE WISKUNDE EN NATUURWETENSCHAPPEN
AAN DE KATHOLIEKE UNIVERSITEIT TE NIJMEGEN,
OP GEZAG VAN DE RECTOR MAGNIFICUS
DR. G. BRENNINKMEIJER,
HOOGLERAAR IN DE FACULTEIT DER SOCIALE WETENSCHAPPEN,
VOLGENS BESLUIT VAN DE SENAAT
IN HET OPENBAAR TE VERDEDIGEN OP
WOENSDAG 17 JUNI 1970 DES NAMIDDAGS
TE 4 UUR
DOOR

JAN JOZEF HUBERT HENRI MARIA DE PONT

GEBOREN TE WEERT

Druk: M. H. Tunnessen Malden/Nijmegen

Aan mijn ouders
Aan Jeanne Marie

The research reported in this thesis was greatly facilitated by the helpfull discussions and the technical assistance by many persons in and around the Department of Biochemistry. In particular I wish to thank the members of the Visual Mechanism research group, especially Dr. F. J. M. Daemen for his inspiring and enthousiastic guidance, Dr. G. Duncan for his collaboration in the ionic movement studies, Drs. J. M. P. M. Borggreven for the many stimulating discussions and Miss Eleonore Kühlman, Miss Jet Loeffen, Mrs. Ans van Prooyen-van Eeden and Miss Anneke Volk for their excellent technical assistance in various parts of this investigation. I am greatly indebted to Mr. R. Jacobs for his help in the initial phase of the retinoldehydrogenase study.

Thanks are due to Mr. C. P. Nicolasen of the department of Medical Illustration (head: Mr. Chr. van Huyzen) for the drawings, to the staff of the department of Medical Photography (head: Mr. A. Reijnen) for the photography, to Mrs. C. Los for correction of the manuscript and to Mrs. J. M. Richards for the typing of the manuscript.

I also wish to thank the staff of the Zoological Station in Naples for their assistance and the Royal Dutch Academy of Sciences for the financial support of the investigations in this Station. Additional financial support was received from the Netherlands Foundation for Chemical Research (SON), division of the Netherlands Organization for Basic Research (ZWO).

CONTENTS

The function of the visual system is the translation of a light stimulus into an electric stimulus, which is conducted to the brain and there leads to a visual sensation. To explain this process, Bonting and Bangham (1967) have proposed a biochemical model in which they assume that light leads to a chemical reaction (an aldimine formation) in the photoreceptor membranes and this reaction increases the permeability for cations of these membranes, leading to a stimulation of the synaps. This theory can be indicated as the aldimine cation channel hypothesis.

In this thesis various aspects of this hypothesis are investigated. For a better understanding of the experiments a short survey of the anatomy of the vertebrate and invertebrate visual system, of the visual pigments and the various models for the visual excitation mechanism is given in chapter 1. The aspects studied in our investigations are:

1. the chemical reaction leading to an increase in permeability for cations by means of experiments with monolayers of rhodopsin and phospholipids (chapter 2);
2. the light induced ionic movements in the retina of Sepia officinalis (chapter 3);
3. the restoration of the original low cation permeability of the photoreceptor membrane, which is interpreted as involving a direct conversion of aldimines of retinaldehyde by retinoldehydrogenase (chapter 4 and 5);
4. the role of the Na-K activated ATPase system in the restoration of the ionic gradients (chapter 6).

The experiments described in this thesis strongly support the aldimine cation channel hypothesis for the visual excitation, which is extended and reformulated in the last chapter (chapter 7).

STRUCTURE AND FUNCTION OF THE VISUAL SYSTEM

1.1. ANATOMY

1.1.1. *Vertebrates*

The retina is a thin sheet of tissue, morphogenetically originating from the brain. It is located inside the eye cup, and covering the rear half of it. In the retina, going from the sclera to vitreous, the following layers can be distinguished: the pigment epithelium, the photoreceptor cells, the bipolar cells, the ganglion cells and the nerve fibre layer. Light has therefore to cross the retina nearly completely before it reaches the photoreceptor cells. The stimulus is translated into an electric signal and this signal is conducted via the bipolar and the ganglion cells through the optic nerve to the visual cortex. In the retina are, moreover, horizontal and amacrine cells in which signals from different receptor cells are partly co-ordinated.

In fig. 1 a schematic diagram of the photoreceptor cells is drawn. At the scleral side is the outer segment in which the visual pigments are localised. This outer segment is connected via a narrow cilium with the inner segment in which a large nucleus, many mitochondria and an endoplasmatic reticulum can be distinguished. At the vitreal side there is a synaptic connection with the bipolar cells.

There are two types of photoreceptor cells, the rods and the cones, which differ in function. The rods are the dim light receptors and do not distinguish colour of light. The cones however function only in bright light and mediate colour vision.

Electron micrographs of the outer segments of rods and cones show lamellar membrane structures. These membranes originate by invagination of the plasma membrane. In the cones this plasma membrane remains continuous with the invaginations, leading to an intracellular compartment, separated by a membrane of large surface area from the extracellular space. In the rods, however, the membranes are pinched off from the plasma membrane, resulting in a large number (500-2500) of regularly stacked, independant sacs enclosed by a plasma membrane. In the rods we must therefore distinguish two compartments: an extrasaccular and an intrasaccular compartment, the latter composed of a large number of independant sacs. Evidence for this difference is mainly obtained by electron microscopy (Cohen, 1961; Dowling, 1967), by the use of lanthanum, which does not penetrate rod sacs (Cohen, 1968) and by injection with radioactive amino

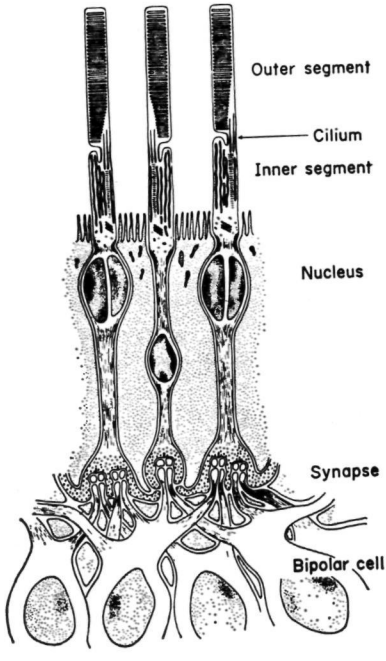


Fig. 1 Structure of rod photoreceptor cell. After Sjöstrand (1961).

acids in vivo. In the rods the labelling was distributed in a small region in the sacs, indicating that each sac is separately formed, and the radioactivity in the cones was randomly distributed suggesting that on all places amino acid exchange occurs (Young, 1967, 1969).

1.1.2. *Invertebrates*

While the vertebrate retina is formed as an ectodermal invagination of the head region, the cephalopod retina is formed as an ectodermal evagination of the forebrain. This results in a retina in which the photosensitive surface is turned towards the light, in contrast to the position in the vertebrate retina. In the cephalopod retina light therefore does not have to pass the neural layer first, but can be absorbed by the photoreceptor cells directly. The invertebrate retina is simpler in construction than the vertebrate retina. The complex neural system, which makes up most of the vertebrate retina, is absent from the cephalopod retina, but is located behind the eye in the lobus opticus (Young, 1962 b).

The retina thus consists of an outer segment layer, a small layer with a large number of pigment granules, an inner segment layer with the nuclei of the photoreceptor cells and also some nuclei of the pigment epithelium

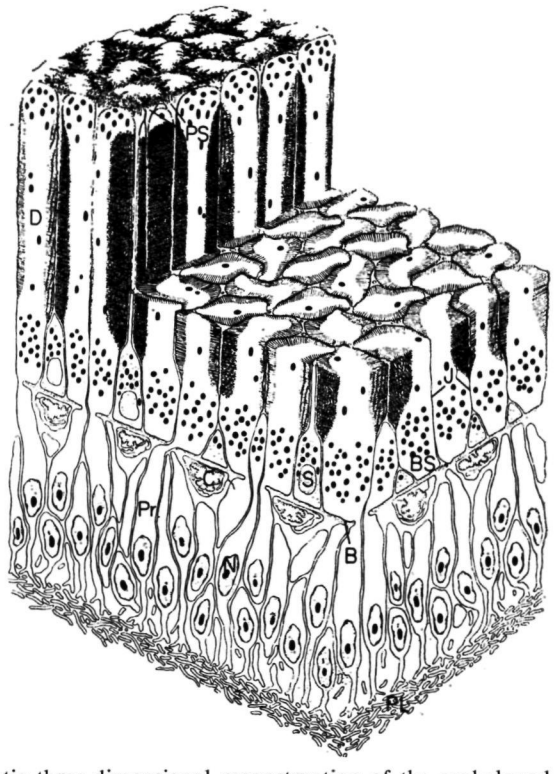


Fig. 2 A schematic three-dimensional reconstruction of the cephalopod retina. The glial fibers and the limiting membrane which covers the vitreal surface (upper side in this diagram) of the retina are not depicted. B, basement membrane; BS, basal spindle portion of the visual cell loaded with numerous pigment granules; C, blood capillary; D, distal segment of the visual cell carrying rhabdomeres (R) on its lateral surfaces; J, plasma membranes between two adjoining basal spindles; N, nucleus of the visual cell; PL, plexiform layer of the retina; Pr, proximal segment of the visual cells; PS, tip portions of processes from the supporting cells (S). After Yamamoto et al (1965).

cells, and the nerve fibre layer, in which the fibres are combined to form a nerve bundle which leads to the lobus opticus (fig. 2).

The outer segments or rhabdomes are parallel to the direction of the incident light. The plasma membrane of these outer segments is evaginated in a large number of microvillous projections, which are transverse to the direction of the incident light. Zonana (1961), in an extensive study of the squid retina, concluded that the microvilli are cylinders with an average length of 1.1 μ and a diameter of 0.1 μ. The tips of the microvilli of one cell seem to abut the tips of the microvilli of the adjacent cells. In side view the microvilli of one retinula cell appear as regular hexagonal arrays in

which each microvillus is surrounded by six others. Zonana (1961) distinguished two types of retinula cells one of which is circular in transverse section and the other is more elliptical. The microvilli are located at two sides of the retinula cells. In the squid 69% of the cells are of the elliptical type and 31% of the circular type. The elliptical cells contain about 700.000 and the circular 200.000 microvilli. The total number of outer segments is of the order of 5×10^6 per cm^2 tissue (Zonana, 1961; Young, 1962 a).

Electron microscopic investigations of the compound eye of Arthropods show that the eye is composed of ommatidia. Each one contains several retinular cells, the differentiated part of which is the rhabdomere. These rhabdomeres may be thought of as being composed of the tightly packed microvilli of the receptor cell surface. The structure is very much like that of the outer segments of cephalopods. Much electrophysiological work is being done with such Arthropods as Limulus, honey bee and dragon fly. Because of the structural analogy many of the conclusions from this work are probably valid for the cephalopods.

For further details on the anatomy of the visual system the reader is referred to the extensive reviews by Sjöstrand (1961), Fernández-Morán (1961), de Robertis and Lasansky (1961), Cohen (1963), Eakin (1965), and Trujillo-Cenoz (1965).

1.2. VISUAL PIGMENTS

1.2.1. *Location*

Rod outer segments have a reddish colour and Boll (1877) discovered that this colour was changed upon illumination. Kühne (1878) could extract a red pigment from these outer segments and this pigment he called sehpurper which name was later changed to rhodopsin. Investigations of König (1894), Hecht and Williams (1922), Dartnall and Goodeve (1937), Crescitelli and Dartnall (1953), and of Wald and Brown (1958) have shown that there is a close correlation between the visual absorption spectra of rhodopsin and the human rod sensitivity spectrum, when the last spectrum is plotted on an equal quantum basis. It is therefore generally assumed that the visual pigments are directly involved in the visual mechanism.

Recent investigations by Dewey and collaborators (1969) of the frog retina with an immunofluorescent technique gave definite proof that the light sensitive visual pigment rhodopsin is located in the membranes of the rod sacs. The large surface area of the rod sac membranes, perpendicular to the incident light, make the change of absorption of light by these molecules maximal.

The anatomical similarity between the invertebrate microvilli and the vertebrate rod sacs makes it very likely that the visual pigments in the invertebrates are located in the microvilli.

1.2.2. *Extraction*

The visual pigments are insoluble in water and are denaturated by many organic solvents. Hence, for chemical studies the pigments of rod and cone outer segments are extracted with aqueous solutions of detergents such as digitonin (Tansley, 1931), sodium cholate, sodium desoxycholate, cetyltrimethylammoniumbromide (CTAB) and chloride (CTAC) (Bridges, 1967), Triton-X-100 (Crescitelli, 1967) and emulgophene BC 720 (Shichi et al, 1969). In these solutions the visual pigment molecules and other membrane components are solubilised by their incorporation into micelles, containing a large proportion of detergent molecules.

Many spectroscopic and chemical investigations are carried out with detergent solutions of the visual pigments. Since the original structural relations between the various membrane components present in the intact membrane are lost during detergent extraction, some important properties of the original configuration may be changed. This must be remembered when results from experiments with visual pigment extracts are extrapolated to the in vivo situation.

1.2.3. *Absorption spectra*

The absorption spectra of the visual pigments and especially the difference in spectra before and after illumination are the most important criteria in the determination of the properties and the concentration of the visual pigments. The absorption spectrum of the visual pigment rhodopsin from cattle rod outer segments, measured in a 1% digitonin solution is given in fig. 3, (solid line). The spectrum after illumination in the presence of hydroxylamine is represented by the broken line.

The spectra of all visual pigments in solution show three recognisable bands: a broad absorption maximum in the visible between 430 and 620 nm, but mostly near 500 nm (α-band); a shallow absorption peak between 340 and 370 nm (β-band) and a high absorption peak at 278 nm (γ-band). The γ-band, which is probably due to protein, does not change upon illumination. The α-band decreases and disappears nearly completely and at the place of the β-band a new peak with an absorption maximum at 360 nm is produced.

1.2.4. *Chromophoric group*

When a pigment solution is illuminated in the presence of hydroxylamine,

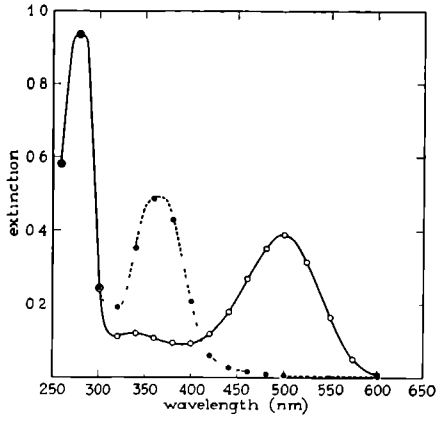


Fig. 3 Absorption spectra of cattle rhodopsin in 1% digitonin, before (solid line) and after (dashed line) illumination in the presence of hydroxylamine.

retinylidene oxime (360 nm) is produced. In the absence of hydroxylamine retinaldehyde (λ_{max} = 381 nm in ethanol) is formed. This compound could be extracted completely from illuminated rhodopsin with hexane (Wald, 1935, 1936); the remaining part of the visual pigment was called opsin. Ball et al (1948) showed that the aldehyde was identical with vitamin A aldehyde. Hydroxylamine converts the aldehyde to retinylidene oxime and this reaction withdraws retinaldehyde from the illuminated rhodopsin complex. Incubation of opsin, obtained by illumination and hexane extraction of rhodopsin with retinaldehyde from a natural source led again to formation of rhodopsin (Hubbard and Wald, 1951). This indicates that retinaldehyde is the chromophoric group of rhodopsin.

1.2.5. *Cis-trans isomerisation*

When opsin was incubated with synthetic retinaldehyde instead of with a natural product, no rhodopsin was formed (Hubbard and Wald, 1952). They explained this by assuming that only one of the isomers occurring in natural retinaldehyde could react with opsin to form rhodopsin. Oroshnik et al (1956) proved that this is the 11-cis isomer of retinaldehyde. The chromophore that could be extracted after illumination of rhodopsin was shown to be all-trans retinaldehyde (Hubbard and Wald, 1952). This indicates, that upon illumination the 11-cis form of retinaldehyde, present in rhodopsin, is isomerised to the all-trans form. This conclusion was confirmed when after thermal denaturation the 11-cis form of retinaldehyde was isolated (Hubbard and Kropf, 1958; Hubbard, 1958, 1959).

1.2.6. *Other components*

Since visual pigments are present in high concentration in the membranes of the outer segments of photoreceptor cells, they can be assumed to be structural components of these membranes. In addition to retinaldehyde there are lipids and proteins present in these membranes and upon extraction of the visual pigments with detergents these lipids and proteins are also extracted.

The presence of protein is deduced from the sharp absorption peak at 278 nm (γ-band), characteristic for the amino acids tyrosine and tryptophan (Collins et al, 1952), the bleaching of rhodopsin by proteolytic enzymes (Radding and Wald, 1958) and the thermodynamic parameters for the heat denaturation of rhodopsin (Lythgoe and Quilliam, 1938; Hubbard, 1958). It is unlikely that all protein present in the rhodopsin complex is essential for the extinction at 500 nm, because rhodopsin is stable between pH 4 and 10 (Radding and Wald, 1956 b). In this pH range an enormous change in protonation of the polar groups of protein occurs.

Amino acid analysis of the protein part of the rhodopsin complex has been carried out by various authors (Shields et al, 1967; Heller, 1968; Shichi et al, 1969). Although they all found a molecular weight near 28,000 for the protein part of the rhodopsin complex, there were significant differences in the reported amino acid compositions. This could be due to the different purification techniques used by these authors.

Lipids constitute about 40% of the dry weight of cattle rod outer segments (Borggreven et al, 1970). Three quarters of these lipids are phospholipids. These phospholipids have been analysed by Eichberg and Hess (1967) in frog, and by Poincelot and Zull (1969) and Borggreven et al (1970) in cattle rod outer segments. These last analyses showed that the aminogroup containing phospholipids, phosphatidylethanolamine and phosphatidylserine, make up about 50% of the phospholipids.

About 80% of the phospholipids could be removed by dialysis, extraction with mild organic solvents and by enzymatic hydrolysis with phospholipase C from Clostridium Welchii (Krinsky, 1958 a). In our laboratory Borggreven (unpublished results) could even remove, with a phospholipase C from Bacillus cereus over 90% of the phospholipids without effecting the spectral properties and the susceptibility to photolysis. However the last 4 molecules of phospholipid per molecule of retinaldehyde could not be removed by this technique. Heller (1968) was able to fractionate CTAB extracts of rhodopsin on a Sephadex G 200 column and he found that the resulting purified rhodopsin contained less than 2% phospholipids. This indicates that only a small part of the phospholipids are essential for the visible absorption maximum

and the photolytic properties of the visual pigment.

1.2.7. *The binding site of retinaldehyde*

The molar extinction of rhodopsin is of the same order as the molar extinction of retinaldehyde. The maximum of the last compound is however displaced about 120 nm to the red. The work of Morton and associates (see Morton and Pitt, 1957) has shown that in rhodopsin 11-cis retinaldehyde is bound via an aldimine band to an amino group in the lipoprotein complex. Since aldimines are very unstable in aqueous solution, sodiumborohydride is generally used to reduce these aldimines to the more stable secondary amines. However, both Bownds and Wald (1965) and Akhtar et al (1965) were unable to reduce the aldimine bond in native rhodopsin. They explained their failure by assuming that the aldimine bond was located in a hydrophobic pocket, thus preventing the polar sodiumborohydride molecule from reducing the aldimine. This location in a hydrophobic environment explains the stability of rhodopsin in detergent solutions.

1.2.8. *Other visual pigments*

Rhodopsin is composed of 11-cis retinaldehyde linked to the lipoprotein opsin from the rod outer segment. Its visible absorption peak is located between 430 and 560 nm, depending on the species. There is another class of rod outer segment visual pigments occurring in amphibians and fresh water fishes (Köttgen and Abelsdorf, 1896; Wald, 1937 a, 1939), which yield upon photolysis the 3-dehydro derivative of retinaldehyde (λ_{max} 401 nm, Farrar et al, 1952). These pigments, called porphyropsins, have absorption maxima at a somewhat higher wavelength (520-640 nm) than the corresponding rhodopsins.

In cone outer segments still other visual pigments with retinaldehyde (iodopsins; Wald, 1937 b; Wald et al, 1955) and with 3-dehydroretinaldehyde (cyanopsins; Wald et al, 1953; Liebman and Entine, 1967) as chromophore are present. The prophyropsins and the cone visual pigments have been less extensively studied than the rhodopsins.

Microspectrophotometric studies indicate that in the cones of one retina three different visual pigments are present (Marks et al, 1964; Wald and Brown, 1964), which explains the function of cones in colour vision. Vision in the rods is achromatic, and hence one visual pigment suffices for these photoreceptors.

1.2.9. *Photolysis of vertebrate rhodopsin*

Illumination of vertebrate rhodopsin leads to a sequence of changes, which

are accompanied by a change in colour from dark red to pale yellow. Hence the process has often been called "bleaching". Since not all visual pigments bleach upon illumination and since we now know that bonds are broken in the process, we shall use the term "photolysis". This process is a very fast one, and has therefore been studied mainly by low temperature absorption spectroscopy. The component reactions can be distinguished by means of the intermediate photoproducts, characterized by their absorption spectra. These photoproducts, their absorption maxima and the temperature above which they can be recognised are given in fig. 4.

The first photoproduct that can be obtained is prelumirhodopsin. Illumination of rhodopsin at —195° C leads to a shift in the absorption maximum from 498 to 543 nm. In this step the cis-trans isomerisation of retinaldehyde occurs (Yoshizawa and Wald, 1963). This is probably the only change occurring in this step. All reactions after this step are thermal reactions, i.e. they do not require light.

On warming prelumirhodopsin to —140° C lumirhodopsin is formed as indicated by a spectral shift to 497 nm. Warming to —40° C yields metarhodopsin I (480 nm, Wald et al, 1950) and this compound is converted above 0° C into metarhodopsin II (380 nm, Matthews et al, 1963). Up to this point agreement exists between different investigators.

In detergent solution metarhodopsin II hydrolyses slowly into free retinaldehyde and opsin. Matthews et al (1963) have described a product,

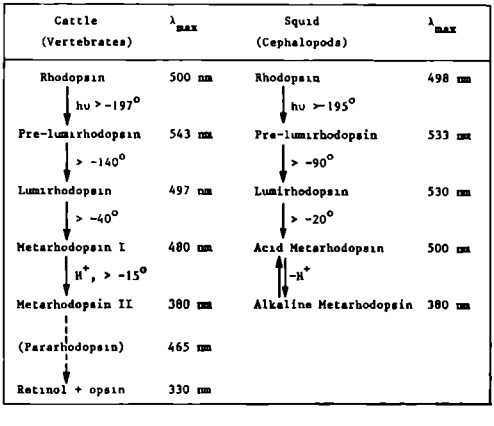


Fig. 4 Scheme of the photolytic process in vertebrates and invertebrates.

which they call pararhodopsin (465 nm), and which they consider as resulting from a side reaction. Ostroy et al (1966) believe this photoproduct to be a real intermediate after metarhodopsin II. This product would preceed N-retinylidene opsin, a simple aldimine between retinaldehyde and opsin, which is thereafter hydrolysed into retinaldehyde and opsin.

Investigation of the kinetic data for the various thermal reactions in the photolytic cycle (Abrahamson and Ostroy, 1967) indicate that by far the largest positive change in entropy and enthalphy of activation occurs in the step metarhodopsin I to metarhodopsin II. Moreover, in this step also the largest spectral shift occurs (from 480 nm to 380 nm). It is also the first step that requires water (Wald et al, 1950) and in this step one proton per molecule of rhodopsin is taken up by frog rod outer segments (Radding and Wald, 1956 a; Falk and Fatt, 1963, 1966 a, 1966 b). Moreover, metarhodopsin II is the first photoproduct that can be reduced by aqueous sodium-borohydride (Akhtar et al, 1965; Bownds and Wald, 1965), indicating that the aldimine band in this compound is located in a more hydrophilic environment. Finally, at physiological temperature metarhodopsin II is formed in about 1 msec. after light absorption, while its decay takes minutes, and visual excitation also takes place in about 1 msec. All this strongly suggests that in the vertebrates the metarhodopsin I $\rightarrow$ metarhodopsin II transition may trigger visual excitation.

Investigations of the photolysis in the excised eye (Hagins, 1956), the isolated retina (Matthews et al, 1963; Baumann, 1966, 1967, 1968; Donner and Reuter, 1967, 1969; Cone and Brown, 1969), rod outer segment suspensions (Bridges, 1962 b; Pratt et al, 1964) or direct microspectrophotometry in individual isolated outer segments (Liebman, 1962) confirms the above sequence and intermediates derived from observations in aqueous detergent solution. However, under these conditions retinol is found as the end product of the visual cycle instead of retinaldehyde, which is the end product in the case of detergent solutions of the pigment.

1.2.10. *Photolysis of cephalopod rhodopsin*

The photolysis of cephalopod rhodopsins differs in some respects from that of the vertebrate rhodopsins. The rhodopsins are similar in having a maximum around 500 nm (Hubbard and St George, 1958; Brown and Brown, 1958), and in their first two photoproducts, pre-lumirhodopsin (Yoshizawa and Wald, 1964) and lumirhodopsin (Kropf et al, 1959), which seem to have the same properties as the corresponding vertebrate compounds. The next compound, a metarhodopsin (λ_{max} = 500 nm), differs from the vertebrate metarhodopsins in being stable at room temperature (Hubbard

and St George, 1958; Brown and Brown, 1958). The cephalopod metarhodopsin, upon addition of alkali, changes its λ_{max} to 380 nm. This is a reversible process with a pK = 7.7. The conversion of acid into alkaline metarhodopsin has a small negative entropy (—8 e.u., Hubbard and St George, 1958). This suggests that in this step no important conformational changes occur. The transition lumirhodopsin → acid metarhodopsin, however, has a high positive entropy of activation (S = 90 e.u., Abrahamson and Erhardt, 1964). Therefore it is very likely that this reaction has the same function in the excitation in cephalopods as the metarhodopsin I → metarhodopsin II step has in the vertebrate system.

1.2.11. *Visual pigment regeneration in the vertebrates*

The final product of the photolysis of rhodopsin in vitro is retinaldehyde, while retinol is the end product in vivo. In rod outer segments a retinol-dehydrogenase is present (Bliss, 1948; Wald and Hubbard, 1949; Futterman and Saslaw, 1961), which can reduce retinaldehyde to retinol in the presence of NADH or NADPH. According to Futterman (1963) the reduction is faster in the presence of NADPH. He also observed that the enzyme is bound to the membranes of the rod outer segments. Other authors have described a soluble enzyme of this type in whole retina extracts (Wald, 1950; Hubbard, 1956; Koen and Shaw, 1966), while horse liver alcoholdehydrogenase will also reduce retinaldehyde to retinol in the presence of NADH or NADPH (Bliss, 1951 a; Zachman and Olsen 1961). The difference between the end product of the photolytic cycle in vivo and in vitro is probably due to the fact that in the detergent extracts no cofactor is present.

After exhaustive bleaching of the pigment in vivo, retinol is found to be stored in the pigment epithelium in the case of frog and rat, mainly in the form of long-chain fatty acid esters (Wald, 1935; Krinsky, 1958 b; Dowling, 1960; Hubbard and Dowling, 1962; Futterman and Andrews, 1964; Andrews and Futterman, 1964). The esterification of retinol may facilitate the transport of retinol through the lipid membranes of the outer segment.

Relatively little is known about the resynthesis of rhodopsin from all-trans retinol, although it has been achieved in retinol homogenates of frog, rat and cattle (Bliss, 1951 b; Hubbard and Wald, 1951; Collins et al, 1953, 1954). The process can be subdivided into three steps: oxidation of retinol to retinaldehyde, isomerisation of all-trans into 11-cis form and recombination of 11-cis retinaldehyde with opsin. This last step can be carried out in vitro by incubation of 11-cis retinaldehyde and opsin between pH 5 and 7 (Radding and Wald, 1956 b). The isomerisation of all-trans retinaldehyde into the 11-cis form is catalysed by an isomerase present in cattle retina

(Hubbard, 1956). The enzymatic isomerisation does not occur with all-trans retinol, indicating that the oxidation step has to preceed the isomerisation step. The physiological significance of the isomerase is not yet clear, because the conversion of all-trans to 11-cis retinaldehyde in the presence of the enzyme requires light which normally would not be able to penetrate the retina (Hubbard, 1956, Rushton, 1957). The oxidation of all-trans retinol to all-trans retinaldehyde can probably be catalyzed by the same retinoldehydrogenase, which assists in the formation of retinol during photolysis. However, the equilibrium for this reaction lies so far in the direction of the alcohol (Bliss, 1951 a; Futterman, 1965) that a following reaction is necessary. This reaction is supplied by the binding of 11-cis retinaldehyde to opsin, which under physiological conditions goes rapidly to completion.

1.2.12. *Visual pigment regeneration in the cephalopods*

Earlier we mentioned (1.2.10.) that photolysis of cephalopod rhodopsin leads to a metarhodopsin, which does not hydrolyze to retinaldehyde and opsin in detergent solution at room temperature (Hubbard and St George, 1958; Brown and Brown, 1958). Recent investigations (Hara and Hara, 1965, 1967, 1968; Hara et al, 1967) show that there is another light sensitive pigment in cephalopods that is not located in the rhabdomes where rhodopsin is localized, but rather in the layer of the retina that is left when the rhabdomes and the pigment layer are removed. This pigment, (λ_{max} = 15 nm higher than rhodopsin) named retinochrome by Hara, contains all-trans retinaldehyde and upon illumination with orange light 11-cis retinaldehyde is released. Retinochrome regeneration has a pH optimum of 6.5 and is complete in 30 seconds at 20° C. (Hara and Hara, 1968). The retinochrome system may therefore represent the regeneration mechanism in cephalopods, in which retinochrome would serve as an isomerization system for all-trans retinaldehyde. In agreement with this is the fact that retinoldehydrogenase activity has not been found in cephalopod retina (Wald, 1960). A difficulty is however to explain the transport of retinaldehyde from the one pigment to the other in view of the stability of the metarhodopsin observed in vitro.

1.3. EXCITATION

1.3.1. *Introduction*

In order to give a satisfactory explanation of the mechanism of visual excitation several points must be taken into account. First there is the amplification factor involved in visual excitation. Absorption of a single photon by a molecule rhodopsin may lead to a nervous excitation, (Hecht, Shlaer and Pirenne, 1942). The energy necessary to stimulate the synapse is

4 to 5 orders of magnitude greater than the energy of a photon. Secondly, the signal excited by the photon must be transferred from the outer segment where the rhodopsin molecules are located, to the synapse with the bipolar cell (fig. 1). Thirdly, explanation is needed for the fact that rods work only at low light levels and that saturation occurs above a certain number of quanta per time unit.

Three different hypotheses have been proposed (Wald et al, 1963): the enzyme hypothesis, the solid state hypothesis and the ionic hypothesis.

1.3.2. *The enzyme hypothesis*

The enzyme hypothesis postulates that rhodopsin is a proenzyme and that one of its photoproducts is the active form of the enzyme. Since one enzyme molecule can catalyse the reaction of thousands of molecules this hypothesis would account for the amplification factor. The only claim in this direction, suggesting that rhodopsin would be an ATPase (Mc Connell and Scarpelli, 1963), was shown to be incorrect (Bonting et al, 1964 a; Frank and Goldsmith, 1965). Moreover, visual excitation is complete within 1 msec., and this is rather fast for any enzymatic process.

1.3.3. *The solid state hypothesis*

The orderly arrangement of the membranes in the photoreceptor outer segments and the high concentration of rhodopsin molecules in these membranes has suggested that this represents a solid state system, in which excitation of one molecule of rhodopsin could stimulate many other molecules in its neighbourhood. The primary stimulation could thus be conducted over a considerable distance, involving at the same time adequate amplification. One form of conduction could be by radiationless energy migration or by resonance. Hagins and Jennings (1959) and Liebman (1962) tested this hypothesis experimentally and they found that this process could not play a role in vision.

The other form would be by a semiconduction process excited by light. Photoconduction has been demonstrated in films of all carotenoid pigments including protonated aldimines of retinaldehyde (Rosenberg, 1958, 1959; Rosenberg and Harder, 1967) and in dried sheep rod outer segments (Rosenberg et al, 1961). For a simple electronic process however the distance between nearest neighbours should be less than 10 Å. In cattle rod outer segments the distance between different rhodopsin molecules is 140 Å and the distance between rhodopsin molecules in different sacs is even greater. Therefore, charge migration would have to take place through the other proteins and phospholipids in the membrane, implying either an electronic

conduction in hydrophobic regions of the membrane or a protonic or ionic conduction in the hydrophilic regions.

Another problem in the photoconduction theory is the function of the cis-trans isomerisation and the various photoproducts in this mechanism. Rosenberg and Harder (1967) observed photoconduction in powdered crystalline 11-cis retinaldehyde, but not in the all-trans isomer. If such a process would occur physiologically, this would mean that the entire photolytic cycle would have no function at all. Moreover, the cis-trans isomerisation takes place within 10^{-10} to 10^{-11} sec, while visual excitation takes some milliseconds. The resulting time interval would then be completely due to charge migration to the site of excitation.

Although the possibility of a photoconduction mechanism can not be entirely ruled out, there are still too many problems attached to it to give it real significance.

1.3.4. *The ionic hypothesis*

The ionic hypothesis is based on the fact that rhodopsin molecules are located in the membranes of the rod outer segments and on the assumption that there exist cation gradients across these membranes. Illumination would then result in an increased permeability for cations, leading to ion fluxes which in turn would stimulate the synapse with the bipolar cell.

The evidence for this hypothesis is mainly based on electrophysiological observations. In invertebrate photoreceptor cells like those of Limulus (Kikuchi et al, 1962; Millecchia and Mauro, 1969 a, 1969 b), crayfish (Eguchi, 1965), honey bee (Fulpius and Baumann, 1969), barnacle (Brown et al, 1969) and squid (Hagins et al, 1962; Hagins, 1965) there is an intracellular potential of about —50 mV and upon illumination depolarisation occurs. This depolarisation is accompanied by a decrease in resistance of the photoreceptor membrane (Fuortes, 1959; Rushton, 1959), indicating that illumination does indeed increase the permeability for cations.

In the frog retina an increased potassium efflux upon illumination (Sekoguti, 1960 a, b) was demonstrated as well as an increased uptake of ^{22}Na (Buckser and Diamond, 1966). In isolated cattle outer segments there was a nearly constant increase in sodium content and an equivalent decrease in potassium content upon illumination over a wide range of intensities (Bonting and Bangham, 1967).

A transient potential change, called electroretinogram (E.R.G.), can be obtained from an electrode placed on the cornea of an intact eye and an indifferent electrode placed on the scalp upon illumination. The first part of this potential, the a-wave, originates from the photoreceptor cells (Tomita

et al, 1960; Brown and Watanabe, 1962 a, 1962 b; Arden and Brown, 1965). The latency of this α-wave depends on the intensity and the duration of the light stimulus, and can be reduced to a few tenths of a millisecond (Arden and Ikeda, 1968). The amplitude of the electroretinogram is dependent on the sodium and potassium concentration at the receptor side of the retina (Hamasaki, 1963, 1964; Hanawa et al, 1967). Moreover, the α-wave could be abolished in 7 minutes by applying ouabain (10^{-4}M) to the receptor side (Frank and Goldsmith, 1967). Ouabain inhibits the enzyme sodium-potassium activated ATPase, which can be assumed to be identical with the sodium pump (Post et al, 1960). The presence of this enzyme in rod outer segments has been reported by Bonting et al (1964 a) and by Frank and Goldsmith (1965).

These observations strongly support the idea that an ionic mechanism is involved in visual excitation. This has led Bonting and Bangham (1967) to put forward an explanation for the visual mechanism based on the ionic mechanism.

1.3.5. *The aldimine cation channel hypothesis*

The increased leakage of potassium and the equivalent uptake of sodium observed upon illumination of isolated cattle rod outer segments suggest that the cation permeability of membranes in these segments is increased by light. Bonting and Bangham (1967) observed a similar effect when retinaldehyde (10^{-4} - 10^{-6}M) was added to these outer segments.

They suggested that both processes were caused by the same phenomenon. Addition of retinaldehyde would lead through aldimine formation to a blocking of the normally protonated amino groups on the surface of these membranes, leading to a loss of protonation and thus to a more negative surface charge, which would result in an increased cation permeability. This was confirmed by experiments with liposomes (phospholipid micelles). Addition of retinaldehyde led to an increased cation permeability in micelles consisting of an amino group containing phospholipid, but not in micelles consisting of lecithin which has no free amino group. Previously, Bangham et al (1965) had observed that the induction of a negative surface charge in lecithin liposomes by addition of anionic detergents led to a marked increase in cation permeability.

Bonting and Bangham (1967) suggested that upon illumination retinaldehyde shifts from its original binding site inside the membrane to an amino group on the surface of the membrane, thus leading to a blocking of a membrane amino group. This transiminisation process would occur in the

step metarhodopsin I to metarhodopsin II, which on several grounds appears to be responsible for excitation (1.2.9.).

The transiminization hypothesis was supported by investigations of Poincelot and co-workers (1969). Upon extraction of dry rhodopsin or metarhodopsin I with anhydrous acid methanol followed by reduction with sodium-borohydride, they were able to obtain retinyl phosphatidylethanolamine in high yield. Previously it had been shown that in metarhodopsin II retinaldehyde is bound to the ε-amino group of lysine in the protein backbone of the rhodopsin complex (Akhtar et al, 1965; Bownds and Wald, 1965). This indicates that there occurs in the metarhodopsin I → metarhodopsin II transition a transiminisation of retinaldehyde from phosphatidylethanolamine to lysine.

In other experiments Bonting and Bangham (1967) spread lyophilized rhodopsin in a monolayer in a Langmuir trough. Upon illumination there was a small increase in film pressure. Penetration of retinaldehyde into a rhodopsin monolayer caused a similar increase in film pressure. Preliminary experiments on penetration of retinaldehyde into phospholipid monolayers suggested that this effect could be due to an aldimine formation with amino groups in the monolayer. The increase in film pressure upon illumination of rhodopsin monolayer could also be explained in a similar way.

Bonting and Bangham (1967) proposed that once a rod sac had been stimulated by absorption of a photon no further stimulation would occur until cation permeability had been restored and recovery of the cation gradients had taken place. For the regeneration of the cation gradients the Na-K activated ATPase system would be responsible. They calculated that at rod saturation level the time required for restoration of the cation gradients is about equal to the time elapsing between absorption of consecutive photons per sac.

1.3.6. *Aim of our investigations*

The aldimine cation channel hypothesis described above has been, for the last few years, the guide-line for the study of the visual mechanism in our laboratory.

Chapter 2 describes an extension of the monolayer experiments of Bonting and Bangham (1967), aimed at proving that the formation of an aldimine with an amino group in the monolayer material can indeed explain the increase in film pressure upon illumination of a rhodopsin monolayer.

The apparent absence of retinaldehyde liberation during photolysis of cephalopod rhodopsin raised the question whether in these animals an analogous excitation mechanism as in vertebrates could occur. In Chapter 3

our studies of the ionic movements in the retina of Sepia officinalis are discussed. The existence of cation gradients across the photoreceptor cell membrane was demonstrated. In addition cation permeability increases upon illumination were established by tracer flux studies. Addition of retinaldehyde gave an increase in cation permeability similar to that of light. Ouabain, an inhibitor of Na-K activated ATPase, reduced the cation gradient and also the effect of light upon cation flux.

The aldimine cation channel hypothesis requires an explanation for the restoration of the low cation permeability during the rod sac regeneration phase. This would have to involve the removal of retinaldehyde from the membrane amino group. However, the hydrolysis of metarhodopsin II, in detergent solution, is too slow to account for the return of the initial low cation permeability.

This led us to study the possibility of a direct enzymatic reduction of aldimines of retinaldehyde to retinol. Since metarhodopsin II was not suitable as a substrate, we used aldimines of retinaldehyde and aliphatic amines. Their preparation and their use as substrates in the enzyme study required a thorough investigation of the equilibrium conditions of aldimine hydrolysis (Chapter 4). Utilization of this information permitted the demonstration of a direct reduction of these aldimines to retinol by rod outer segment retinol-dehydrogenase (Chapter 5). The solubility of the enzyme and its coenzyme specificity were also investigated. Liver alcoholdehydrogenase, which reduces free retinaldehyde, did not reduce aldimines.

The activity, properties and distribution of the enzyme Na-K activated ATPase in retinas of various species, in particular Sepia, are described in Chapter 6. A relation between the relative instability of rod outer segment Na-K activated ATPase, as compared to that in other tissues, with the high content of unsaturated fatty acids in rod outer segments was noticed.

The results of these investigations lend further support to the aldimine cation channel hypothesis proposed by Bonting and Bangham (1967). In the final chapter (Chapter 7) this hypothesis is reformulated in the light of evidence obtained from these and other recent investigations.

LIGHT INDUCED FILM PRESSURE INCREASE IN RHODOPSIN MONOLAYERS

2.1. INTRODUCTION

In studies aimed at elucidating the biochemical mechanism of the visual process Bonting and Bangham (1967) employed monomolecular films of rhodopsin at an air-water interface as a model for the structure of the rod-sac membrane. Illumination of the rhodopsin monolayer caused a small increase in film pressure, indicating an expansion of the molecules in the monolayer. It was believed that this effect might be due to penetration into the monolayer of retinaldehyde liberated during photolysis of the rhodopsin. When the penetration of retinaldehyde into monolayers of rhodopsin (bleached or unbleached) and phospholipids was studied, a curious phenomenon was observed. With rising initial film pressure the increase in film pressure caused by penetration of retinaldehyde decreased in all cases. However, at film pressure of 40 dyne/cm, close to the collapse pressure of these films, the phosphatidylcholine-cholesterol monolayer became impenetrable to retinaldehyde, while a residual penetration of retinaldehyde occurred in monolayers of rhodopsin as well as of phosphatidylethanolamine. It was postulated that this residual penetration of retinaldehyde into phosphatidylethanolamine films might be due to aldimine formation between the aldehyde group of retinaldehyde and the phosopholipid aminogroup. The possibility of this reaction had earlier been suggested by Krinsky (1958 a). The absence of an aminogroup in phosphatidycholine and cholesterol would explain why no residual penetration occurred in the phosphyatidylcholine-cholesterol films.

In the present study we have first repeated the experiments of Bonting and Bangham (1967) and we were able to confirm their findings. Thereafter we have tested the hypothesis that aldimine formation was the origin of the observed phenomena. Three different observations confirmed the hypothesis: the absorption spectrum of a phosphatidylethanolamine monolayer after penetration of retinaldehyde indicated the presence of an aldimine link; retinol showed no residual penetration at high film pressure into phosphatidylethanolamine films; at a subphase pH below 6, excluding aldimine formation, no residual penetration of retinaldehyde was observed in these films,

2.2. MATERIALS AND METHODS

2.2.1. *Chemicals*

Phosphatidylethanolamine, containing 50% palmitic acid, 30% oleic acid and 20% linoleic acid, as confirmed by gas chromatographic analysis, was synthesized according to Daemen (1967). Phosphatidylcholine was isolated from egg yolk according to Pangborn (1951). Gas chromatographic analysis showed the following fatty acid composition: 30% palmitic acid, 14% stearic acid, 26% oleic acid, 12% linoleic acid, 13% linolenic acid and 4% arachidonic acid. Both phospholipids were pure when examined by thin-layer chromatography. Stock solutions of the phospholipids (1.6 mM) in n-hexane (Merck) were stored at —20° under nitrogen. All-trans-retinol and all-trans-retinaldehyde were obtained from Distillation Products (Rochester, N.Y., U.S.A.). For penetration experiments 4.6 mM solutions in absolute ethanol were prepared fresh each week and stored in the dark at —20°.

2.2.2. *Isolation of cattle rod outer segment preparations*

Retinae from 60-80 dark adapted cattle eyes were briefly homogenized in saline (0.5 — 1 ml per retina) in a Potter Elvehjem homogenizer by moving the loosely fitting teflon pestle (1.8 mm clearance) 40 times slowly up and down to detach the outer segments from the rest of the retina (Mc Connell, 1965). After filtration through a 120-mesh stainless steel wire screen the filtrate was mixed with 66.7% (w/w; 2.52 M) aqueous sucrose solution to a final concentration of 0.42 M. From this suspension and an aqueous 1.28 M sucrose solution continuous gradients with a density range of 1.05-1.16 (0.42-1.28 M) were prepared in four 45 ml centrifuge tubes. Centrifugation at 13200 x g for 90 min gave a purple layer containing rod outer segments (ROS) in the middle of the tubes. Occasionally this layer was separated into two closely adjacent layers with identical spectral properties. The ROS containing layers were isolated and diluted with 5 volumes of saline.

The insoluble material was collected by centrifugation, and washed and sedimented twice with distilled water. The final sediment was generally lyophilized. One retina yielded 1-1.5 mg of lyophilized material. All manipulations were carried out in dim red light or darkness. In the rest of this chapter this material is referred to as rhodopsin, although it contains also other rod outer segment membrane components. Opsin was obtained by illumination of this preparation.

2.2.3. *Monolayer experiments*

Monolayer experiments were carried out in a teflon trough of 20 cm x

5 cm x 1 cm. Surface tension was measured with a conventional torsion balance (V.D.F., United, 250 mg range, Nijmegen, The Netherlands) and a microscopic coverslip (18 mm x 18 mm x 0.15 mm) attached to a platinum-iridium wire was used as a Wilhelmy plate.

The pH of the subphase was varied by means of citric acid — phosphate buffer (0.12 M, pH 3.0), phosphate buffer (0.07 M, pH 5.3, 6.3 and 7.4) or borax-HCl buffer (0.06 M, pH 9.0). Twice-distilled water from an all-glass still was used throughout. Before each experiment the water surface was cleaned by moving the two teflon barriers towards each other, removing any surface film by suction and subsequently returning the barriers to their initial position. This procedure was repeated until the surface tension of the cleaned surface reached a value of 71-72 dyne/cm.

Monolayers of rhodopsin were prepared in dim red light by bringing approx. 0.5 mg dry rhodopsin onto the fluid surface in the trough, which resulted in an immediate large drop in surface tension. Monolayers of opsin were prepared in the same way, but the procedure was carried out under normal laboratory light conditions. Illumination experiments were carried out with a normal tungsten lamp (75 W, placed 30 cm above the trough). The time of illumination was 30 seconds.

Penetration of retinaldehyde or retinol was studied by bringing, with a microsyringe (Hamilton nr. 701 N), predetermined volumes of their ethanolic solutions beneath a rhodopsin, opsin or phospholipid monolayer of pread-justed film pressure and by measuring the resulting increase in film pressure. The addition was once repeated. The quantity of penetrating substance was adjusted according to the surface left between the barriers (normally between 25 and 50 cm^2). Per cm^2 0.05 µl of a 4.6 mM solution of retinaldehyde or retinol in absolute ethanol was applied each time. Application of solvent alone had no effect on the film pressure.

Since the results of penetration experiments upon phospholipid monolayers in dim red light were the same as those obtained under normal laboratory illumination (fluorescent light, 100 lux), all further experiments with phospholipid films were carried out in the light.

Force-area curves (Gaines, 1966) of the phospholipid films were measured as follows. With a micropipette 5 µl of a 1.6 mM phospholipid solution were applied onto the subphase surface. After evaporation of the solvent the barriers were moved along the length of the trough in steps of 1 cm and the film pressure (surface tension of pure subphase minus surface tension of subphase with monolayer) was measured as a function of the area left for the phospholipid.

The amount of retinaldehyde remaining in the aqueous subphase was

determined by extracting 20 ml of the subphase with freshly distilled chloroform, evaporating the solvent and adding to the residue 500 µl propanol. In 300 µl of this solution retinaldehyde was determined by the thiobarbituric acid reaction using the method of Futterman and Saslaw (1961) at a tenfold reduction in scale. The extinction at 530 nm was corrected by the reading from an equal volume of subphase not penetrated by retinaldehyde. The measured amount was referred to the total subphase volume and was expressed as a fraction of the total amount of retinaldehyde used for penetration.

2 2.4. *Absorption spectra*

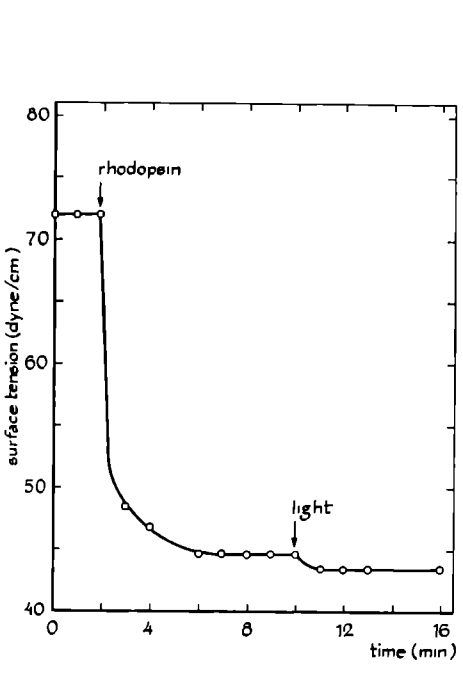
Absorption spectra of the monolayer material could be obtained in two ways. In the first procedure, the surface was compressed into the smallest possible area, transferred with a 50 µl micropipette to a microcuvette, where it was mixed with an equal volume of aqueous, neutralized 1% digitonin solution. In an alternative procedure the absorption spectrum was obtained from a set of 25 parallel microscopic slides (held at 3 mm intervals in a slotted teflon block), which had been dipped into the trough (Trurnit and Colmano, 1959). With both methods difference spectra of monolayers, either penetrated or nonpenetrated by retinaldehyde, were obtained by means of a Zeiss PMQ II spectrophotometer. The coating technique was applied because it appeared to offer less opportunity for aldimine formation after sampling of the monolayer than in the procedure, whereby monolayer material is suspended in digitonin solution. With both techniques similar spectra for retinaldehyde were recorded, indicating that the results obtained are comparable.

2.3. RESULTS

2.3.1. *Monolayers of rhodopsin and opsin*

Application of lyophilised rod outer segment material on a water-air interface markedly decreased the surface tension of the water phase (i.e. increased the film pressure) resulting in a monolayer with a film pressure of 15-40 dyne/cm. After 10-15 minutes equilibrium was reached which was indicated by constancy of the film pressure. Illumination resulted in a further small increase in film pressure as illustrated in fig. 5.

Penetration of retinaldehyde into rhodopsin and opsin monolayers also caused an increase in film pressure (fig. 6). The effect did not differ considerably between rhodopsin and opsin monolayers. At the high initial film pressure of 40 dyne/cm, which is near the collapse point of the monolayers, there was still an increase in film pressure.



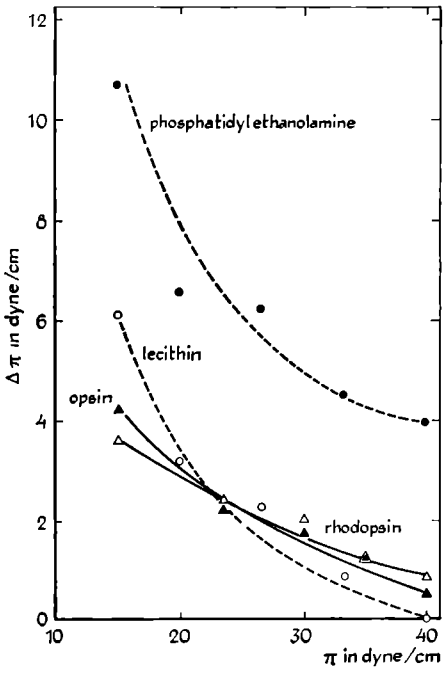
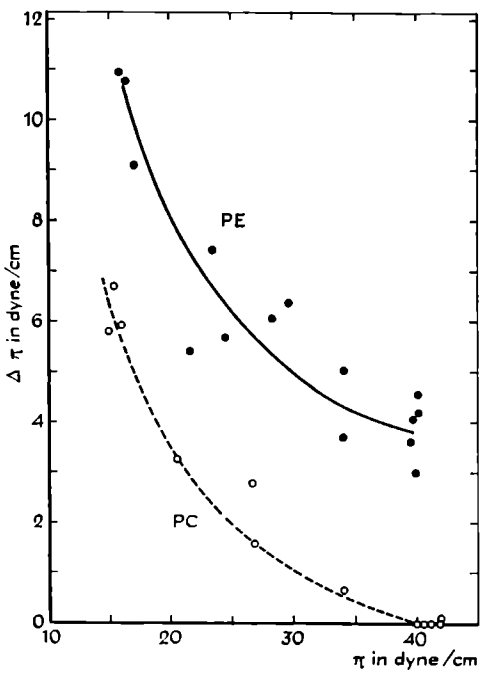


Fig. 5 Effect of monolayer formation and photolysis of rhodopsin on surface tension. The pH of the subphase was 7.4.
Fig. 6 Increase of film pressure ($\Delta\Pi$) upon penetration of all-trans-retinaldehyde into monolayers of rhodopsin, opsin, phosphatidycholine (lecithin) and phosphatidylethanolamine. The pH of the subphase was 7.4.

2.3.2. *Phospholipid monolayers*

The residual penetration of all-trans retinaldehyde at high initial film pressure (Π = 40 dyne/cm) into a phosphatidylethanolamine monolayer reported by Bonting and Bangham (1967) was confirmed. The increase of film pressure ($\Delta\Pi$) at a subphase pH of 7.4 was 3.77 dyne/cm (S.E. 0.42, 6 detns), while for penetration of all-trans retinaldehyde in a phosphatidylcholine monolayer the increase was not significantly above zero ($\Delta\Pi$ = 0.15 dyne/cm, S.E. 0.15, 6 detns.).

Extending the measurements of the penetration of all-trans retinaldehyde into monolayers of phosphatidylethanolamine and of phosphatidylcholine over the range Π = 15 to 40 dyne/cm gave the curves shown in fig. 7. The increase of film pressure over the entire range was higher for phosphatidylethanolamine monolayers than for phosphatidylcholine monolayers. Analysis of retinaldehyde present in the aqueous subphase showed that in no case more than 6% of the retinaldehyde remained in the subphase.



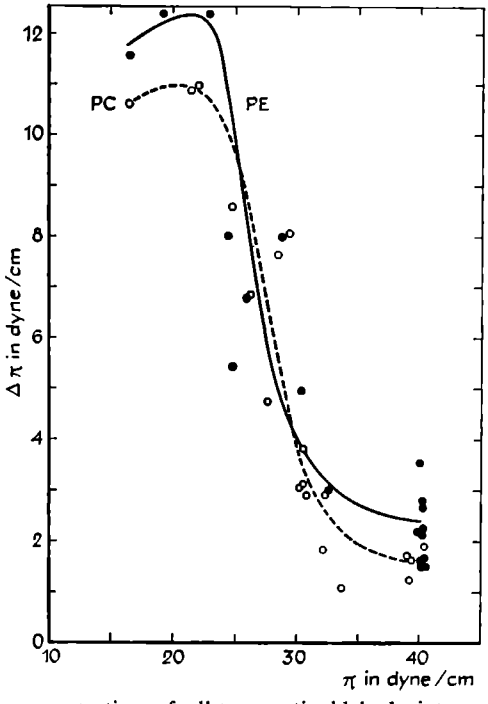


Fig. 7 Increase ($\Delta\Pi$) of film pressure upon penetration of all-trans-retinaldehyde into monolayers of phosphatidylethanolamine (PE) and phosphatidylcholine (PC) as function of initial film pressure (Π). The pH of the subphase was 7.4.
Fig. 8 Increase ($\Delta\Pi$) of film pressure upon penetration of all-trans-retinol as a function of initial film pressure (Π). The pH of the subphase was 7.4. For abbreviations see Fig. 7.

When these two types of monolayers were penetrated by the same amounts of all-trans retinol there was virtually no difference in behavoir of the two phospholipids (fig. 8). Comparison of figs. 7 and 8 shows moreover that the shape of the penetration curves for retinaldehyde and retinol was quite different.

2.3.3. *Variation of the subphase pH*

It seemed desirable to study the effect of changing the subphase pH upon penetration, since formation of an aldimine depends on pH. Alkaline pH favours aldimine formation, because this reaction requires a non-protonated amino group. Therefore, the pH of the subphase was varied between 3.0 and 9.0. Fig. 9 indicates that only at pH $>$ 7.5 the extra penetrating effect of retinaldehyde into monolayers of phosphatidylethanolamine occurs maximally. This applies for initial surface pressures of 20, 30, and 40 dyne/cm.

For monolayers of phosphatidylcholine there was no dependence of penetration upon the pH of the subphase (Fig. 9). In this case $\Delta\Pi$ was about the same as for the penetration of retinaldehyde in phosphatidylethanolamine monolayers at pH < 6. This effect is not due to a greater pH dependence of the phosphatidylethanolamine monolayer itself as compared to phosphatidylcholine. This could be concluded from the following observations: first, penetration of retinol into phosphatidylethanolamine monolayers was not pH dependent (Fig. 10) and secondly, force-area curves of this phospholipid coincided over a subphase pH range of 3.0-9.0.

2.3.4. *Spectral studies*

It is known that aldimine formation displaces the absorption maximum of retinaldehyde to shorter wavelengths. Therefore, difference spectra of phosphatidylethanolamine, penetrated with retinaldehyde, as compared to the corresponding nonpenetrated material, were obtained from suspensions

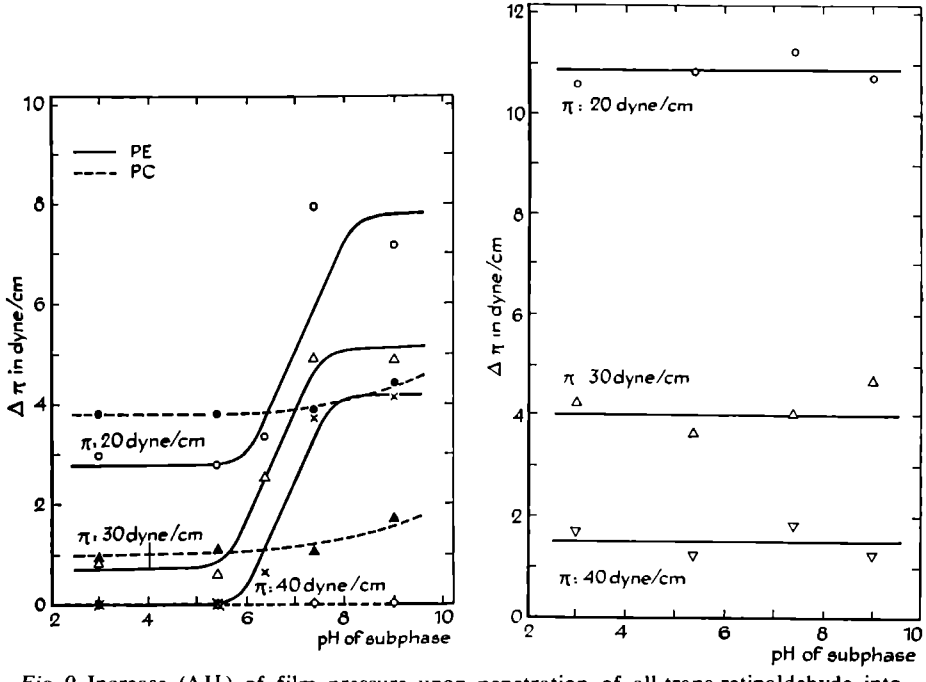


Fig. 9 Increase ($\Delta\Pi$) of film pressure upon penetration of all-trans-retinaldehyde into phospholipid monolayers as a function of subphase pH at initial film pressures (Π) of 20,30 and 40 dyne/cm. For abbreviations see Fig. 7.

Fig. 10 Increase ($\Delta\Pi$) of film pressure upon penetration of all-trans-retinol into phosphatidylethanolamine monolayers as a function of subphase pH at initial film pressures (II) of 20,30 and 40 dyne/cm.

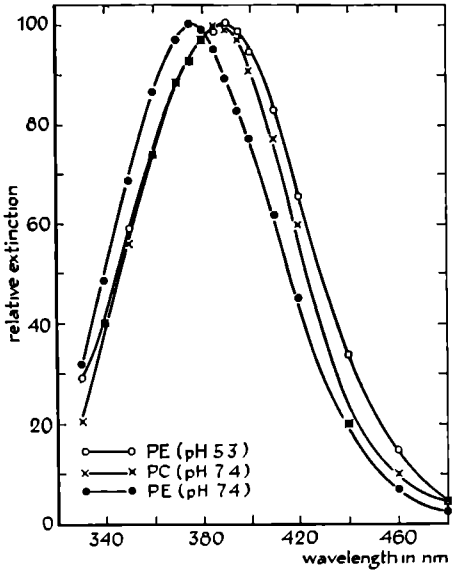


Fig 11 Absorption spectrum of phospholipid monolayer material (dissolved in an equal volme of 1% digitonin) after penetration of all-trans-retinaldehyde on subphases of pH 5 3 and 7 4 The spectra shown are difference spectra of penetrated and non-penetrated material Absorbance at the absorption maxima is arbitrarily set at 100, absorbance at 500 nm at 0 For abbreviations see Fig 7.

of monolayer material in an equal volume of 1% aqueous, neutralized digitonin (Fig. 11). After penetration on a subphase of pH = 5.3 a difference spectrum was obtained with a maximum between 385 and 390 mm, which coincides with the maximum for free retinaldehyde in aqueous digitonin solution. Similar difference spectra, after penetration on a subphase of pH = 7.4, gave an absorption maximum between 370 and 375 mm After penetration of retinaldehyde into phosphatidylcholine films the difference spectrum, independent of the subphase pH, again coincided with the spectrum for free retinaldehyde A similar shift in absorption maximum was obtained after penetration of retinaldehyde into a phosphatidylethanolamine monolayer at a subphase pH of 9 0 after coating the monolayer material on a set of parellel glass slides (Fig. 12).

2.4. DISCUSSION

2.4 1. *Phospholipid monolayers*

Penetration of retinol into monolayers of phosphatidylcholine and phosphatidylethanolamine gave approximately equal increases ($\Delta\Pi$) in film pressure (Fig 8) Replacing retinol by retinaldehyde, i e. changing an alcohol

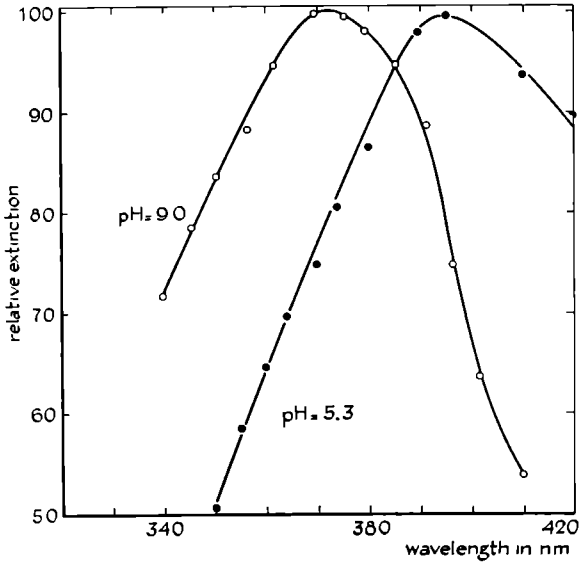


Fig 12 Absorption spectra of phosphatidylethanolamine monolayer material (coated on parallel glass slides) after penetration of all-trans-retinaldehyde on subphases of pH 5 3 and 9 0 Difference spectra were obtained as described in Fig 11

by an aldehyde group, yielded a much higher value for $\Delta\Pi$ upon penetration into a monolayer of phosphatidylethanolamine than upon penetration into a phosphatidylcholine film (Fig 7) An interaction between the two polar end groups, viz the aldehyde group of retinaldehyde and the amino group of phosphatidylethanolamine, could explain this phenomenon The most likely type of interaction would be the formation of an aldimine between the aldehyde group of retinaldehyde and the amino group of phosphatidylethanolamine The quaternary ammonium group of phosphatidylcholine, which replaces the amino group of phosphatidylethanolamine, cannot form an aldimine

Spectroscopic evidence for the formation of an aldimine upon penetration of retinaldehyde in phosphatidylethanolamine film was obtained as follows. From the work of Pitt et al (1955) it is known that a retinylidene imine, depending upon the medium used, has an absorption maximum at 360-365 nm, about 25 nm below the absorption maximum of free retinaldehyde (385-390 nm) Measurement of the absorption spectra of monolayers, penetrated with retinaldehyde at a subphase pH of 7 4 and 9 0, showed a similar shift in the case of phosphatidylethanolamine, but not of phosphatidylcholine This result was obtained whether the monolayer material was present in 0 5% digitonin in a cuvette (Fig 11) or as a coat on a set of parallel

glass slides (Fig. 12). Acidification of the suspension in digitonin returned the absorption maximum to 390 nm in the case of phosphatidylethanolamine, which is due to the instability of the retinylidene imine at moderate acid pH (Morton and Pitt, 1955).

Further evidence in favour of aldimine formation follows from Fig. 9 where $\Delta\Pi$ for the penetratation of retinaldehyde into a phosphatidylethanolamine monolayer is plotted as a function of the pH of the subphase. This figure shows that the film pressure began to increase at subphase $pH > 6$, reaching a maximal value at subphase $pH > 7.5$. In accordance with this observation no spectral shift was noticed after penetration of retinaldehyde into phosphatidylethanolamine monolayers at a subphase of pH 5.3 (Fig. 11 and 12). Neither of these pH effects occurred with phosphatidylcholine monolayers (Fig. 9). Morton and Pitt (1955) showed that a retinylidene imine can only be formed with a non-protonated amino group, which requires a $pH > 6$. Therefore, we must conclude that aldimine formation cannot occur in a monolayer on a subphase with $pH < 6$, which explains the absence of the extra increase in film pressure at subphase $pH < 6$ and the absence of a shift in the absorption maximum at subphase pH 5.3. At first sight the apparent pK of about 7, indicated by Fig. 9 for the aldimine formation, might seem in contradiction to the pK of 9 (Garvin and Karnovsky, 1956) for the protonation of the aminogroup of phosphatidylethanolamine. The explanation for this apparent discrepancy must be that the removal of non-protonated phospholipid by retinaldehyde shifts the equilibrium between the protonated and non-protonated forms of the phospholipid to the latter form (see also chapter 4).

An alternative explanation for the effect of the subphase pH upon retinaldehyde penetration in a phosphatidylethanolamine monolayer would be that the subphase pH might affect the packing of the phospholipid molecules. However, this explanation is quite unlikely, since neither the force-area curves for phosphatidylethanolamine films, nor the penetration of retinol into these films (Fig. 10) were affected by the subphase pH. Moreover, in the absence of aldimine formation deprotonation of the phospholipid amino group and a related change in packing would occur at a pH of about 9, rather than at pH 7. On the basis of these observations we may conclude that upon penetration of retinaldehyde into phosphatidylethanolamine films at neutral pH a retinylidene imine is formed, and that this reaction can explain the residual penetration of retinaldehyde into these monolayers at high film pressures.

2.4.2. *Rhodospin monolayers*

Can the residual penetration of all-trans retinaldehyde into rhodopsin monolayers at high film pressures (fig. 6) also be explained in terms of aldimine formation? The retinaldehyde content of these rod outer segment preparations is 0.14% (Borggreven et al, 1970). This means that per gram rod outer segment preparation 4.9 μmoles of retinaldehyde are present. The phospholipid content is 31.5% and half of these phospholipids contain amino groups (Borggreven et al, 1970). Assuming an average phospholipid molecular weight of 750, there are 210 μmoles phospholipid amino groups present per gram dry weight of rod outer segments. Hence, per retinaldehyde residue 43 phospholipid amino groups are present in the rhodopsin monolayers. Since there are no free N-terminal-amino groups in rhodopsin (Albrecht, 1957; Heller, 1968), the only free amino groups present are the ε-amino groups of lysine. Per rhodopsin molecule about 10 molecules of lysine are found (Shields et al, 1967:13; Heller, 1968:10; Shichi et al, 1969:8).

Disregarding the presence of amino groups in protein other than that in rhodopsin, and adding the phospholipid and protein amino groups together, there are 53 free amino groups per molecule of retinaldehyde. In the rhodopsin monolayers (0.5 mg) about 2.5×10^{-9} moles visual pigment are present. In the penetration experiments 2-4 times as much retinaldehyde is used. This means that per penetrating molecule of retinaldehyde about 18 molecules free amino groups are available. Even if only part of these molecules would be accessible at the air-water interface, there would still be an excess of free amino groups available for reaction with retinaldehyde. Considering the easy formation of an aldimine in the phosphatidylethanolamine monolayers, where the number of amino groups was only slightly higher than the number of retinaldehyde molecules added, it appears very likely that aldimine formation can explain the residual penetration of retinaldehyde in rhodopsin and opsin monolayers.

It is also quite likely that the effect of light upon rhodopsin monolayers (fig. 5) is due to aldimine formation between retinaldehyde and a membrane aminogroup. Poincelot et al (1969) showed that illumination of rhodopsin leads to a transiminisation of retinaldehyde from its original binding site (a phosphatidylethanolamine molecule) to an ε-amino group of lysine in the metarhodopsin I → metarhodopsin II transition. The latter amino group is, in contrast to the first one, located in a hydrophilic environment, since metarhodopsin II in a detergent solution hydrolyses into retinaldehyde and opsin (Matthews et al, 1963) and since metarhodopsin II can be reduced with aqueous sodiumborohydride (Ahktar et al, 1965; Bownds and Wald,

1965). Neither hydrolysis nor reduction occur in rhodopsin and metarhodopsin I, which indicates that in these compounds the aldimine is located in a hydrophobic environment. Such a transiminsation would also be expected to occur in the rhodopsin monolayers upon illumination, and leading to an aldimine formation between retinaldehyde and an ε-amino group of lysine at the air-water interface. This reaction would then cause the increase in film pressure observed under these conditions.

Two alternative explanations of the light effect in rhodopsin monolayers, namely the cis-trans isomerisation of retinaldehyde and a protein conformational change, appear to be less likely. Cis-trans isomerisation in films of free retinaldehyde leads to a decrease in film pressure, rather than an increase (Maeda and Isemura, 1967). Moreover, considering the small size of the retinaldehyde group relative to the opsin moiety, the mere cis-trans isomerisation of the retinaldehyde residue would be expected to have little if any effect on the packing of the rhodopsin molecules in the film. Protein conformational changes during photolysis of rhodopsin, as observed by Crescitelli et al (1966) and by Kito and Takezaki (1966), would also be expected to have little effect on the film pressure of the rhodopsin monolayer, which has a predominantly phospholipid character, as indicated by its collapse pressure of approx. 46 dyne/cm and the absence of a hysteresis effect upon compression and decompression. A conformational change could explain the increase of film pressure upon illumination only if the arrangement of the phospholipids in the phospholipid-rhodopsin complex would be altered.

2.4.3. *The in vivo situation*

Since formation of an aldimine between retinaldehyde and a phospholipid amino group has been shown to occur in a phosphatidylethanolamine monolayer at room temperature and neutral pH and probably also in a rhodopsin monolayer upon illumination, it seems reasonable to assume that the same reaction can occur in vivo in a rod sac upon photolysis of rhodopsin. This assumption is supported by the fact that the visual pigments in a membrane are located in a type of monomolecular arrangement. However, if this reaction is to be involved in visual stimulation, as proposed by Bonting and Bangham (1967), then it must occur in a time span of about one millisecond. The monolayer techniques employed in the present study cannot record such fast changes in surface tension, hence we are not able to answer this question definitively. All that can be said at the moment is that this aldimine would have to be formed at the metarhodopsin II stage of the photolytic process, and its observed absorption maximum (λ = 370-375 nm) agrees with that reported for metarhodopsin II (λ = 370-380 nm).

2.5. SUMMARY

Illumination of a rhodopsin monolayer leads to a small increase in film pressure. Penetration of all-trans retinaldehyde into rhodopsin and opsin monolayers gives an increase in film pressure, even at high initial film pressure.

Penetration of all-trans retinaldehyde into phosphatidylethanolamine monolayers gives a much higher increase of film pressure than penetration of the same substance into phosphatidylcholine monolayers. No such difference was observed upon penetration of all-trans retinol into monolayers of these two phospholipids. The extra penetration effect into phosphatidylethanolamine monolayers occured at a subphase pH above 7 and was always accompanied by a shift towards shorter wavelenghts of the absorption maximum of the monolayer material as compared to the spectrum of retinaldehyde itself.

These observations indicate the formation of an aldimine between retinaldehyde and phosphatidylethanolamine in the monolayer under the conditions mentioned. Formation of an aldimine between retinaldehyde and free amino groups could also explain the observed increase in film pressure upon penetration of retinaldehyde into an opsin or a rhodopsin monolayer and probably also the increase in film pressure obtained upon illumination of rhodopsin.

LIGHT INDUCED ION MOVEMENTS IN THE RETINA OF THE CUTTLEFISH (SEPIA OFFICINALIS)

3.1. INTRODUCTION

It has often been suggested that the light stimulated potentials observed in photoreceptor cells of invertebrates such as Limulus (Kikuchi, Naito and Tanaka, 1962; Stieve, 1965; Millecchia, Bradbury and Mauro, 1966), crayfish (Eguchi, 1965), squid (Hagins, Zonana and Adams, 1962; Hagins, 1965), barnacle (Brown et al, 1969) and the honeybee (Fulpius and Baumann, 1969) are a direct consequence of an increase in the sodium permeability of the photoreceptor cell membranes. However the evidence for this supposition has been exclusively provided by electrophysiological experiments, in the absence of information on the ion content of photoreceptor cells, except for one brief report (Adams and Hagins, 1960).

In a second short note Hagins and Adams (1960) reported experiments in which the effect of light on the ^{42}K efflux from the dark adapted squid retina was studied. Surprisingly, they found little or no effect of light pulses and moreover, they also reported that "light adaptation that reduced the retinal sensitivity 30-fold left the fluxes unaltered and the potassium content of the cells essentially undisturbed".

The absence of an effect on the efflux is surprising, since in most tissues (Sjodin and Mullins, 1967; Duncan, 1969 b) the potassium movement is sensitive to changes in the transmembrane potential. Since light causes a depolarization of the squid photoreceptor cell (Hagins et al, 1962) an increased potassium efflux would be expected. Furthermore, Duncan and Bonting (1969) have argued that if this depolarization is a result of a change in the passive permeability of the membranes and not a result of a change in the eletrogenicity of a membrane pump, as suggested by Smith et al (1968), a change in cation levels must then be invoked to explain the steady state component of the receptor potential. Therefore, as an electrogenic pump does not seem to contribute to the photoreceptor potential (Millicchia and Mauro, 1969 b; Brown et al, 1969), there should be a change in the photoreceptor cation levels if the ionic mechanism is to hold true.

We have therefore investigated the cation distribution in the photoreceptor cells of the invertebrate Sepia officinalis. This species was chosen, partly because of its availability to us, and partly because of the ease with which

the retina of this cephalopod is removed from the eye as compared to other cephalopods. These retina's are rich in photoreceptor cells and the outer segment or rhabdome layer constitutes two thirds of the retinal volume (Zonana, 1961; Young, 1962 a). We have also used in this study the isotope ^{86} rubidium. This ion behaves like potassium, which should contribute most to the resting potential and resting conductance of the photoreceptor membrane. First we have studied the movement of rubidium in darkness and subsequently upon illumination. The effect of ouabain and of some retinaldehyde derivatives on this movement are also described in this chapter.

3.2. MATERIALS AND METHODS

3.2.1. *Dissection*

After a dark adaptation period of at least one hour in oxygenated seawater, the cuttlefish (Sepia officinalis) was killed by decapitation and the eyes were removed under dim red light. A deep cut was then made round the perimeter of the cornea and the lens and vitreous were pushed out. After removing the cornea completely, the eyeball was opened by incisions in the sclera. The retina was freed from the sclera while being immersed in artificial seawater (A.S.W.) in a petri dish. In contrast to Loligo and Octopus vulgaris, the retina could be easily removed without seriously damaging it. The isolated retina was divided into six or eight pieces, which were then transferred to a beaker containing 50 ml of A.S.W. Total time elapsing between death and isolation of retina was about 5 minutes.

3.2.2. *Solutions*

All experiments were carried out in modified artificial seawater (Katz and Miledi, 1968), containing 466 mM NaCl, 10 mM KCl, 11 mM $CaCl_2$, 54 mM $MgCl_2$ and 3 mM $NaHCO_3$, to which was added 10 mM Tris buffered to pH 7.8 with HCl. The solution was saturated with pure oxygen before use. In experiments, where preincubation in sodium-free solutions was required, the NaCl and $NaHCO_3$ were replaced by 469 mM choline chloride.

3.2.3. *Radioactive solutions*

Since these experiments were carried out at the Zoological Station, Naples, where ^{42}K was not available, ^{86}Rb was used because of its similar behaviour. It was obtained in aqueous solution with a specific activity 2-10 mC/mg Rb (Code RGS_1, Radiochemical Centre, Amersham, England). Approximately 50 µl of isotope were added to 50 ml A.S.W. to give a final activity of 2 µC/ml.

3.2.4. *Weight determinations*

The retina, held between forceps, was gently blotted with a corner of tissue paper and then transferred to a preweighed piece of aluminium foil which was then quickly reweighed to ± 0.1 mg to obtain the wet weight of tissue. After evaporating to dryness under an infrared lamp, the foil and retina were reweighed. A constant weight was obtained after about 5 hours.

3.2.5. *Flamephotometric measurements*

After this weighing procedure, the retina and foil (manipulated throughout with clean forceps) were placed in a dry polyethylene tube and 2 ml concentrated nitric acid were added. After overnight destruction of the tissue, 10-40 ml of double distilled water were added according to the weight of the retina. The sodium and potassium concentrations were determined by means of a Beckman model DU spectrophotometer with flamephotometer attachment, calibrated with standards containing equimolar amounts of sodium and potassium in the range 5×10^{-4} — 5×10^{-5}M. Addition of nitric acid to the standards in the same concentration as in the samples had no effect on the emission.

3.2.6. *Radioactivity measurements: Rubidium influx and efflux*

Pieces of retina were placed in pre-oxygenated seawater containing isotope. At various intervals of time, varying from 1-300 min, pieces were removed from the incubation medium, briefly washed for about ½ min in two changes of tracer-free A.S.W., lightly blotted and transferred to a preweighed piece of aluminium foil. After determination of the dry weight, followed by digestion in nitric acid, 20 ml distilled water were added, and after thorough mixing a 1 ml aliquot was transferred to a Nuclear Chicago counting planchette and dried under the infrared lamp. The planchettes were counted in the Geiger chamber of a Nuclear Chicago automatic low background counter (Model 0110-B with model 161 A Scaling unit). One-ml aliquots of the ^{86}Rb loading solution were made up to 100 ml with distilled water, and 1 ml of this dilution was dried and counted in the same way. Assuming that rubidium behaves identically to potassium, the ion uptake was calculated in mmole rubidium per lit. cell water, from the water content of the retina, the radioactivity of the retina in cts/min/ml and the potassium concentration of the loading solution in mM.

The efflux of rubidium was determined by means of a method previously developed for the toad lens (Duncan, 1969 a). Retinal pieces, which had been incubated for 1½-5 hours in radioactive A.S.W., were briefly washed in two dishes of inactive medium, attached to a thin metal hook, and then

transferred to the first of a series of planchettes, each containing 2 ml of oxygenated A.S.W. The retina remained in each dish for exactly one minute and after the last dish, the retina was gently blotted, weighed and the isotope content analysed as before. The radioactivity in each planchette was determined after drying. The activity in the retina at the beginning of the efflux period was found by adding the counts in each of the dishes to the activity present in the retina at the end of the experiment. Subtracting counts in dish 1 from the total gave the activity in the retina at the end of 1 minute; subtracting the counts in dish 1 and 2 gave the activity after 2 minutes. In this way the activity in the retina could be determined at any given time during the efflux period.

3.2.7. *Light experiments*

Prior to illumination, rubidium influx and efflux experiments were carried out in darkness or in dim red light. After six minutes, the rhabdomal side of the retina was illuminated for 15-60 sec with white light from a tungsten filament microscope lamp. The energy of the light was measured with a YSI Kettering model 65 Radiometer.

At the maximum intensity the total light energy was 8×10^5 erg/cm²/sec. From the known emission spectrum of a tungsten lamp at 3000° K, we calculated that 5.5% of this energy consists of light of wavelengths 400 to 600 nm. This is equivalent to 9.5×10^{15} photons/cm²/sec. Correction of this figure by means of the relative absorption by the Sepia rhodopsin between 400 and 600 nm (Brown and Brown, 1958) gives a number of 5.6×10^{15} effective photons absorbed per cm² per sec at the highest intensity. Lower light intensities, down to 6×10^{-4} of this intensity, were obtained by the use of calibrated neutral density filters.

The number of photons per microvillus in our experiments can be calculated as follows. The cephalopod retina contains about 5×10^6 photoreceptor cells (Young, 1962 a), with an average of 5×10^5 microvilli per cell (Zonana, 1961). This means that at the lowest light intensity (5×10^2 erg/cm²/sec or 3.5×10^{12} effective photons/cm²/sec) during 30 sec illumination each microvillus would receive approximately 42 effective photons. The squid retina contains 1.5×10^{16} molecules of rhodopsin per cm² (Hubbard and St. George, 1958); hence at the lowest light intensity 0.7% of the rhodopsin and at the highest intensity all the rhodopsin could be subject to photolysis. Illumination even at the highest intensity did not significantly increase the temperature of the A.S.W. In most experiments, the retina was illuminated from the 15th to the 45th second of the minute in dish seven. The rate constant in this minute was determined as described in 3.2.6. The rate constant for the

first 45 seconds after switching on the light was calculated by assuming that the rate constant in the first 15 sec (darkness) was the same as in the preceding minute.

3.2.8. *Chemicals*

Analytical grade reagents were used in the preparation of all solutions. Ouabain (Merck, Darmstadt) was stored at 0° C and was dissolved directly in oxygenated A.S.W. when required. Retinaldehyde, retinol and retinoic acid; Eastman Kodak) were stored in small portions at —20° C in vacuo in the dark. These chemicals and also 2,4-dinitrofluorobenzene (DNFB) were dissolved in absolute alcohol immediately before use, and 20 µl were added to the efflux dishes containing 2 ml A.S.W. directly after transferring the retina to the dish.

3.3. RESULTS

3.3.1. *Water and ion content*

The water content of the Sepia retina, 81 (S.E. = 1)% of the total wet weight, agrees well with that found by Adams and Hagins (1960) for the squid retina. The retina was incubated in sodium-free A.S.W. solution (choline replacing Na^+, all other ions present in normal concentration) in order to determine the intracellular concentrations of the major cations. During the first 15 min incubation in Na-free A.S.W. the Na^+ concentration decreased considerably, whilst the K^+ concentration remained virtually constant (Fig. 13). The relatively rapid loss of a large fraction of sodium

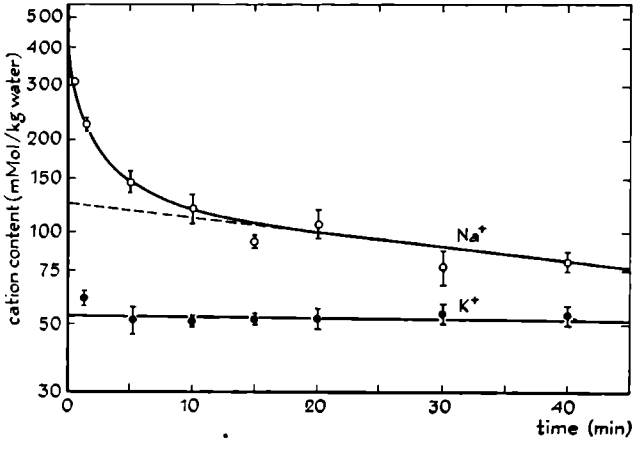


Fig. 13 Loss of sodium and potassium from sepia retina incubated in a sodium-free A.S.W. solution.

ions ($t_{1/2}$ = 1½′) was followed by a slow loss ($t_{1/2}$ = 60′). The rapid initial loss must be due to the washing out of extracellular Na^+, while the slow loss appears to be an exchange of intracellular Na^+ by choline ions.

The mean total sodium concentration of the retina was initially 420 mM while the mean concentration after loss of the extracellular compartment, but extrapolated back to t = 0, was 130 mM (Fig. 13). Now if x is the fraction of the retinal volume occupied by cells and (1 — x) is the extracellular fraction, then

$$xNa_i + (1 - x)Na_e = 420\text{mM} \quad (1)$$

and

$$xNa_i = 130\text{ mM} \quad (2)$$

where Na_i and Na_e are the concentrations of sodium in the intracellular and extracellular spaces respectively. If Na_e is taken as equal to that of A.S.W., i.e. 470 mM, then x = 0.38 and Na_i = 340 mM.

Similarly, for potassium

$$xK_i + (1 - x)K_e = 61\text{ mM} \quad (3)$$

and

$$K_e = 10\text{ mM, hence } K_i = 145\text{ mM} \quad (4)$$

3.3.2. *^{86}Rb influx*

The concentration of ^{86}Rb in the tissue was calculated from the radioactivity in the tissue and the incubation medium and the potassium concentration in the incubation medium assuming that the ^{86}Rb/K ratio does not differ between incubation medium and tissue. The concentration is expressed as mM rubidium. For the calculation of the intracellular values of ^{86}Rb in the dark, the extracellular contribution of 6.2 mM was subtracted from the total amount of ^{86}Rb in the retina and the resulting number was multiplied

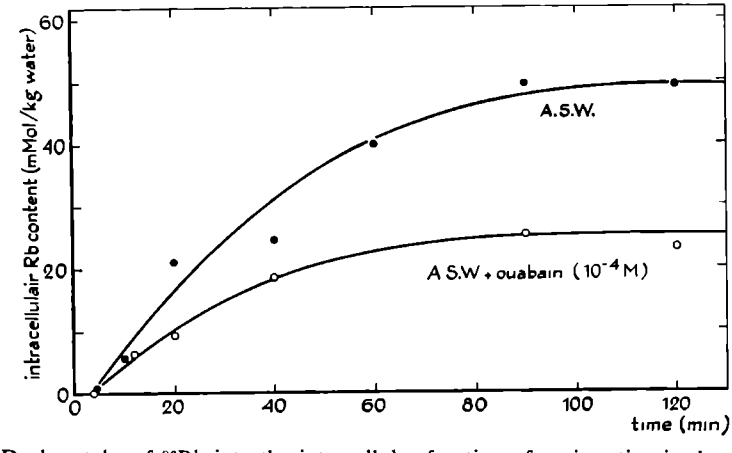


Fig. 14 Dark uptake of ^{86}Rb into the intracellular fraction of sepia retina in the absence and presence of ouabain.

TABLE I
EFFECT OF INCUBATION IN A.S.W. ON THE ^{86}Rb CONTENT OF THE RETINA

Time	90′	120′	150′	180′	240′	300′	average
Total ^{86}Rb content in A.S.W.	27.9 (4)	24.3 (9)	24.6 (9)	26.7 (12)	26.7 (13)	21.4 (5)	25.5 ± 0.7 (52)
Intracellular ^{86}Rb content in A.S.W.	57.7 (4)	47.6 (9)	48.2 (9)	54.0 (12)	54.0 (13)	17.2 (3)	50.8 ± 1.8 (52)
Total ^{86}Rb content in A.S.W. + ouabain	16.0 (1)	15.2 (2)	15.2 (3)	16.0 (8)	19.5 (2)	29.2 (3)	16.3 ± 0.4 (19)
Intracellular ^{86}Rb content in A.S.W. + ouabain	25.8 (1)	23.7 (2)	23.7 (3)	25.8 (8)	35.0 (2)	41.6 (5)	26.7 ± 1.0 (19) *

* 10^{-4}M ouabain: 27.9 ± 1.4 (12)
10^{-3}M ouabain: 24.7 ± 1.5 (7)

A.S.W. = artificial seawater.
All concentrations given in mmol/kg water.
The intracellular values are calculated by subtracting 6.2 from the total values and by multiplying the difference by 1/0.38, i.e. the ratio of total to intracellular volume.

with 1/0.38, i.e. the ratio of total to intracellular volume. Thus was assumed that all the potassium in the extracellular space can be exchanged by ^{86}Rb in a few minutes, which is reasonable because the $t_{1/2}$ for the loss of extracellular sodium in sodium-free A.S.W. was only $1\frac{1}{2}$ min. Fig. 14 shows the uptake of ^{86}Rb into the intracellular fraction in the dark. It is obvious that rubidium is taken up against its concentration gradient. Such an accumulation should require an active pump mechanism, and indeed we have demonstrated in the photoreceptor layer of this retina a high activity of Na-K activated ATPase (2.67 moles ATP hydrolyzed per hour per kg dry weight) (see 6.3.1. Table XIII). It was therefore not unexpected to find (Fig. 14) in a parallel experiment in the presence of ouabain that the latter substance markedly inhibits the Rb uptake by blocking the pump mechanism. The half-time for uptake in normal as well as in ouabain-containing A.S.W. was approximately 30 min. The level of ^{86}Rb in normal A.S.W. remained constant from 90 to 300 min (Table I). This latter observation indicates that the retina is not significantly degenerating during prolonged incubation in A.S.W.

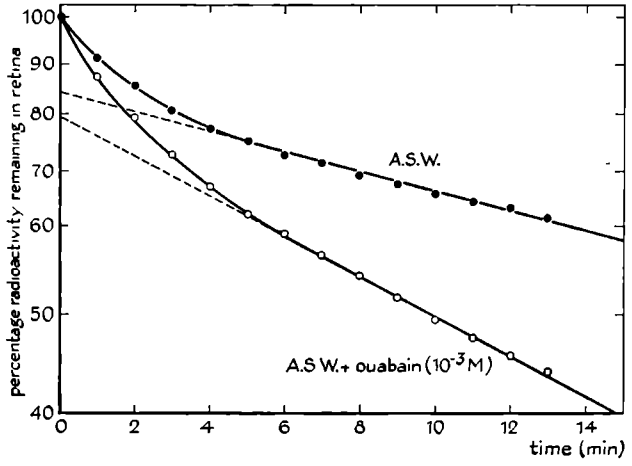


Fig. 15 Dark efflux of ^{86}Rb from sepia retina previously incubated for about two hours in radioactive A.S.W. The graphs show two exponential processes presumably corresponding to efflux of extracellular and intracellular rubidium, respectively. Application of ouabain doubled the efflux rate constant.

3.3.3. *^{86}Rb efflux in darkness*

The efflux of ^{86}Rb from retinas preincubated in radioactive solutions reveals that the Rb is distributed between two compartments, presumably corresponding to the intracellular and the extracellular space (Fig. 15).

The linearity of the log of the ^{86}Rb concentration vs. time after 5 min indicates that the intracellular space behaves as a single compartment. Thus we may write:

$$\frac{dRb_i}{dt} = -k(Rb_i)_t \qquad (5)$$

where Rb_i is the radioactivity in the intracellular compartment at time t and k is the rate constant in min^{-1}.

Integration gives

$$(Rb_i)_t = (Rb_i)_0 \exp(-kt) \qquad (6)$$

or $\ln(Rb_i)_{t_1} - \ln(Rb_i)_{t_2} = -k(t_1 - t_2)$, where $(Rb_i)_{t_1}$ and $(Rb_i)_{t_2}$ are the activities in the intracellular compartment at the beginning and end of an efflux period. As in our case $t_1 - t_2$ was 1 min, the rate constants can be immediately found from the internal activities at the beginning and end of each minute. The average rate constant from t = 6 to t = 13 for three experiments is 2.0×10^{-2} (S.E. 0.03×10^{-2}) min^{-1}, equivalent to a $t_{1/2}$ = 34 min. The mean rate constant for the efflux agrees with that for the influx, which suggests that the system is in a steady state with no net loss of potassium taking place. The rate constant for the slow efflux com-

ponent of the retinas incubated in the presence of ouabain is 4 x 10^{-2} (S.E 0.44 x 10^{-2}), 4 detns.) min ^{1}. This is significantly higher than the rate constant in normal A.S.W. (2 0 x 10 ^{2}, S.E. 0.03 x 10 ^{2}, 3 detns.)

When the radioactivity in the compartment represented by the slow efflux at any time during the first 5 minutes is subtracted from that in the whole retina, then the ^{86}Rb content of the extracellular compartment is obtained. When the log of the activity is plotted as a function of time (Fig. 16), it is seen that the loss is again a single exponential process The rate of loss in ouabain solutions is not different from that in normal seawater, nor from that of sodium in choline seawater, replotted from Fig. 13, and so it may be assumed that in each case the ions originate from the same compartment of the retina.

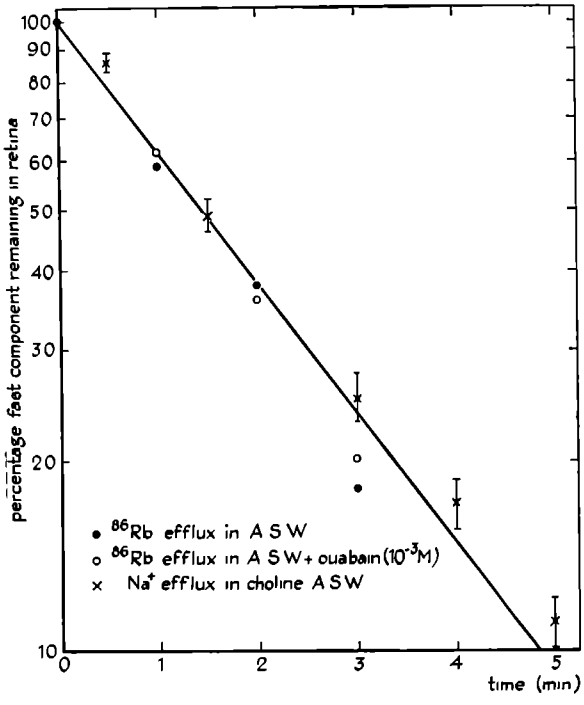


Fig 16 Initial fast efflux of sodium and ^{86}rubidium calculated from the graphs of figs 13 and 15 The coincidence of the graphs indicates that the sodium and rubidium originate from the same retinal fraction, presumably the extracellular space

3.3 4. *Rb efflux in light*

Illumination of the Sepia retina with intensities ranging from 3.5 x 10^{12} — 5.6 x 10^{15} effective photons/cm^{2}/sec resulted in every case in an increased rubidium efflux. Fig. 17 shows this effect for two retinas illuminated with

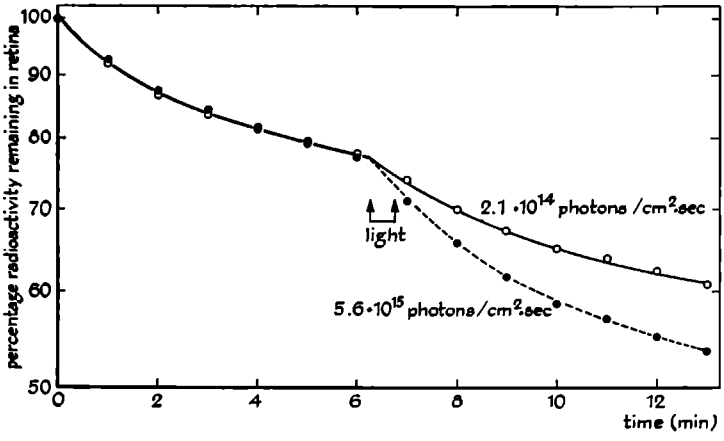


Fig. 17 The effect of light on the dark efflux of ^{86}Rb from sepia retina. The retinas were illuminated for 30 seconds with white light containing 5.6×10^{15} effective photons/cm²/sec (-●-●-●-) and 2.1×10^{14} effective photons/cm²/sec (-o-o-o-).

light of intensities 2.1×10^{14} and 5.6×10^{15} effective photons/cm²/sec respectively. After the light-stimulated increase the rate decreased again in the dark and approached the value for the dark adapted retina prior to illumination. The recovery process was found to take approximately 5 minutes at the highest light intensity and less at lower intensities.

A second illumination caused another increase in the rate constant, although the effect was usually much smaller than the initial one (Fig. 18). When the illumination period was short, or the intensity was low, responses to 3 or 4 consecutive flashes could be observed. The effect of varying the light intensity on the rate of loss of rubidium is presented in Table II. The efflux increased about 3-fold with a 1600-fold increase in light intensity.

TABLE II
EFFECT OF INTENSITY OF LIGHT ON RATE CONSTANTS of ^{86}Rb EFFLUX

;ht intensity in nber of "effective")tons/cm²/sec	0	3.5×10^{12}	3.5×10^{13}	3.5×10^{14}	1.4×10^{15}	5.6×10^{15}
te Constant ($\times 10^2$)	2.0	3.4	4.1	4.8	5.3	9.5
:. ($\times 10^2$) and nr. detns.	0.03 (3)	0.22 (4)	1.47 (4)	1.47 (3)	1.20 (2)	0.43 (3)

The retina was illuminated from the 15th to 45th second of minute 6, and the rate constants were calculated for the first 45 seconds after onset of illumination. The dark control rate constants were calculated for the period minute 6 to minute 12 from a separate set of experiments.

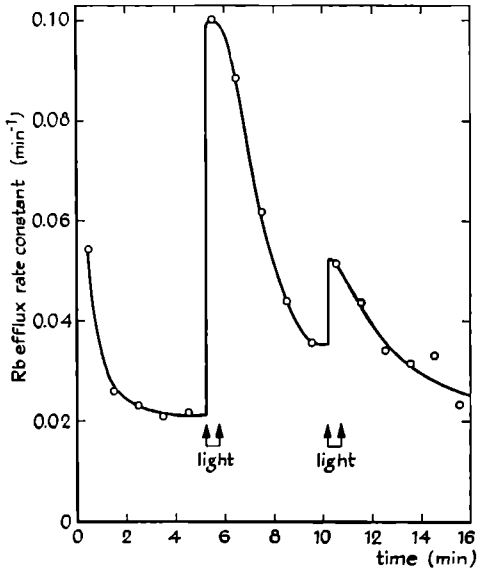


Fig. 18 The effect of light on the rate constant for Rb efflux. The efflux was carried out in the dark except for the periods 5'15 to 5'45 and 10'15 to 10'45 when the sepia retina was illuminated with white light of energy 5.6 x 10^{15} effective photons/cm²/sec.

Fig. 17 indicates that there was a change in the rubidium content of the retina after light stimulation. The extent of the change was calculated in the following way: extrapolating back to the ordinate, the line drawn through the three dark efflux points immediately prior to illumination gave the internal dark adapted rubidium level (at t = 0), while extrapolating the last three points on the graph gave the steady state level after illumination. In nine experiments in which light energies between 3.5 x 10^{12} and 1.4 x 10^{15} effective photons/cm²/sec were used, a net amount of 6.4 (S.E. 1.4)% of the initial dark adapted intracellular rubidium level was lost. While our data were not sufficiently accurate to differentiate among the various light intensities in this range, illumination with light of a higher intensity (5.6 x 10^{15} effective photons/cm²/sec) caused a significantly greater loss of rubidium. This can be attributed to the greater increase in efflux rate at this intensity.

3.3.5. *Effect of reagents*

The effect of light on the ^{86}Rb efflux could be imitated to a certain extent by 10^{-4}M retinaldehyde, while this effect was not obtained with lower concentrations of retinaldehyde (10^{-5} — 10^{-6}) or a 10^{-4}M solution of retinoic acid (Table III). Addition of 10^{-4}M retinol to the A.S.W. resulted in a

TABLE III
EFFECT OF ADDITION OF VARIOUS SUBSTANCES ON THE RATE CONSTANT OF ^{86}Rb EFFLUX

lded ɔstance	—	Retinalde-hyde (10^{-4}M)	Retinalde-hyde (10^{-5}M)	Retinalde-hyde (10^{-6}M)	Retinol (10^{-4}M)	Retinoic Acid (10^{-4}M)	DNFB ($2x10^{-3}$M)
te nstant & Ɛ. ($x10^2$)	2.0 ± 0.03 (3)	2.9 ± 0.18 (3)	2.1 (1)	2.1 (1)	2.5 (1)	2.1 (1)	2.7 ± 0.10 (2)

The test compounds were added to dishes 7-11 immediately after transferring the retina. The 5 rate constants during the test period were averaged and the mean and standard error were calculated from the averages of different experiments.
DNFB = 2 4 dinitro-fluoro-benzene.

smaller increase than that caused by 10^{-4}M retinaldehyde. Addition of DNFB (2×10^{-3}M, uncorrected for hydrolysis) also resulted in a significant increase in the rate constant.

In paragraph 3.3.3. we reported that addition of ouabain to the medium bathing the retina in darkness resulted in a marked fall in the rubidium uptake and also in an increased unidirectional-efflux. Illumination (5.6×10^{15} effective photons/cm^2/sec) of retinas treated with 10^{-4} or 10^{-3}M ouabain for 2-3 hrs resulted in a further increase in rate constant (Table IV). However, this increase was significantly less than that resulting from retinas illuminated in A.S.W. without ouabain.

TABLE IV
EFFECT OF LIGHT AND OUABAIN ON THE RATE CONSTANTS OF ^{86}Rb EFFLUX

	Rate constants and S.E. ($x10^2$) Dark	Light	Light/Dark
A.S.W.	2.0 ± 0.03 (3)	9.5 ± 0.43 (3)	4.75
A.S.W. + Ouabain	4.0 ± 0.44 (4)	7.0 ± 0.40 (7)	1.75

In the light experiments the retina was illuminated for 30 sec with white light of energy 5.6×10^{15} effective photons/cm^2/sec after a dark efflux of six minutes in isotope-free A.S.W. The rate constants were calculated for the first 45 sec after the onset of illumination. In the dark controls the rate constant of ^{86}Rb efflux was calculated for the sixth to the twelfth minute. Ouabain (10^{-3} or 10^{-4}M) was added to both influx and efflux solutions. After each value with its standard error the number of experiments is given between parentheses.

3.4. DISCUSSION

3.4.1. *Ion content of the retina*

The intracellular concentrations of sodium (Na_i = 340 mM) and potassium (K_i = 145 mM) and the extracellular space (x = 0.38), calculated from our choline experiments, differ rather considerably from those reported briefly by Adams and Hagins (1960) for squid (K_i = 300 mM, Na_i = 110 mM, x = 0.65). It would appear that this discrepancy cannot be due to a rapid degeneration of the Sepia retina with a large loss of K^+ and gain of Na^+, since the initial total K concentration of 61 mM (S.E.: 4, 2 detns. on 16 samples) was not significantly different from that after 90 min incubation in oxygenated A.S.W. (54 mM, S.E.: 6, 2 detns. on 16 samples). The constancy of the ^{86}Rb level from 90 to 300 min after isolation of the retina (Table I) indicates that this holds true even for periods up to 5 hrs. It seems rather unlikely that large cation changes would occur during the first minute after excising the retina and not thereafter. The low resistance (140 ohm cm^2) across the isolated squid retina (Hagins, 1965) suggests that during the excision of the retina a considerable number of extracellular channels between the photoreceptor cells are formed through which ions carrying the transretinal current can pass. This phenomenon might explain our low value of x for the excised retina. The exchange of sodium for choline, which might underlie the slow sodium loss ($t\frac{1}{2}$ = 60 min), cannot have affected the value of x, because the sodium values used in the calculation of x were extrapolated to t = 0.

The efflux rate constant (Fig. 16) will also give a measure of the diffusion coefficient of sodium and potassium in the extracellular space of the retina. The retinal pieces used in our experiments approximate to infinite sheets, as the thickness of 0.4 mm was much smaller than the surface diameter of approximately 10 mm. The time for loss of 50% of the radioactivity from the extracellular space will therefore be given by $t\frac{1}{2} = 0.1 \times r^2/D$, where r is the halfthickness of the retina and D the self-diffusion coefficient of the ions in the extracellular space (Briggs et al, 1961). In our experiments r was 200 μ and $t\frac{1}{2}$ was 1.5 min, hence D is 4×10^{-7} cm^2 sec^{-1}. As the free solution value for sodium and potassium is approximately 6×10^{-6} cm^2 sec^{-1} (Dainty and House, 1966), this 15 x lower value suggests that either the ions have to take a tortuous path from the cells to the outside medium, or that the diffusion coefficient itself is less, perhaps because of the frictional interaction with other molecules.

From the intracellular values for sodium and potassium it is possible to predict a value for α, the ratio of the membrane permeabilities of sodium (P_{Na}) and potassium (P_K). If it is assumed that the chloride ions are in

equilibrium across the membrane, the following equation (Hodgkin and Katz, 1949) holds true:

$$E = -2.30 \frac{RT}{F} \log \frac{K_i + \alpha Na_i}{K_o + \alpha Na_o} \qquad (7)$$

where $\alpha = P_{Na}/P_K$ and $2.30\,RT/F = -58$ mV at 20° C. Substituting E = -48 mV (Hagins et al, 1962; Millecchia and Mauro, 1969 a) and the cation values from Fig. 13 then α is 0.03, which compares with a value of 0.05 for squid nerve (Baker, Hodgkin and Meves, 1964). If it is assumed that a considerable part of the K^+ ions are bound (see next paragraph), the value of α would be even much lower. The low value of α indicates that the membrane potential is essentially determined by the K^+ gradient across the membrane, which is in agreement with the relationship between this potential and the external K^+ concentration observed by Millecchia and Mauro (1969 a).

3.4.2. *Isotope uptake and efflux in the dark*

Equilibrium is reached after 1½ hours exposure to ^{86}Rb (Fig. 14). The intracellular concentration of ^{86}Rb is then only 50 mM and the isotope has therefore replaced only about 35% of the total potassium (145 mM) of the retina. This is half the value of 70% given by Hagins and Adams (1960) for ^{42}K uptake. This discrepancy does not appear to be due to a difference in the inward pumping rate of rubidium, as we find that the Rb^+ and K^+ activation of the sodium pump enzyme Na-K activated ATPase are identical (6.3.2. Fig. 31). In mammalian heart muscle rubidium replaces only 40% of the potassium, although the permeabilities of potassium and rubidium are identical in this tissue (Van Zwieten, 1968). This phenomenon could be explained by assuming that part of the potassium inside the cells is in a complexed form, as previously suggested for a considerable fraction of potassium in the sacs of vertebrate rod outer segments (Duncan, Bonting and Daemen, 1970).

3.4.3. *Effect of ouabain in darkness*

The lowering of the rubidium influx by ouabain (Fig. 14, Table I) is understandable in view of the relatively high activity of the ouabain-sensitive sodium pump enzyme located in the outer segments. However, in first instance, it is surprising to find that rubidium still accumulates against a concentration gradient, although the Na-K ATPase is completely inhibited in the presence of 10^{-4}M ouabain (see 6.3.2. Fig. 33) and the same ^{86}Rb contents are found with 10^{-4} and 10^{-3}M ouabain (Table I).

After 120 min the internal concentration of Rb is 26 mM, whereas the outside concentration is 10 mM. This cannot be explained by assuming that ouabain is not equally distributed around the microvilli, because there is no further decrease in rubidium concentration between 90 and 300′ incubation in ouabain containing A.S.W. The phenomenon could be explained, if it is assumed that the membrane potential has not been completely abolished during this time. In the absence of a pump, rubidium ions will be distributed across the membrane passively and in a concentration ratio predictable from the Nernst equation:

$$E = -\frac{RT}{F} \ln \frac{Rb_i}{Rb_e} \qquad (8)$$

From equation (8), if Rb_i = 26 mM and Rb_e = 10 mM (Table I) then the membrane potential E should be —24 mV. This potential would cause the rubidium ions to be accumulated to a concentration higher inside than outside.

The doubling of the passive ^{86}Rb efflux by ouabain is also unexpected, as it has been generally found that ouabain has little effect on the potassium efflux from various tissues (Caldwell and Keynes, 1960; Becker and Cotlier, 1965; Duncan, 1969 b) and hence that it has no effect on membrane permeability. A possible explanation for an effect of ouabain on membrane permeability can be derived from the application of the Kimizuka-Koketsu (1964) equation:

$$k = P_K \frac{A}{V} \exp(FE/2RT) \qquad (9)$$

where k is the rate constant, P_K the potassium permeability, E the membrane potential in artificial seawater and A/V the surface to volume ratio of the cells. An identical equation as (9) can be obtained with ouabain in the artificial seawater. Dividing the first equation by the second gives:

$$\frac{k'}{k} = \frac{P_K}{P'_K} \exp \frac{F}{2RT} (E - E') \qquad (10)$$

The prime indicates new values achieved by a change of the resting potential from E to E′. This equation predicts changes in efflux rate upon change of potential in an adequate fashion in the toad lens (Duncan, 1969 b). If we suppose that ouabain does not directly change the permeability, then equation (10) simplifies to:

$$\frac{k}{k'} = \exp \left(\frac{F}{2RT} \cdot \Delta E \right) \qquad (11)$$

A change in the rate constant from 2.0×10^{-2} to 4.0×10^{-2}, as observed in our experiments, would require a potential change of E = 35 mV. Since the resting potential of cephalopod photoreceptors is about — 48 mV in the dark (Hagins et al, 1962), a fall to approximately —13 mV after one hour in ouabain solutions would produce the observed increase in rate constant. This requires a larger fall in potential than was predicted from our influx studies (E = 24 mV). However, a mere 25% decrease in K^+ permeability would remove the discrepancy.

An alternative way of explaining the discrepancy would be to assume that in the absence of ouabain a certain amount of back pumping of isotope into the cell occurs, which is abolished in the presence of ouabain.

3.4.4. *Calculation of the permeability constant*

From the rate constant for Rb efflux in A.S.W. it is possible to obtain a value for P_K, the potassium permeability of the membrane system limiting the movement of potassium, assuming that the two cations have the same permeability. If it is assumed that the limiting membranes are those of the photoreceptor cells, which make up the bulk of the Sepia retina, and if their surface to volume ratio (A/V) is known, the application of equation (9) will give P_K. From the electronmicroscopical data of Zonana (1961) for the squid photoreceptor the surface to volume ratio of circular cells (type I) was found to be 30 μ^{-1} and that of elliptical cells (type II) was 22 μ^{-1}. This relatively large surface to volume ratio is due to the large numbers of microvilli emanating from each cell and should be compared with values of 0.03 μ^{-1} for a sphere of radius 100 μ and 8×10^{-3} μ^{-1} for the average cephalopod giant axon. A large surface to volume ratio appears to be a feature common to all photoreceptor cells (Moody, 1964). Taking a resting potential of —48 mV and the value of k of 0.02 min^{-1} and the average A/V ratio of 26 μ^{-1}, P_K is found to be 3.3×10^{-9} cm. sec^{-1}, which compares with values of 5×10^{-7} for frog muscle (Kimizuka and Koketsu, 1964) and 3×10^{-7} cm. sec^{-1} for squid axon (calculated by applying equation (11) to the potassium efflux data of Caldwell and Keynes (1960). This low value for the potassium permeability indicates that invertebrate photoreceptor membranes are highly resistant, and indeed Millecchia and Mauro (1969 b) have recently reported values of 50,000-100,000 Ω cm^2 as against 1,000 Ω cm^2 for the squid axon (Cole and Curtis, 1939).

Our data on ion content and ion movements in Sepia photoreceptors, taken together with electrophysiological data indicate that these invertebrate photoreceptor cells behave in the dark like many other cell types. There is an asymmetrical distribution of ions across the membranes maintained by

an active cation pump and the asymmetry apparently causes diffusion potentials across the membranes. The major contribution to the resting potential appears to come from the diffusion of potassium ions, which are at least 30 times more permeable than sodium ions.

3.4.5. *Effect of illumination*

The resting potential of the cephalopod photoreceptor in the dark is —48 mV (Hagins et al, 1962), while the sodium equilibrium potential is calculated to be +8 mV from the values Na_e = 470 mM and Na_i = 340 mM determined previously (see 3.3.1.). Hence the maximum depolarisation which can be produced by light through an increased sodium permeability is +56 mV and from the Kimizuka-Koketsu flux equations (see 3.4.3.) this would be expected to result in a 3.2.-fold increase in the potassium efflux rate constant. Table I indicates that we observed 1.7-4.8 fold increase in the rubidium efflux in the light intensity range used.

It is of interest to compare our net decrease in potassium content with the number of charges moving per absorbed photon as calculated by Hagins (1965). We may assume that the microvilli are the lightsensitive membrane structures in the invertebrate photoreceptor, as are the sacs in the vertebrate rod. In our experiments illumination, representing from 42-16.800 effective photons received by each microvillus, gave a nearly constant decrease in rubidium content of 6.4%. This suggests that absorption of a single photon by each microvillus would give about the same decrease. Assuming that the microvillus is a cylinder of length 1.1 μ and diameter 0.1 μ (Zonana, 1961), then its internal volume is 8.6×10^{-18} l. As the exchangeable intracellular potassium concentration is 50 mM (Fig. 14; Table I), each microvillus contains 2.6×10^5 exchangeable potassium ions. A 6.4% decrease would represent a number of 1.7×10^4 ions moved from a microvillus by a single stimulation. This figure is in reasonable agreement with the value of 5.7 (S.E.: 1.1) $\times 10^4$ charges per photon calculated by Hagins (1965) from photocurrent measurements. This suggests that cations are carrying the photocurrent and that the cations moved upon stimulation by a single photon can come from the stimulated microvillus and the immediately adjacent cell space. It does not contradict or confirm the conclusion of Hagins et al (1962) that at the site of illumination Na^+ ions enter the cell, while K^+ ions leave the cell at more proximal sites.

3.4.6. *Effect of retinaldehyde*

Using isolated vertebrate photoreceptor cells, Bonting and Bangham (1967) have shown that retinaldehyde causes ion movements identical in magnitude

and direction to those induced by light. This finding supports the hypothesis (Bonting and Bangham, 1967; Bonting, 1969) that light stimulated ion movements are a result of the retinaldehyde molecule attaching itself to a specific amino group on the surface of the photoreceptor-membranes during visual pigment photolysis. In phospholipid micelles, Daemen and Bonting (1969 b) have demonstrated the specificity of the retinaldehyde effect on cation permeability compared to other vitamin A analogues, and they have also shown that the amino group reagent 2,4-dinitrofluorobenzene leads to a similar increase in permeability. Table III shows that both retinaldehyde (10^{-4}M) and DNFB cause significant increases in the dark rubidium efflux rate in Sepia retina. As in the vertebrate receptors (Daemen and Bonting, 1968), retinol had a much smaller effect and retinoic acid had no effect. The absence of an effect with low concentrations of retinaldehyde (10^{-6} and 10^{-5}M) is probably due to the slower penetration of this substance in the solid Sepia retina than in isolated rod outer segments.

3.4.7. *Effect of ouabain in the light*

We showed that in the presence of ouabain the internal rubidium concentration was significantly reduced, but that there still existed a rubidium gradient of 26 mM inside to 10 mM outside and hence a potential difference across the photoreceptor membrane (see 3.4.3.) This residual potential difference explains why a light-stimulated increase in rubidium efflux, though smaller than in the absence of ouabain, was found under these conditions (Table IV).

Since after exposure to ouabain illumination increased the rate constant by only 37% of the increase found in the absence of ouabain, this does not mean that under these circumstances the retina would still have given a receptor potential. Smith et al (1968) observed abolition of the receptor potential in Limulus after prolonged exposure to ouabain, even though a residual cation gradient was present, as suggested by the fact that the cell was not completely depolarized.

The data presented in this chapter indicate that the Sepia photoreceptor cells have cation gradients similar to those of most excitable tissues. Fitting the intracellular values to the Goldman equation yields permeability ratios in the dark-adapted state very close to those previously found in squid nerve, i.e. with potassium much more permeable than sodium. Upon illumination we have observed an increased rubidium efflux, in contrast to the negative findings of Hagins and Adams (1960), and we have also established a reduction in the rubidium concentration.

3.5. SUMMARY

The intracellular cation concentrations in isolated Sepia retina were obtained by flamephotometric cation determinations before and after incubation in artificial seawater with choline replacing sodium. Intracellular Na and K concentrations of 340 and 145 mmole/l water respectively were found against extracellular Na and K concentrations of 470 and 10 mmole/l. The influx kinetics of ^{86}Rb shows that rubidium is taken up against its concentration gradient. Ouabain (10^{-4}-10^{-3}M) markedly inhibits this uptake.

The efflux kinetics revealed a fast-exchanging extracellular compartment ($t_{1/2}$ = 1.5 min) and a slowly-exchanging intracellular compartment ($t_{1/2}$ = 34 min). A doubling of the efflux rate by ouabain in darkness was explained partly in terms of cell depolarization although inhibition of back pumping from the extracellular space was also possible.

Upon illumination with light of intensities ranging from 3.5×10^{12} to 5.6×10^{15} effective photons/cm^2/sec, there was a 1.7-4.8 times increase in the efflux rate and a simultaneous fall of 6% in the rubidium content. The presence of ouabain reduced the effect of light on the efflux rate. This effect could be explained by the decreased potential over the membrane. Addition of retinaldehyde (10^{-4}M) or dinitrofluorbenzene (2×10^{-3}M) gave 45% and 35% increase in the dark efflux rate. Retinol (10^{-4}M) gave a smaller effect and retinoic acid (10^{-4}M) was without effect.

EQUILIBRIUM CONDITIONS IN THE FORMATION OF RETINYLIDENE IMINES

4.1. INTRODUCTION

A satisfactory technique for the preparation of aldimines (Schiff bases) of retinaldehyde in aqueous solution at near neutral pH was required for two reasons. First, we wanted to investigate the possibility of a direct conversion of these aldimines into retinol without primary formation of free retinaldehyde, since this process may play an important role in the visual mechanism (chapter 5). Secondly, the study of possible transiminization reactions in the photolysis of rhodopsin also required a suitable preparation of retinylidene imines.

Previously the formation of retinylidene imines has been studied by Morton and Pitt (1955). They prepared an aldimine of retinaldehyde and methylamine and showed that this compound in aqueous medium hydrolyzed at pH above 3. In the presence of excess methylamine no hydrolysis occurred at pH 8.6, but at pH 6.3 hydrolysis still took place. They explained these results by assuming that only the unprotonated form of methylamine reacts with retinaldehyde:

$$R-\overset{H}{C}=O + R'NH_2 \overset{K_1}{\rightleftarrows} R-\overset{H}{C}=N-R' + H_2O \qquad (1)$$

At alkaline pH more of the amine is in the unprotonated form because of the equilibrium:

$$R'NH_3^+ \overset{K_2}{\rightleftarrows} R'NH_2 + H^+ \qquad (2)$$

At acid pH there is insufficient unprotonated methylamine present to shift equilibrium (1) to the right. Similar conclusions were reached by Cordes and Jencks (1963) for the pH dependence of the hydrolysis rate of *p*-and *m*-substituted benzylidene-1,1-dimethylethylamines.

Our preliminary experiments showed that when a less polar amine was used instead of methylamine hydrolysis did not occur at pH 6-7, even when a much smaller excess of the amine was used. These results indicated that the apparent equilibrium constant for reaction (1) is dependent on the type of amine used. This was an unexpected finding, since the equilibrium

constant for the protonation of amines (K_2) is approximately the same for a variety of aliphatic primary amines (Hoeir et al, 1943). An additional complication arose from the fact that aldimines can also be protonated:

$$R-\overset{H}{C}=\overset{H}{N^+}-R' \quad \overset{K_3}{\rightleftarrows} \quad R-\overset{H}{C}=N-R' + H^+ \qquad (3)$$

At neutral pH the amine is largely protonated while the aldimine is largely unprotonated. The overall reaction is then:

$$R-\overset{H}{C}=O + R'NH_3^+ \quad \overset{K_4}{\rightleftarrows} \quad R-\overset{H}{C}=N-R' + H^+ + H_2O \qquad (4)$$

This reaction can be considered as the sum of the reactions (1) and (2).

The determination of the equilibrium constant K_4 for the formation of aldimines from retinaldehyde and various amines and the equilibrium constant K_3 for the protonation of the aldimine are described in this chapter. From K_4 and the known values for K_2 the equilibrium constant K_1 for reaction (1) was calculated. In the course of this work a method for the determination of aldimine concentration in a mixture of free retinaldehyde and its aldimine was devised, which is also described. The results explain in quantitative fashion our ability to obtain, in aqueous detergent solution at neutral pH and with only a slight excess of amine, a nearly complete formation of retinylidene imines with various aliphatic amines.

4.2. MATERIALS AND METHODS

4.2.1. *Chemicals*

All-trans retinaldehyde was purchased from Distillation Products Industries and was stored in the dark at —20° C, sealed in vacuo in small amounts. Aliphatic amines (Fluka) and Triton-X-100 (Rohm and Haas) were used without further purification. All organic solvents were freshly destilled before use. pH measurements were made with a glass electrode attached to a Radiometer 22 pH-meter. Spectroscopic determinations were carried out in a Zeiss PMQ II spectrophotometer with automatic cuvette changer.

4.2.2. *Preparation of aldimines*

Aldimines of retinaldehyde and various amines could be prepared in excellent yield in anhydrous media by mixing anhydrous alcoholic solutions of retinaldehyde with a solution of amine in dry benzene. The mixture was evaporated to dryness. After addition of small amounts of dry benzene this

procedure was twice repeated. The dry residues were kept for maximal three hours in vacuo in the dark.

4.2.3. *Spectrophotometry*

Absorption spectra of the protonated and unprotonated aldimines of retinaldehyde were obtained in the following way. A retinaldehyde solution was mixed with an excess of the appropriate amine in 1% aqueous Triton-X-100 solution. An aliquot of this solution was brought to pH 13 with 2.5 M NaOH in order to give the unprotonated aldimine. Afterwards the sample was rapidly brought to pH 1 with excess concentrated HCl in order to obtain the protonated form (Morton and Pitt, 1955). Spectra were measured before and after acidification, using 1% Triton-X-100 solution as a blank. A correction was made for dilution. The spectrum of retinaldehyde, which was pH-independent, was obtained in a similar way by omitting the amine. Spectra for retinaldehyde and for retinylidene octylamine at pH = 1 and pH = 13 are given in Fig. 19. The spectra of the aldimines of retinaldehyde and the other amines used in this investigation are essentially the same, and the molar extinctions of the various protonated aldimines at 440 and 380 nm (see Fig. 20) do not differ more than 2%.

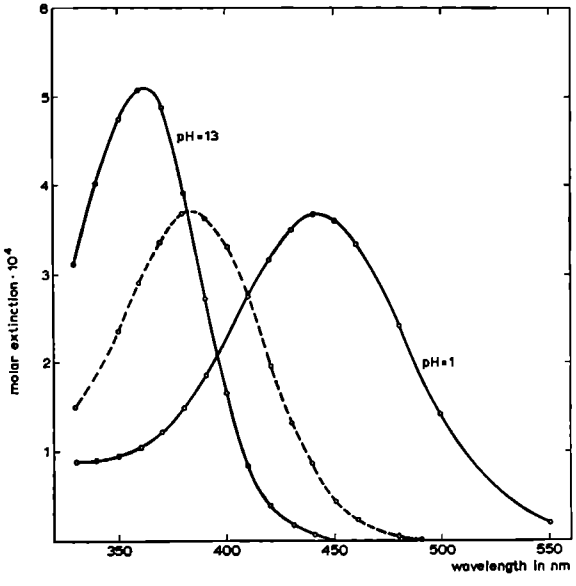


Fig. 19 Spectra of retinaldehyde (- - -) and of retinylidene-octylamine (——) at pH 1 and pH 13.

4.2.4. *Equilibrium constants*

The equilibrium constants quoted in this paper are given by the following expressions:

$$K_1 = \frac{[R-\overset{H}{\overset{|}{C}}=N-R']}{[R-\overset{H}{\overset{|}{C}}=O]\,[R'NH_2]} \tag{5}$$

$$K_3 = \frac{[R'NH_2]\,[H^+]}{[R'NH_3^+]} \tag{6}$$

$$K_2 = \frac{[R-\overset{H}{\overset{|}{C}}=N-R']\,[H^+]}{[R-\overset{H}{\overset{|}{C}}=\overset{H}{\overset{|}{\overset{+}{N}}}-R']} \tag{7}$$

$$K_4 = \frac{[R-\overset{H}{\overset{|}{C}}=N-R']\,[H^+]}{[R-\overset{H}{\overset{|}{C}}=O]\,[R'NH_3^+]} \tag{8}$$

4.2.5. *Equilibrium constants of aldimine protonation*

The equilibrium constant K_3 for the protonation of the aldimine was determined in the following way. The dry residue of the aldimine preparation from 4 μmoles retinaldehyde and 40 μmoles amine was dissolved in 5 ml 25% Triton-X-100 in absolute alcohol. Aliquots of this solution were transferred to 5 ml of 0.1 M acetate buffer (pH varying between 4.4 and 6.0). The extinction at 500 nm, at which wavelength virtually only the protonated aldimine absorbs (Fig. 19), was measured at intervals of one minute after mixing. Since the extinction at 500 nm decreased due to hydrolysis of the Schiff base, the amount of protonated aldimine at the moment of mixing was calculated by extrapolation to time zero. In a similar experiment the buffer was replaced by 0.1 N HCl, in which case all of the aldimine was transformed into the protonated form, which does not hydrolyze measurably (Morton and Pitt, 1955; Cordes and Jencks, 1963). The difference between this extinction and the extinctions of the mixture in the buffered solutions (all at 500 nm) is a measure of the amount of unprotonated aldimine at the time of mixing. K_3 was calculated from these extinction values at eight different pH values in the range pH 4.4-6.0.

4.2.6. *Equilibrium constants of aldimine formation*

Equation (8) can be converted to contain quantities, which are known or can be determined. The total concentration of amine ($C_{am.\ tot.}$) added to the reaction medium is equal to the sum of the concentrations of protonated and unprotonated amine and of protonated and unprotonated aldimine:

$$C_{am.\ tot.} = [R'NH_3^+] + [R'NH_2] + [R-\overset{H}{\underset{}{C}}=N-R'] + [R-\overset{H}{\underset{}{C}}=\overset{H}{\underset{}{N^+}}-R'] \qquad (9)$$

Substituting (6) and (7) into (9) yields the following expression:

$$[R'NH_3^+] = \frac{C_{am.\ tot.} - [R-\overset{H}{\underset{}{C}}=N-R'](1+\frac{[H^+]}{K_3})}{1+\frac{[H^+]}{K_2}} \qquad (10)$$

Substitution of (10) in (8) gives:

$$K_4 = \frac{[R-\overset{H}{\underset{}{C}}=N-R'](K_2+[H^+])}{[R-\overset{H}{\underset{}{C}}=O]\left\{C_{am.\ tot.} - [R-\overset{H}{\underset{}{C}}=N-'R](1+\frac{[H^+]}{K_3})\right\}} \qquad (11)$$

Equation (11) was used to calculate K_4, taking the values for K_2 given in the litterature (Hoeir et al, 1942; Fyfe, 1955; Robinson and Kiang, 1956) and the values for K_3 determined for each amine as described above. The total amine and hydrogen ion concentrations were known. This leaves only the concentrations of the protonated and unprotonated aldimines and free retinaldehyde to be determined.

These concentrations could in principle be determined by measuring the spectrum of the reaction mixture. However, accurate measurement was difficult because of the close proximity of the maxima of the unprotonated aldimine (~ 360 nm) and free retinaldehyde (~ 380 nm). Moreover the dilution required for the spectral measurements caused a shift in the equilibrium. The problem was solved by acidifying the reaction mixture, leading to immediate and complete protonation of the aldimine (equation 3) as well as of the free, unprotonated amine (equation 2). This prevents the shift in the equilibrium upon dilution. After acidification the maximum at 360 nm has disappeared and only the maxima at 380 and 440 nm remain.

Accurate calculation of the concentrations of these compounds was possible by measuring the extinction at 380 and 440 nm and applying the

following equations

$$E_{440} = \varepsilon_{ret\,440}\, C_{ret}\, D + \varepsilon_{ald^+\,440}\, C_{ald^+}\, D \qquad (12)$$

$$E_{380} = \varepsilon_{ret\,380}\, C_{ret}\, D + \varepsilon_{ald^+\,380}\, C_{ald^+}.D \qquad (13)$$

E_{440} and E_{380} are the measured extinctions at these two wavelengths, $\varepsilon_{ret\,440}$ and $\varepsilon_{ret\,380}$ are the molar extinction of retinaldehyde and $\varepsilon_{ald^+\,440}$ and $\varepsilon_{ald\,380}$ the molar extinctions of the protonated aldimine at 440 and 380 nm These molar extinctions can be read from the absorption curves in Fig 19 D is the lightpath (in cm) of the cuvette and C_{ret} and C_{ald^+} are the concentrations of free retinaldehyde and its aldimine A simplified calculation of the latter concentrations is possible by substituting for C_{ret} the expression $1\text{-}C_{ald^+}$ in equations (12) and (13) and then dividing these two equations The resulting expression, using the known molar extinctions of retinaldehyde and its aldimine at 380 and 440 nm, can be plotted as percent aldimine against the ratio E_{440}/E_{380} (Fig 20)

The concentration of free retinaldehyde remained unchanged by acidification The concentration of the protonated aldimine after acidification was equal to the sum of the concentrations of the protonated and unprotonated forms The concentrations of these two forms at each moment of the experiment were calculated from the previously determined value of K_3 and the known pH K_4 was calculated by means of equation (11), substituting

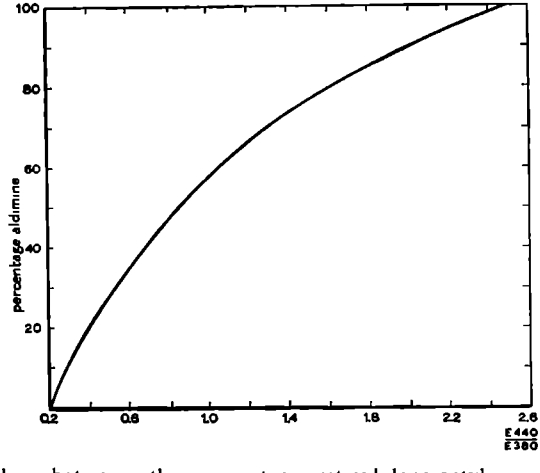


Fig 20 Relationship between the percentage retinylidene-octylamine and the ratio $E_{440/380}$ after acidification with concentrated hydrochloric acid Aldimine concentration is expressed as percentage of the initial concentration of retinaldehyde

the concentrations of the protonated and unprotonated aldimine and free retinaldehyde, existing at equilibrium (no further change with time). K_1 was obtained by dividing K_4 by K_2.

4.3. RESULTS

4.3.1. *Equilibrium constants of aldimine formation*

The aldimine formation equilibrium was studied in two ways. For amines with chainlength of 12 C-atoms or less the formation of the aldimine was followed, while for amines with longer chainlength the hydrolysis of preformed aldimine was observed. In the former case, an aqueous solution of retinaldehyde in 1% Triton-X-100 was shaken with a buffered solution of the aliphatic amine (C_2-C_{12}) at 37° C under nitrogen. Aldimine formation, indicated by a shift of the absorption maximum of the solution from 380 to 360 nm, was followed quantitatively by the spectroscopic procedure described under 4.2.5. Fig. 21 shows the results obtained with octylamine in various initial concentrations at pH 7.3. The establishment of equilibrium requires periods of 2 hrs. or more. Similar curves were obtained with this technique for all amines up to tetradecylamine.

The amines with a chainlength of more than 12 C-atoms are too insoluble to permit use of this method, and hence the hydrolysis of the preformed aldimine (see under 4.2.2.) was followed until equilibrium was reached. The validity of this approach is shown by the fact that identical equilibrium

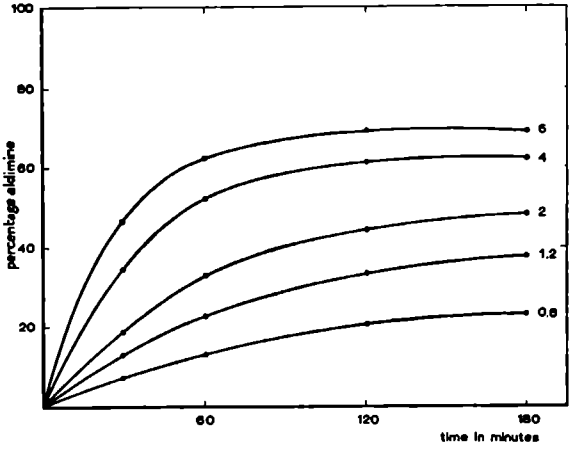


Fig. 21 The formation of retinylidene-octylamine in a 1% Triton-X-100 solution at pH 7.3 as function of time. The initial concentration of retinaldehyde is 0.33 mM. The concentration of octylamine is given in mM for each slope. Aldimine concentration is expressed as percentage of the initial concentration of retinaldehyde.

TABLE V

DETERMINATION OF pK_4 OF RETINYLIDENE OCTYLAMINE UNDER VARIOUS CONDITIONS

Concentrations		pH	Percent Aldimine	pK_4
Retinaldehyde (mM)	Octylamine (mM)			
0,33	0,6	7,54	28,5	4,64
0,33	2	7,52	62	4,56
0,33	6	7,49	82	4,59
0,33	20	7,50	95	4,52
0,33	20	7,40	94,5	4,47
0,33	20	6,88	85	4,43
0,33	20	6,37	63,5	4,47
0,33	20	5,92	42	4 42
0,33	20	5,53	24	4,47
0,033	0,6	7,45	23	4,57
0,033	1,2	7,45	39,5	4,67
				av. 4,53
				SE 0,03

Determination of pK_4 of retinylidene octylamine after preparation under different conditions. The concentrations mentioned are the amounts of retinaldehyde and octylamine added in the experiments. Aldimine concentration is expressed as percentage of the initial concentration of retinaldehyde.

concentrations were found with these two approaches for octylamine, decylamine and dodecylamine.

In addition, the effects of pH and retinaldehyde concentration on aldimine formation were studied for octylamine (Table V). From the aldimine concentration at equilibrium and the other known parameters, values of pK_4 were calculated for each set of conditions by means of equation (11). The results in Table V show little variation, the standard error being only 0.03 for an average pK_4 of 4.53. Therefore, the pK_4 for the other amines was determined from experiments in which only the amine concentration was varied.

The values of pK_4 thus obtained for the homologous series of aliphatic amines are plotted as a function of the chainlength of the amine in Fig. 22. With increasing chainlength there is a decrease in the measured pK_4 of about 0.5 unit for each additional C-atom. At chainlengths of more than 10 C-atoms the decrement diminishes and there is no difference in pK_4 for dodecylamine and octadecylamine.

4.3.2. *Equilibrium constants of aldimine protonation*

The equilibrium constants for the protonated aldimines and different

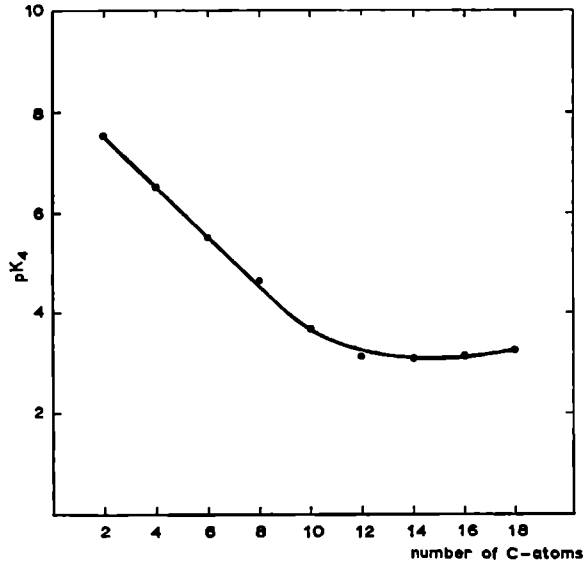


Fig. 22 pK_4 as a function of the chainlength of the aliphatic amine. Each value represents the average of at least 5 experiments, in which different concentrations of amine were used.

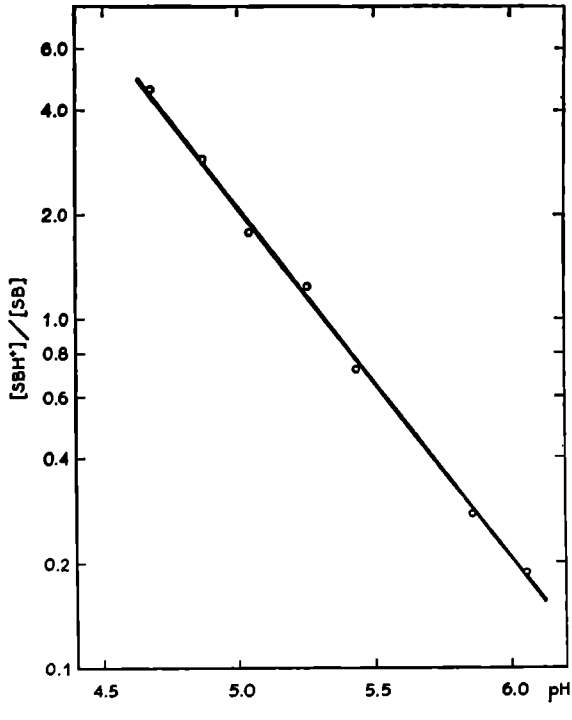


Fig. 23 Relation between the protonated and unprotonated form of retinylidene octylamine in an aqueous (1% Triton-X-100) solution as a function of the pH of the solution.

amines (K_3) were obtained by utilizing the fact that hydrolysis of protonated aldimines follows first order rate kinetics (Cordes and Jencks, 1963) and thus the logarithm of the concentration of the protonated aldimine is linear with time. The concentration of this compound at the moment of mixing with the buffer, determined by extrapolation to time zero, gives a measure of pK_3, the pK of the protonation of the aldimine. In Fig. 23 the logarithm of the ratio of the concentrations of protonated and unprotonated retinylidene-octylamine is plotted as a function of the pH. From this curve pK_3 can be calculated. This pK_3 value showed some dependence on the reaction conditions. With increasing excess of amine lower values for pK_3 were obtained. In Table VI these equilibrium constants, measured under indentical circumstances, are listed for the amines studied. The value of pK_3 decreases with increasing chainlength, but the change is small compared to that in pK_4 (1.2 units vs. 4.4 units from C2 to C16), and the decrement is more constant over the entire range. The values of pK_1, pK_2 and pK_4 are also listed in this table.

TABLE VI

VALUES OF pK_1, pK_2, pK_3 and pK_4 FOR THE ALDIMINES OF RETINALDEHYDE AND VARIOUS AMINES

primary amine number of C-atoms	pK_1	pK_2 *	pK_3	pK_4
2	-3.15	10.67[2]	6.25	7.52
4	-4.08	10.61[1]	5.99	6.53
6	-5.03	10.64[1]	5.70	5.61
8	-6.12	10.65[1]	5.32	4.53
10	-6.98	10.64[1]	5.35	3.66
12	-7.53	10.63[1]	5.21	3.10
14	-7.56	10.62[1]	5.03	3.06
16	-7.51	10.61[1]	5.02	3.10
18	-7.53	10.60[1]	5.23	3.25
benzylamine	-4.13	9.35[3]	5.13	5.22

* Values from the literature: 1. (Hoeir et al, 1942). 2. (Fyfe, 1955). 3. (Robinson and Kiang, 1956).

4.3.3. *Evaluation of the experimental data*

For the calculation of pK_4 and pK_1 in this paper litterature values for pK_2 were used, which were determined by extrapolation from conductometric values in aqueous-alcoholic solutions (Hoeir et al, 1943). In principle it would have been better to use values determined in a 1% Triton-X-100 solution by titration with HCl but an attempt to determine such values did

not lead to consistent results. This is probably due to a difference between the H^+ concentration in the bulk phase and on the micellar surface, which is large at the end of the titration when the amine is protonated, but which is small or absent at the start of the titration when the amine is unprotonated. Since for all amines used in this study the value for pK_2 is much higher than the pH employed for the determination of pK_4, the influence of the value of pK_2 on the value of pK_4 can be considered negligible (see equation 11). On the other hand, pK_2 has a large influence on the calculation of pK_1 (equations 5 and 6) and the determined values for pK_1 (Table VI) should therefore be considered with some reservation.

4.4. DISCUSSION

4.4.1. *Influence of the amine chainlength on the equilibrium constant of aldimine formation*

With the techniques developed here for the study of the equilibrium of the aldimine formation:

RETINALDEHYDE + PROTONATED AMINE $\rightleftarrows$ ALDIMINE + H^+ + H_2O

it was possible to obtain reliable values for the equilibrium constant pK_4. When a single amine was used and the pH as well as the concentrations of retinaldehyde and amine were varied, a remarkably constant value of pK_4 was obtained (Table V).

When, however, pK_4 was determined for different amines, a striking dependence of this constant on the amine chainlength was observed (Fig. 22). This was an unexpected finding, since variation in pK_4 with amine chainlength would in first instance be expected to depend on pK_2, the equilibrium constant for the amine protonation. However, for amines with chainlength varying from 2 to 18 C-atoms pK_2 is constant within 0.1 unit (Hoeir et al, 1943), while our pK_4 values decreased in this direction over 5 units. If pK_2 values for micellar solutions of the amines would have been used, the spread in these values would be expected to be somewhat larger, but not larger than that in pK_3 (1.2 unit). Thus, the spread in the pK_4 values for the various amines would remain much larger than could be explained from the variation in pK_2.

We have been able to explain this paradoxical finding by taking into account that the reaction occurs in a micellar environment. Retinaldehyde is very insoluble in water and must therefore have been overwhelmingly present in the micelles formed by the non-ionic detergent Triton-X-100, an octylphenyl oxyethylene adduct. The aliphatic amines will be distributed between the aqueous and micellar phases, with increasing chainlength favouring the

micellar phase. Consequently the effective values of $[R'NH_3^+]$ in equation (8) and for $C_{am.\ tot.}$ in equation (11) are lower for the short-chain amines, which are predominantly present in the aqueous phase. This results in lower values of K_4 and higher values of pK_4 for the short-chain amines as compared to the long-chain amines. A semi-quantitative treatment of the micellar aspect of the equilibrium, given in the next paragraph, shows that the variation in pK_4 with amine chainlength can mainly be ascribed to the variation in the partition coefficient for the various amines, and is not due to differences in H^+ ion concentration between the two phases.

4.4.2. *Explanation in terms of the distribution of the amine between micellar and aqueous phase*

Since the reactions are practically completely taking place at the micellar surface, a new equilibrium constant K_5 might be formulated, which contains the micellar concentrations of the reactants:

$$K_5 = \frac{[R-\overset{H}{\overset{|}{C}}=O]_m\,[R'NH_3^+]_m}{[R-\overset{H}{\overset{|}{C}}=N-R']_m\,[H^+]_m} \qquad (14)$$

The subscript m means that between brackets the concentration of the reactant at the micellar surface is given. Retinaldehyde and the aldimine are assumed to be completely present in the micelle because their concentrations are low and they are very lipophilic. $[R-\overset{H}{\overset{|}{C}}=N-R']_m$ and $[R-\overset{H}{\overset{|}{C}}=O]_m$ can thus be replaced by $p.[R-\overset{H}{\overset{|}{C}}=N-R']$ and $p.[R-\overset{H}{\overset{|}{C}}=O]$ in which p is the ratio between the total volume and the volume of the micellar phase. The concentration of protonated amine at the micellar surface $[R'NH_3^+]_m$, is related to the total amine concentration, $[R'NH_3]^+$, by the equation:

$$[R'NH_3^+]_m = p.q\,[R'NH_3^+] \qquad (15)$$

In this equation p is again the ratio between the total volume and the volume of the micellar phase and q is the fraction of the amine present in the micelles. The fraction q is of course dependent on the amine. The H^+ concentration at the surface of a charged micelle at 25° is given by an equation formulated

by Hartley and Roe (1940):

$$PH_s = pH_b + \frac{\zeta}{60} \qquad (16)$$

In this formula s and b denote the micellar surface and the bulk of the solution, respectively, while ζ is the surface potential of the micelle in millivolts. This surface potential is caused by the presence of positively charged amines in the non-ionic micelles. The magnitude of this potential is not known but must be related to the concentration and the orientation of the amines in the micellar interface (Muherjee and Bonerjee, 1964). Substituting the values thus obtained in equation (13) gives:

$$K_5 = \frac{[R-\overset{H}{\overset{|}{C}}=N-R'][H^+].\ 10^{\frac{-\zeta}{60}}}{[R-\overset{H}{\overset{|}{C}}=O]\ p.q\ [R'NH_3^+]} \qquad (17)$$

Substitution of equation (8) in equation (17) gives:

$$K_5 = \frac{K_4\ .\ 10^{\frac{-\zeta}{60}}}{p.q} \qquad (18)$$

The factor p is constant for different amines, because the properties of a micelle composed of a non-ionic and a cationic detergent are determined by the former, when this comprises more than 10 molar % of the micelle (Schick, 1966) as was the case in our experiments. Hence, there are only two factors which influence the dependence of K_4 on the chainlength. One is the factor $10^{\frac{-\zeta}{60}}$ stemming from the difference in H^+ in the bulk of the solution and at the micellar interphase; the other is q, the fraction of the amine present in the micelle.

The first factor is the least important one, as is shown by the small effect of the chainlength on the protonation of the aldimine (0.1 pK unit for each additional CH_2 group, Table VI). The second factor is much more important and determines nearly completely the dependence of pK_4 on the chainlength. According to Traube's rule (Traube, 1891) the partition coefficient $\varkappa$ between lipid and waterphase for homologous aliphatic acids, alcohols and amines increases by a factor 3 for each additional CH_2 group.

The relation between this coefficient $\varkappa$ and the value q cited above is given by equation (19).

$$\frac{1}{q} = \frac{p}{\varkappa} + 1 \qquad (19)$$

When $\frac{p}{\varkappa} >> 1$ multiplication of $\varkappa$ with a factor 3 results in an identical multiplication of q. Because the micellar phase is only a small part of the total volume this is indeed the case when $\varkappa$ is small. Therefore the factor q should increase with a factor 3 for each additional CH_2 group. Fig. 22 shows that K_4 increases also by a factor 3 for the lower homologue of the series. From dodecylamine to octadecylamine there is little change, which is probably due to the fact that for these compounds q approaches its maximal value of 1. These considerations suggest that the true equilibrium constant K_5 is nearly constant for the homologous series of aliphatic amines, as would be expected from the absence of an effect of the chainlength on the protonation of the amine.

4.4.3. *Consequences of this investigation*

The pK_4 values determined in this study are useful in determining the conditions under which aldimines of retinaldehyde can be formed in high yield in neutral aqueous detergent solution. Our results explain why Morton and Pitt (1955) could only obtain a stable aldimine of retinaldehyde with methylamine in aqueous medium at alkaline pH (8.6) and a large (20,000-fold) excess of amine. Our results also explain why we were able to prepare aldimines of retinaldehyde in 90% yield (based on an initial retinaldehyde concentration of 0.3 mM) at neutral pH in the presence of only a small (2-fold) excess of dodecylamine. This finding is also in agreement with our previous demonstration that aldimine formation between retinaldehyde and phosphatidylethanolamine in a monolayer is possible at neutral pH and with only a slight excess of the latter substance (chapter 2). Moreover in the next chapter these findings are applied in preparing aldimines as substrates for a study of their enzymatic conversion to retinol.

There are also consequences to be derived from this investigation for our understanding of visual pigment regeneration. Aldimine formation plays a role in the regeneration of the visual pigment, because rhodopsin appears to be an aldimine of 11-cis retinaldehyde with an amino group of the lipoprotein opsin. Since in vivo this particular aldimine formation would have to take place at a pH near 7, and in vitro it is found to proceed only in the pH range 5-7, Wald and Brown (1952) have raised the question whether in this pH range aldimine formation could occur at all. Our experiments, however, show that this is indeed possible with an amine that is sufficiently lipophilic. Recent findings by Poincelot et al (1969) and Akhtar and Hirtenstein (1969) indicate that the amine to which retinaldehyde is bound in rhodopsin, is the lipophilic substance phosphatidylethanolamine. The hydrophobic conditions

which exist in the micelles in our studies and which also are expected to prevail in the rod sac membrane at the site of rhodopsin regeneration, would also favour aldimine formation at neutral pH.

4.5. SUMMARY

In order to determine the conditions under which aldimines (Schiff bases) of retinaldehyde can be obtained in high yield, equilibrium constants for the formation and protonation of aldimines between retinaldehyde and aliphatic amines were determined in 1% Triton-X-100 solution. A spectroscopic technique for the determination of aldimine concentrations in an equilibrium mixture was worked out. The formation constants are highly dependent on the chainlength of the amine. The pK for the overall reaction (RETINALDEHYDE + PROTONATED AMINE $\rightleftarrows$ ALDIMINE + H^+) decreases from 7.52 for ethylamine to 3.10 for dodecylamine and remain approximately constant for the higher homologues (C14-C18). This dependence on the amine chainlength is not due to variation in the pK for the protonation of the amine, but is apparently a result of the fact that the reaction takes place in a micellar lipid-water interface. The results indicate how aldimines of retinaldehyde can be obtained in good yield in aqueous detergent solution at neutral pH, using a relatively moderate excess of amine. Lipophilic aldimines of retinaldehyde thus prepared can be used as substrates for enzymatic studies.

ENZYMATIC CONVERSION OF RETINYLIDENE IMINES BY RETINOLDEHYDROGENASE FROM ROD OUTER SEGMENTS

5.1. INTRODUCTION

Illumination of the visual pigment rhodopsin leads — in this order — to the following photoproducts: pre-lumirhodopsin, lumirhodopsin, metarhodopsin I and metarhodopsin II (Fig. 4). Evidence has been advanced that in the last substance retinaldehyde is connected via an aldimine (Schiff base) bond with an ε-amino group of lysine in the lipoprotein complex, called opsin (Akhtar et al, 1965; Bownds and Wald, 1965). There is no unanimity about the reactions in the photolytic process after metarhodopsin II. Already in 1878 Kühne discovered that in intact rods and also in a bile salt solution of the visual pigment in about an hour complete discoloration occurred and a new product was formed that showed green fluorescence under ultraviolet radiation. Wald (1935) confirmed this finding and identified the end product as retinol. He noticed that the formation of retinol was reversibly inhibited by lowering the temperature. Bliss (1948) demonstrated that this reaction was enzymatic, and this was confirmed by Wald and Hubbard (1949).

Since photolysis of rhodopsin in detergent extracts yields retinaldehyde rather than retinol as the product of the visual cycle (Wald, 1938; Bridges, 1962 a), retinaldehyde has generally be assumed to be the substrate for the enzymatic conversion into retinol. Various investigators have studied the enzymatic conversion in vitro. This reaction occurs with enzyme preparations from rod outer segments (Wald and Hubbard, 1949; Futterman and Saslaw, 1961; Futterman, 1963, 1965), whole retina (Wald, 1950; Koen and Shaw, 1966) and with horse liver alcoholdehydrogenase (Bliss, 1949, 1951 a; Zachman and Olsen, 1961; Futterman and Saslaw, 1961; Futterman, 1963, 1965; Koen and Shaw, 1966). NADH was generally used as cofactor, but Futterman (1963) discovered that NADPH was the better cofactor for the rod outer segment enzyme.

In recent investigations of the photolytic process in the intact isolated retina retinol was found as the product, without any indication for the occurrence of free retinaldehyde (Matthews et al, 1963; Baumann, 1967, 1968; Cone and Brown, 1969; Reuter, 1966; Donner and Reuter, 1967, 1969). Bridges (1962 b) showed that upon illumination of fresh isolated rods retinol was the end product, but that aged or washed rods formed retinalde-

hyde as the end product, unless NADH was added, when retinol was again the end product.

This suggests that normally metarhodopsin II is converted into retinol either directly or via an intermediate, in which retinaldehyde is bound by an aldimine link and that free retinaldehyde is only formed in the absence of the cofactor NADPH or of retinoldehydrogenase activity. The purpose of the present study was to investigate whether retinylidene imines can be converted into retinol without intermediary formation of free retinaldehyde. It could be shown that retinoldehydrogenase from rod outer segments, but not alcoholdehydrogenase from horse liver, is capable of catalyzing this reaction.

5.2. MATERIALS AND METHODS

5.2.1. *Chemicals*

All-trans retinaldehyde was purchased from Distillation Products Industries and was stored in the dark at —20° C, sealed in vacuo in small amounts. Aliphatic amines and Triton-X-100 were obtained in the purest form and used without further purification. Horse liver alcoholdehydrogenase was obtained from Boehringer (Mannheim, Germany). Retinyloctylamine was prepared by reduction of retinylidene-octylamine. To a dry residue of retinylidene-octylamine (see 4.2.2.) a few milligrams of sodiumborohydride was added followed by methanol. After 5 minutes the excess sodiumborohydride was destroyed with 1 N acetic acid. Extraction with chloroform yielded a chromatographically pure product with a Rf value clearly different from that of retinol. 11-cis Retinol was obtained by alkaline hydrolysis of 11-cis retinylacetate (gift of Dr. U. Schwieter, Hoffmann-La Roche, Basle, Switzerland).

5.2.2. *Enzyme preparation*

Cattle rod outer segment preparations (ROS) were prepared as described in 2.2.2. with the exception that all steps were carried out at 4° C. The material was prepared in daylight except when indicated otherwise. The rod outer segments were lyophilized and used as enzyme preparation. In other experiments whole cattle retina's were homogenized in a fourfold volume of water or 0.067 M phosphate buffer (pH 7.0) in a Potter-Elvehjem homogenizer and centrifuged for 5 minutes at 500 x g at 4° C. The sediment was homogenized and centrifuged again and the supernatants were combined. The turbid supernatant was then centrifuged for two hours at 100,000 x g at 4° C. The resulting sediment was lyophilized and used as enzyme preparation.

5.2.3. *Enzyme assay*

The enzymatic assay method of Futterman (1963) was used with slight modifications. If retinaldehyde was to be the substrate, a 50-µl aliquot of a 12.5 mM solution of retinaldehyde in a 1 : 1 mixture of dioxan and 5% aqueous Triton-X-100 was mixed with 25 µl of a 40 mM solution of NADPH or NADH and the volume was brought to 725 µl with a 0.067 M phosphate buffer (pH 5.5-7.6) containing 1% Triton-X-100.

If a retinylidene imine was to serve as substrate, one of two approaches was used depending on the chainlength of the amine. For amines up to C 12, varying amounts of amine were dissolved in the phosphate buffer solution, the pH was adjusted by addition of HCl, and the mixture with the above amounts of retinaldehyde and cofactor was shaken in glass-stoppered tubes under nitrogen for two hours to permit formation of the aldimine. For amines of more than 12 C-atoms the aldimine was preformed by twice evaporating *in vacuo* a solution of 0.63 µmol of retinaldehyde, varying amounts of amine, and 7.5 µl of Triton-X-100 in absolute methanol or benzene. A 100% yield of aldimine could thus be obtained with equimolar amounts of dodecylamine and hexadecylamine. The preparation was taken up in 700 µl 0.067 M phosphate buffer (pH 7.0) and 25 µl of 40 mM NADPH was added.

To the 725 µl buffered substrate medium 25 µl of an enzyme suspension was added, while the control tube received 25 µl water. The enzyme concentration was chosen in such a way that the conversion proceeded for at least 30 minutes linearly with time. During incubation with the enzyme shaking was continued at 37° C under nitrogen and every 10 minutes a 100 µl sample was removed and added to 4.9 ml n-propanol.

5.2.4. *Retinaldehyde determination*

Retinaldehyde was determined in the samples added to propanol by the thiobarbituric acid method of Futterman and Saslaw (1961). This method determines retinaldehyde, both in the free form in the free form and in the aldimine form. In the presence of excess amine color development was complete within a few minutes, while in its absence a developing time of 20 min was necessary; the final extinction was, however, the same in both cases. The enzymatic activity was calculated from the average decrease in retinaldehyde concentration, corrected for the controls during the first three ten minute periods. The method of Zachman and Olsen (1961), which continuously records the decrease in extinction at 410 nm, was only used in some experiments with liver alcoholdehydrogenase. Its drawbacks for the other experiments were: 1) the incubation mixture cannot be shaken during incubation, which is undesirable because the insoluble enzyme tends to

sediment. 2) the high turbidity of the incubation mixture severely limits the accuracy of the determination of the decreases in extinction.

5.2.5. *Aldimine assay*

The amount of aldimine present in the incubation mixture was determined by acidifying a 200-µl aliquot with 10 µl concentrated HCl and recording the absorption spectrum of the mixture after appropriate dilution. From the E_{440}/E_{380} ratio the percentage aldimine was calculated as described in section 4.2.6.

5.2.6. *Spectrophotometric analysis*

In some cases the reaction was followed by direct absorption spectrophotometry. Aliquots were removed from the test tubes at various times and were diluted with 1% Triton-X-100 solution, containing the same concentration of amine as present in the incubation mixture. This prevented virtually completely a shifting of the free retinaldehyde to aldimine ratio, if amine was present in excess. In other cases aliquots were brought to pH 2 with concentrated HCl and further diluted with 1% Triton-X-100 solution. Under these circumstances the aldimine was protonated, thus protecting it from hydrolysis. Spectra were measured between 290 and 600 nm at the beginning and the end of the reaction period (2 hrs., in order to obtain maximal conversion) against blanks in which retinaldehyde or aldimine was omitted.

5.2.7. *Fluorimetric determination of retinol*

Incubation samples (100 µl) were added to 4.9 ml n-propanol to stop the enzymatic reaction. Retinol was estimated fluorimetrically in the resulting solutions, using a Beckman ratio fluorimeter (excitation wavelength 360 nm, fluorescence filter cutting off light below 405 nm).

5.3. RESULTS

5.3.1. *Rod outer segment preparation*

The lyophilized cattle rod outer segment preparation could reduce free retinaldehyde to retinol in the presence of the cofactor NADPH or NADH. At pH 6.5 the activity with NADH amounted to 0.297 (S.E.: 0.024; 8 detns.) moles retinaldehyde per kg dry weight per hour (MKH). Because of the low solubility of retinaldehyde suboptimal concentrations of retinaldehyde (0.83 mM) had to be used. From the Michaelis-Menten constant of 0.8 mM the theoretical maximal velocity can be calculated to be about 2 times as high. The activity with the cofactor NADH was barely significant: in 13 determinations the average activity was 14% (S.E.: 6.5; $P = 0.046$) of the

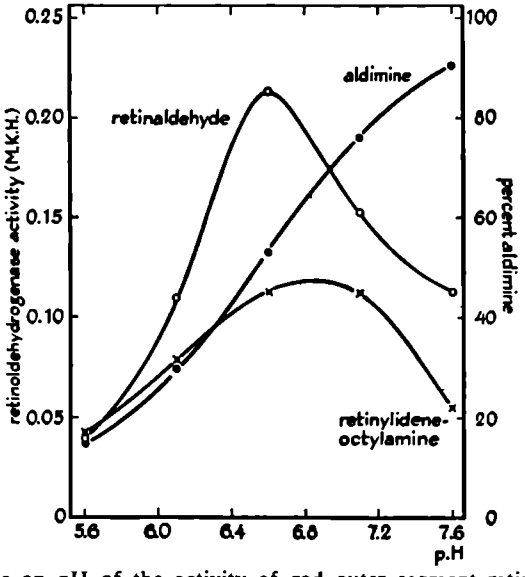


Fig. 24 Dependence on pH of the activity of rod outer segment retinoldehydrogenase with retinaldehyde (-o-o-o-) and retinylidene-octylamine (-x-x-x-) as substrate. The activity of the enzyme is expressed as moles converted per hour per kg dry weight rod outer segment. NADPH was used as cofactor. The aldimine concentration, determined before the enzymatic incubation, is expressed in percent of the initial concentration of retinaldehyde (-•-•-•-).

activity with NADPH. The pH optimum of the activity with NADPH as cofactor was pH 6.5 (Fig. 24). By means of a Lineweaver-Burk plot the Michaelis-Menten constant was found to be 0.8 (S.E.: 0.1; 3 detns.) mM at pH 7.0. This pH was chosen, because it is more suitable for use with the aldimines as substrate.

Aldimines of retinaldehyde with benzylamine, octylamine, decylamine, dodecylamine and hexadecylamine were also converted to retinol by this enzyme preparation. The aldimines with benzylamine, octylamine and decylamine were prepared by mixing retinaldehyde with excess amine in phosphate buffer (pH 7.0), containing 1% Triton-X-100. The mixtures were incubated for about one hour until equilibrium was reached. The aldimines with dodecylamine and hexadecylamine were prepared by evaporating *in vacuo* the mixture of retinaldehyde and amine in absolute methanol or benzene. Half of the samples were incubated with either NADH or NADPH and with added enzyme suspension. The other half were incubated with buffer only in order to determine the aldimine concentration as it would have been at the end of the 30 min. incubation period without enzymatic conversion.

TABLE VII

ENZYMATIC CONVERSION OF VARIOUS ALDIMINES BY ROD OUTER SEGMENT RETINOLDEHYDROGENASE

Amine	intial concentration amine	percent measured	percent calculated	relative reaction rate as compared to free retinaldehyde as substrate
No amine	0 mM	0%	0%	100%
benzylamine	150 mM	91%	90%	55%
octylamine	30 mM	92%	90%	41%
decylamine	5 mM	91%	90%	52%
dodecylamine	4 mM	95%	96%	62%
hexadecylamine	4 mM	94%	96%	66%

Benzylamine, octylamine and decylamine were mixed with retinaldehyde (0.83 mM final concentration) in 0.067 M phosphate buffer (pH = 7.0), containing 1% Triton-X-100. After equilibrium was reached, NADPH (1.3 mM) and enzyme suspension were added and 30′ incubated at 37° C. The percentage aldimine, measured in the enzyme-free control tubes before and after 30′ incubation, was constant. In the case of dodecylamine and hexadecylamine, the aldimine was prepared in organic solvent containing 7.5 µl Triton-X-100 and after evaporating of the solvent the buffer (pH 7.0), NADPH and the enzyme suspension were added. The percentage of aldimine was measured in the enzyme-free control tubes after 30 min incubation. Retinol formation was investigated by thin layer chromatography (see Table IX). In identical experiments in which the same amount of NADH was added, no enzymatic conversion could be observed, either from extinction measurements or by thin layer chromatography.

Table VII (columns 3 and 4) shows that the measured aldimine concentrations agree rather closely with the calculated equilibrium values. In the enzyme incubation tubes the initial reaction rate was measured and expressed as percentage of the initial reaction rate for free retinaldehyde as substrate. The reaction rate for the conversion of the aldimines with NADPH as cofactor was on the average 55% of that when free retinaldehyde was used as substrate (Table VII, last column). When NADH was used as cofactor no enzymatic conversion could be observed with the aldimines as substrate while for free retinaldehyde only a slight conversion (14% as compared to NADPH as cofactor) was obtained.

In order to determine whether the conversion of the aldimines could be due simply to reduction of the free retinaldehyde present in the equilibrium mixture, similar experiments were carried out in which preformed aldimines with dodecylamine and hexadecylamine in different concentrations were used as substrates (Table VIII). The initial reaction rate was again expressed as percentage of the initial reaction rate for free retinaldehyde as substrate. The reaction rate expected if the aldimine conversion were simply due to reduction of free retinaldehyde present in the equilibrium mixture was

calculated in the following way. The Michaelis-Menten expressions for the reaction rates for each pair of experiments were divided:

$$\frac{V_1}{V_2} = \frac{\dfrac{V_{max}\ [S_1]}{K_m + [S_1]}}{\dfrac{V_{max}\ [S_2]}{K_m + [S_2]}} = \frac{[S_1]\ (K_m + [S_2])}{[S_2]\ (K_m + [S_1])}$$

In this equation V_1 is the initial reaction rate with free retinaldehyde ($[S_1]$ = 0.83 mM) as substrate, and V_2 is the initial reaction rate for the free retinaldehyde concentration $[S_2]$ present in the aldimine equilibrium mixture. For K_m the measured value of 0.8 was substituted. Since the rates during the first 30 min proved to be linear, the rates in Table VIII represent initial rates. It is assumed in these calculations that the establishment of the hydrolysis equilibrium is faster than the reduction reaction. Table VIII (last column) shows that the observed initial reaction rate was from 2.2 to 3.6 times as high as the calculated value for the case in which only free retinaldehyde

TABLE VIII

EXPERIMENTAL PROOF THAT RETINYLIDENE IMINES ARE DIRECTLY CONVERTED BY RETINOLDEHYDROGENASE

Initial molar ratio retinaldehyde/amine	percent aldimine observed at t=30′ in control tube	percent aldimine calculated for equilibrium	initial reaction rate observed	initial reaction rate calculated on free retinaldehyde concentration
1 : 0 dodecylamine	0	0	100	100
1 : 1	76	64	86.2 ± 5.9 (4)	39
1 : 2	84	87	72.1 ± 2.2 (4)	26
1 : 5 hexadecylamine	94	96	61.7 ± 9.3 (4)	18
1 : 5 hexadecyl trimethyl ammoniumbromide	95	96	66.0 ± 3.6 (6)	18
1 : 5	0	0	98.1 ± 18.1 (3)	100

The initial total concentration of retinaldehyde (free or in aldimine form) was in all cases 0.83 mM and of NADPH 1.3 mM. The observed reaction rate was measured as described under Materials and Methods, Enzyme assay. The calculated reaction rate was obtained by dividing for each experiment the Michaelis-Menten expression for v ($= \frac{v_{max}[S]}{K_m+[S]}$) by the same expression for v for the experiment with free retinaldehyde. For K_m the value of 0.8 mM was substituted and for [S] in each case the substrate concentration measured after 30′ incubation without enzyme addition.

would be converted. If the establishment of the hydrolysis equilibrium would be slower than the reduction, these discrepancies would only be larger. Therefore the aldimines cannot have been converted via the free retinaldehyde present in the equilibrium mixture.

The pH dependence of the enzymatic conversion of retinylidene octylamine is shown in Fig. 24. With 10^{-2}M octylamine it was possible to obtain 90% aldimine at pH 7.6, while under the same circumstances at pH 5.6 only 12% of the retinaldehyde was present in this form. Hence the activity curve for the aldimine really represents the pH-activity for an aldimine-aldehyde mixture of varying composition. Although the absolute activities were somewhat lower than the comparable values for free retinaldehyde, the pH maximum (pH 6.8) was nearly the same as for free retinaldehyde (pH 6.5). The Michaelis-Menten constant at pH 7.0 with 75-85% of retinaldehyde in aldimine form was 0.9 mM (S.E.: 0.2) mM, which is the same as the K_m value for free retinaldehyde (0.8 mM, S.E.: 0.1). At pH 7.1 and 7.6 the observed initial reaction rates were 1.8 and 2.7 times as high as would be the case if only the free retinaldehyde present in the equilibrium mixture would be reduced.

When the enzyme preparation and the assay were carried out in the dark, the activity for the conversion of retinaldehyde at pH 6.5 with NADPH as cofactor was 0.291 MKH (S.E.: 0.019, 4 determinations), while the activity in the light under these circumstances was 0.297 MKH (S.E.: 0.024, 8 determinations). Hence the enzyme activity was not significantly affected by light.

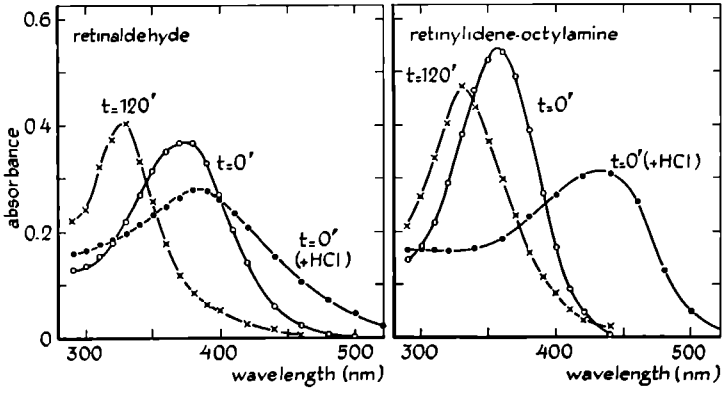


Fig. 25 Spectra of the reaction mixtures in which retinaldehyde (left) and retinylidene-octylamine (right) were being converted into retinol by rod outer segment retinoldehydrogenase in the presence of NADPH. The spectra were measured against blanks in which retinaldehyde or aldimine was omitted. Samples were taken after 0 (-o-o-o-) and after 120 min of incubation (-x-x-x-). Samples taken at t = 0 were, moreover, acidified with concentrated HCl (-•-•-•-).

5.3.2. *Identification of the reaction product*

A spectrophotometric analysis of the enzyme incubation mixture before and after incubation is presented in Fig. 25. The maximum for free retinaldehyde was located at 375 nm and was changed only slightly by acidification. The slight shift is probably due to a small amount of aldimine formation (± 15%) with amino groups present in the outer segment preparation. The maximum for retinylidene-octylamine was found at 365 nm at neutral pH and was shifted to 440 nm upon acidification. After two hours of incubation retinaldehyde as well as the aldimine had been nearly completely converted into retinol, as indicated by the peak observed at 330 nm.

The reaction product was also identified by thin layer chromatography. Samples of the incubation mixture before and after 2 hours of incubation were extracted with chloroform-methanol (Bligh and Dyer, 1958) and the organic layer was subjected to thin layer chromatography on silicagel with ether-hexane (1 : 1) or chloroform-methanol (10 : 1) as developing solvents. Table IX describes the spots found with the samples and with all-trans retinol, all-trans retinaldehyde, 11-cis retinol and retinyloctylamine as refe-

TABLE IX

IDENTIFICATION OF REACTION PRODUCTS
OF THE RETINOLDEHYDROGENASE REACTION

developing solvent	Ether/ Hexane (1:1)	Chloroform/ Methanol (10:1)
compound:		
all-trans-retinaldehyde	0.72**	0.89**
all-trans-retinol	0.40*	0.82*
11-cis-retinol	0.49*	—
retinyl-octylamine	0.10*	0.61*
reaction mixture with retinaldehyde t=0 hr	0.72**	0.89**
reaction mixture with retinaldehyde t=2 hr	0.40*	0.82*
reaction mixture with retinylidene-octylamine t=0 hr	0.72**	0.89**
reaction mixture with retinylidene-octylamine t=2 hr	0.40*	0.82*

Rf values after thin layer chromatography with silica gel of retinaldehyde, retinol, retinyl-octylamine and chloroform/methanol extracts of reaction mixtures in which either retinaldehyde or retinylidene-octylamine was incubated with rod outer segment retinoldehydrogenase and NADPH. The extracts were obtained before and after two hours of incubation.

* fluorescent with UV light and blue color with Carr-Price reagent.

** yellow color; green color with Carr-Price reagent.

rence compounds. At t = 0 only all-trans retinaldehyde was present, while at t = 2 hours all-trans retinol was the major component. The absence of retinyloctylamine in the reaction mixture indicates that the enzyme does not react by reducing the aldimine bond to a secondary amine. The absence of 11-cis retinol as an end product indicates that no isomerase activity was present in our ROS retinoldehydrogenase preparation. This is interesting in view of an observation briefly reported by Tolbert (1968) of the conversion of all-trans retinol to 11-cis retinaldehyde by a bovine retinoldehydrogenase.

The formation of retinol was also followed quantitatively by means of fluorimetry. The cofactors NADPH and NADH interfered, because they fluoresce at the same wavelength. However, the fluorescence of NADPH decreased by more than 90% upon standing overnight in n-propanol when benzylamine was present in the incubation mixture. The fluorescence of retinol did not change under these circumstances. Since the fluorescence of NADH was but slightly reduced upon applying this technique, retinol determinations were only carried out in experiments with NADPH as cofactor. In this way the amount of retinol formed could be determined quantitatively. Fig. 26 shows that retinol was formed in amounts equimolar to the decrease in the amount of aldimine.

5.3.3. *Whole retina preparation*

The retinoldehydrogenase activity present in homogenates of whole retina

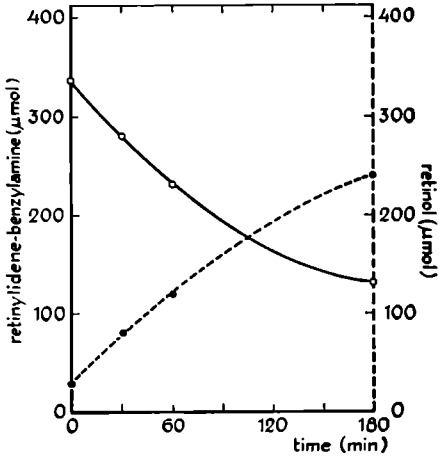


Fig. 26 Enzymatic conversion of retinylidene-benzylamine by rod outer segment retinoldehydrogenase as a function of time. The pH was 7.4 and the cofactor NADPH. The amounts of aldimine converted and of retinol formed were equimolar (210 μmoles).

was also studied. Whole cattle retina was homogenized and centrifuged twice for 5′ at 500 x g. The supernatant contained 70% of the total activity after removal of most of the retinal debris. Upon centrifuging this supernatant for 10′ at 10,000 x g 25% of the activity was still found in the supernatant. However, after centrifugation at 100,000 x g the resulting supernatant was completely devoid of enzyme activity. Repeated washing with water or with dilute buffer or repeated freezing and thawing of the pellet did not solubilize the enzyme activity. This indicates that the activity in whole retina does not represent a soluble enzyme as previously claimed (Wald, 1950; Koen and Shaw, 1966) but it has a particulate character like the enzyme activity present in the rod outer segments.

This whole retina preparation could also reduce both retinaldehyde and retinylidene imines to retinol. Retinaldehyde was converted by this preparation at pH 6.5 with NADPH at a rate of 0.213 MKH (S.E.: 0.22; 3 determinations). There is a shallow pH optimum in the presence of NADPH around 6.5 which is also the pH optimum for the outer segment enzyme. Retinylidene imines were converted 0.6 to 0.9 times as fast as free retinaldehyde by this preparation. However, in this case both NADPH and NADH could serve as cofactor, with the reaction rate in the presence of NADH 1.03 (S.E.: 0.16; 5 determinations) times that in the presence of NADPH. Thin layer chromatography showed that the end product was retinol, as was also the case for the ROS enzyme preparation.

5.3.4. *Liver alcoholdehydrogenase*

We confirmed the finding of other authors (Bliss, 1949, 1951; Zachman and Olsen, 1961; Futterman and Saslaw, 1961; Futterman, 1963, 1965; Koen and Shaw, 1966) that alcoholdehydrogenase from horse liver converts free retinaldehyde into retinol, with NADPH and NADH serving equally well as cofactor. The pH optimum was 5.2 and the K_m value was 0.9 mM. The latter value was 3-4 times as high as the value reported by Zachman and Olsen (1961), who used Tween 80 instead of Triton-X-100 as detergent.

However, the liver alcoholdehydrogenase did not reduce imines of retinaldehyde, as shown by the experiments summarized in Table X. At five different pH values octylamine was added in three different concentrations to the reaction mixture containing retinaldehyde, and the mixtures were incubated until equilibrium was reached. Setting at each pH value the reaction rate in the absence of octylamine at 100%, there is a rough parallelism between the conversion rate and the free retinaldehyde concentration. At the highest pH (7.6) and the highest octylamine concentration (9.1 mM), in which experiment the highest aldimine concentration was obtained (95%), there was no con-

TABLE X

ENZYMATIC CONVERSION OF RETINALDEHYDE AND RETINYLIDENE-OCTYLAMINE BY LIVER ALCOHOLDEHYDROGENASE

pH	octylamine conc. (mM)				octylamine conc. (mM)			
	0	1.82	4.55	9.10	0	1.82	4.55	9.10
	percentage free retinaldehyde				relative initial reaction rate			
5.5	100	96	91	86	100 (100)	71 (97)	32 (93)	29 (89)
6.0	100	95	87	73	100 (100)	70 (96)	37 (90)	18 (78)
6.5	100	90	77	61	100 (100)	48 (92)	35 (82)	21 (68)
7.0	100	78	55	34	100 (100)	37 (83)	14 (62)	9 (41)
7.5	100	48	24	5	100 (100)	40 (55)	0 (30)	0 (7)

At the left side the percentage free retinaldehyde is given at different pH values and octylamine concentrations. The percentage free retinaldehyde was calculated by subtracting from the initial retinaldehyde concentration (0.3 mM) the measured percentage aldimine. At the right side of the table the relative reaction rate is given, setting the reaction rate for the conversion of free retinaldehyde at each pH value as 100%. In parentheses the theoretical reaction rate is given for the case that only free retinaldehyde was converted by the enzyme. These theoretical values were calculated from Michaelis-Menten equations (see text). In all experiments NADH (1.3 mM) was used as cofactor.

version of the aldimine and chromatographic analysis gave no indication for the formation of retinol. The relative reaction rates, calculated on the assumption that only free retinaldehyde could be converted, were always higher than the observed reaction rates. This indicates that there is an inhibition of the enzyme by octylamine. This effect is most striking at pH 5.5, where only very little aldimine formation occurs, but where the reaction rate is drastically reduced.

5.3.5. *Search for aldimine hydrolase activity*

At first sight it would seem reasonable to assume the presence of an aldimine hydrolase in the rod outer segments in order to explain the conversion of aldimines to free retinol. The following experiments was carried out in order to test this supposition. Retinaldehyde and octylamine were mixed in a 1 : 1 ratio in absolute benzene or methanol to which 7,5 µl Triton-X-100 was added. Retinylidene octylamine was formed in nearly 100% yield upon evaporating of the solvent. To the dry residue consisting of 0.63 µmole aldimine 1 mg rod outer segment preparation suspended in 750 µl phosphate buffer (0.067 M; pH = 7.0) was added. To the control tube buffer solution without ROS was added. The tubes, which contained no NADH or NADPH were incubated under nitrogen at 37° C. Every 5 minutes 50 µl samples were withdrawn, in which the percentage aldimine

was determined by acidification and measurement of the E_{440}/E_{380} ratio (see 4.2.6.).

Fig. 27 shows that there is a much faster hydrolysis of the aldimine in the presence of the rod outer segment preparation, but the final equilibrium composition was not influenced. Since the same accelerated hydrolysis was obtained with an outer segment preparation which had been heated at 100° C for 30′, the effect cannot be ascribed to the presence of a hydrolase in the outer segments. Neither could the effect be due to a catalysis by metallic ions, possibly present in the rod outer segment preparation, since addition of EDTA (10^{-4}M, final concentration) to the rod outer segments did not decrease the hydrolysis rate. Addition of EDTA to the control tube had no effect on the hydrolysis rate.

We considered the possibility that a structural component in the outer segment material was responsible for the effect. This possibility was investi-

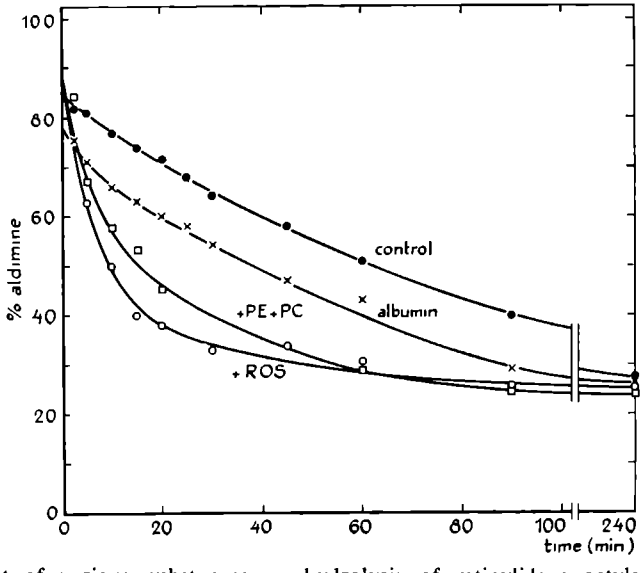


Fig. 27 Effect of various substances on hydrolysis of retinylidene octylamine in a phosphate buffer (0.067M; pH = 7.0, containing 1% Triton-X-100). In the experiment indicated by (-o-o-o-) 1 mg of lyophilized rod outer segments (ROS) was added. An identical curve was obtained when EDTA (10^{-4}M) was added with the outer segments, or when the outer segments were heated at 100° for 30′ prior to addition. In the experiment indicated with (-□-□-□-) 1 mg of a lipid extract of ROS containing as major components phosphatidylethanolamine (PE) and phosphatidylcholine (PC) was applied. In the experiment indicated with (-x-x-x-) bovine serum albumin (1 mg) was added; in the control experiment (-●-●-●-) buffer alone was applied. Addition of EDTA (10^{-4}M) or phosphatidylcholine to the aldimine gave the same hydrolysis curve as in control experiment.

gated by adding in stead of rod outer segments phosphatidylcholine, bovine serum albumin and a lipid extract of the rod outer segments respectively to the aldimine. Phosphatidylcholine had no effect; addition of albumin increased the hydrolysis rate only slightly, but addition of the lipid extract, containing phosphatidylethanolamine had a large effect on the rate of hydrolysis. Thus, it seems reasonable to assume that the presence of a phospholipid with an amino group in the outer segments was responsible for the increased rate of hydrolysis. This suggests that transiminization of retinaldehyde from octylamine to such a phospholipid amino group may occur and that this new aldimine may hydrolyze faster than the retinylidene octylamine.

In our experiments with retinoldehydrogenase (5.3.1.) we started always with the aldimine in equilibrium position. The effect described here only concerns the rate with which the equilibrium is approached, but it does not influence the position of the equilibrium. It does, therefore, not contradict the conclusions from these experiments, which are that aldimines are directly converted into retinol (see 5.4.1.). However this catalytic effect might play an important role, *in vivo,* in accelerating the conversion of metarhodopsin II.

5.4. DISCUSSION

5.4.1. *Proof for the enzymic conversion of aldimines to retinol*

As explained in 5.1., there is an important difference between the reaction products after photolysis of rhodopsin extracts or of rhodopsin in situ. In the former retinaldehyde is the end product (Wald, 1938; Bridges, 1962 a), while in the latter retinol is the end product and there is no indication for the occurence of free retinaldehyde as an intermediate (Matthews et al, 1963; Baumann, 1967, 1968; Cone and Brown, 1969; Reuter, 1966; Donner and Reuter, 1967, 1969).

It was suggested that in situ the retinoldehydrogenase system together with the cofactor NADPH, both present in the rod outer segment, convert metarhodopsin II to retinol, either directly or via an intermediate, in which retinaldehyde is bound by an aldimine link to the opsin complex. This intermediate could be pararhodopsin (λ_{max} = 465 nm) (Matthews et al, 1963; Cone and Brown, 1969), which may be a protonated aldimine of retinaldehyde with the opsin complex and actually might be a retinoldehydrogenase-substrate complex.

The fact that free retinaldehyde is not observed as an intermediate during the photolysis of rhodopsin in situ could be explained in 2 ways: 1. The enzymatic conversion of retinaldehyde into retinol is so fast compared to the hydrolysis of the aldimine that the latter is the rate-limiting step. The reduction of retinaldehyde to retinol then withdraws retinaldehyde from the

aldimine hydrolysis equilibrium and no free retinaldehyde accumulates. 2. The aldimine is directly converted to retinol by the retinoldehydrogenase of the outer segments.

Our experiments were aimed at distinguishing between these two possibilities. We have prepared stable aldimines of retinaldehyde in aqueous detergent solution with the aid of long-chain aliphatic amines (see 4.3.1.). We have demonstrated that these aldimines are converted to retinol by rod outer segment retinoldehydrogenase in the presence of NADPH. The rate of this conversion was only slightly lower than that for the conversion of free retinaldehyde under the same circumstances. The identity of all-trans retinol as the end product was proved by spectroscopic and fluormetric analysis as well as by thin layer chromatography. By means of the latter technique retinyloctylamine, the reduced form of the aldimine, was specifically excluded as the end product.

In view of the fact that in aqueous detergent solution there is an equilibrium between the aldimine and its hydrolysis products, there was always some free retinaldehyde present in the reaction mixture. However, calculations with the aid of the Michaelis-Menten equation showed that if only the free retinaldehyde present would be converted, the initial reaction rate would be 2-3 times slower than the observed rate. This finding strongly supports the second possibility mentioned above: the direct conversion of the aldimine to retinol. In further support of this conclusion is the fact that liver alcoholdehydrogenase does not convert these aldimines, although it does reduce free retinaldehyde. The above conclusion could be invalidated if excess amine present with the aldimine would markedly activate the enzyme. This does not seem likely for the following reasons. The experiments with liver alcoholdehydrogenase indicate that in this case excess octylamine had an inhibitory rather than a stimulatory effect (Table X). Secondly, hexadecyltrimethylammonium bromide in the same concentration as hexadecylamine had no effect on the reduction of free retinaldehyde (Table VIII). Finally, Fig. 24 shows that at pH 5.6-6.1, where there was a large excess of amine (10 mM) in the aldimine equilibrium mixture (15-29% of the retinaldehyde bound as aldimine), the activity was the same or less than for the free retinaldehyde without amine.

Assuming therefore that the aldimine of retinaldehyde is directly converted to retinol, two further possibilities can be distinguished. The first possibility would be that the enzyme preparation contains an aldimine hydrolase, in addition to a retinoldehydrogenase. However, we showed (5.3.5.) that no aldimine hydrolase was present and that the increased rate of aldimine hydrolysis observed upon addition of rod outer segments without cofactor to

the aldimine did not lead to a change in the position of the equilibrium. Since in our experiments equilibrium mixtures of aldimine were used as substrate this increase in the hydrolysis rate could not have influenced the reaction rate. The other possibility is that hydrolysis and reduction are carried out by a single enzyme system. This enzyme might react with the aldimine leading to the formation of an enzyme-substrate complex via transiminization. This enzyme-substrate complex would dissociate and form retinol by stimultaneous reduction. Such a transiminization reaction occurs in pyridoxal-catalysed transaminase reactions (Jenkins and Sizer, 1957).

5.4.2. *Solubility and cofactor specificity of retinoldehydrogenase*

In the literature there are some contradictions concerning the solubility of retinoldehydrogenase. Wald and Hubbard (1949) used in their early experiments a suspension of rod outer segments, but in later investigations (Wald, 1950; Hubbard, 1956) they used whole retina extracts. Koen and Shaw (1966) found activity in a supernatant of frozen-thawed retina's and they were able to separate on starch gel electrophoresis the enzyme activity in different bands. On the other hand, Futterman (1963) was unable to extract the enzyme from a suspension of rod outer segments, in contrast to the soluble enzymes glucose-6-phosphate dehydrogenase, lactic dehydrogenase and hexokinase. The enzyme was found in the same particulate fraction as rhodopsin. We confirmed his results, but found that in the case of whole retina homogenates centrifugation at 10,000 x g for 20′ did not completely sediment all enzyme activity. This is probably why Wald (1950), Hubbard (1956) and Koen and Shaw (1966) found enzyme activity in the supernatant after centrifugation. Centrifugation for 2 hrs at 100,000 x g completely sedimented the enzyme activity in our experiments.

We could confirme Futterman's (1963) finding that the retinol dehydrogenase from rod outer segments works more specifically with NADPH than with NADH. The activity with NADH was barely significant and in some experiments we found no activity at all with NADH. On the other hand, the whole retina enzyme preparation was about equally active with NADH and with NADPH. This suggests that the outer segment enzyme works specifically with NADPH and that there is another enzyme elsewhere in the retina that can either use both cofactors or only NADH. Impurity of the rod preparations could then result in an enzyme preparation with some activity towards NADH. It is very improbable that the enzyme activity towards NADH is due to the presence of an NADPH-forming enzyme in the retina, since the enzyme preparations were washed and hence would lack ATP, which is needed for the conversion of NADH to NADPH (Korn-

berg, 1950). Another indication for the presence of a different enzyme in the retina outside the outer segments is given by the following consideration. The activity of the whole retina preparation is 70% of the activity of the ROS preparation on a dry weight basis, while the rod outer segment layer constitutes about 14% of the weight of the retina (Lowry et al, 1956). This indicates that only a minor part of the retinoldehydrogenase activity in whole retina can be due to the activity present in the rod outer segments.

5.4.3. *Consequences for the in vivo process*

Bonting and Bangham (1967) have advanced a theory of the visual mechanism, according to which a transiminization of retinaldehyde from its original binding site to a membrane amino group during the metarhodopsin I → metarhodopsin II transition would lead to an increase in the cation permeability of the rod sac membrane (see 1.3.5.). Rod sac regeneration would then involve two processes:

1. restoration of the original low cation permeability of the membrane by removal of retinaldehyde form the amino group.
2. restoration of the initial ionic gradients by means of the Na-K activated ATPase cation pump system in the rod sac membrane (Bonting et al, 1964 a).

We would like to propose, that the first process can be carried out by the outer segment retinoldehydrogenase, converting the aldimine of retinaldehyde in the rod sac membrane to retinol. Calculations showed that in cattle and frog rods the gradient restoration rate could be rate limiting and thus explain the rod saturation phenomenon (Bonting and Bangham, 1967; Bonting, 1969; Duncan et al, 1970). This would suggest that the conversion of the aldimine in the membrane would have to occur at a faster rate. We find in vivo a theoretical maximal conversion rate of 0.6 mole per hour per kg dry weight cattle rod outer segments. Assuming a dry weight of cattle rod outer segments of 1.6×10^{-14} kg and an average number of 990 sacs per rod, this would yield a conversion rate of 1.6×10^{3} molecules aldimine per rod sac per second. At rod saturation level each sac receives 4.4 quanta per sec. The rate of aldimine conversion would thus be 370 times as fast as the rate of its formation, and would therefore not be rate limiting in the rod sac regeneration process.

5.5. SUMMARY

An insoluble retinoldehydrogenase present in cattle rod outer segments can reduce all-trans retinylidene imines in the presence of NADPH directly to free all-trans retinol. The rate of this conversion is only slightly smaller than

that for the reduction of the free retinaldehyde. A retinoldehydrogenase preparation obtained from whole retina can also convert these aldimines, but this enzyme preparation works both with NADPH and with NADH.

Horse liver alcoholdehydrogenase reduces free retinaldehyde, but not retinylidene imines.

It is suggested, that the retinoldehydrogenase in the rod outer segments may play an important role in the regeneration of the rod sac after stimulation by light.

THE ROLE OF SODIUM-POTASSIUM ACTIVATED ATPase IN THE VISUAL PROCESS

6.1. INTRODUCTION

The enzyme sodium-potassium activated adenosinetriphosphatase (Na-K ATPase), which is localized in nearly all cell membranes over which a gradient for sodium and potassium exists, is generally accepted to be identical with the sodium pump. A first indication for the presence of this enzyme in the retina and in outer segments was obtained by Sekoguti (1960 c) who found that the addition of potassium stimulated the ATPase activity with sodium present in the assay medium. A high activity of this enzyme in human and cat retina was found by Bonting et al (1961). In a more detailed investigation Bonting et al (1964 a) found high activity in the retina and in isolated rod outer segments of cow, calf, rabbit and frog. They disproved moreover, the claim of McConnel and Scarpelli (1963) that rhodopsin would be an ATPase. Further evidence against this claim was reported by Frank and Goldsmith (1965), who found a high activity of Na-K ATPase in isolated pig outer segments.

The high activity of this enzyme in rod outer segments was one of the reasons which led Bonting and Bangham (1967) to their hypothesis on the biochemical mechanism of the visual process. The sodium pump, present in the rod sac membrane containing the visual pigment, must play a role in restoring the original cation gradients so that each sac in the rod outer segment can be excited again. Bonting and Bangham (1967) calculated that with the pump working at maximal capacity the time required for the restoration of the original cation gradients is of the same order as the time elapsing between absorption of individual photons by the rod sac at rod saturation level.

Since we have investigated the dark and light stimulated ionic movements and the effect of ouabain, a specific inhibitor of the sodium pump, on these movements in the retina of Sepia officinalis, we have investigated the properties, distribution and activity of Na-K ATPase in this retina. In addition, we have reinvestigated and extended earlier studies of the activity of Na-K ATPase in the retinae and rod outer segments of different species. In the course of this investigation we noticed, that Na-K ATPase activity in lyophilized rod outer segments decreases upon exposure to air. This could be the

reason why Matschinsky (personal communication) found upon application of the quantitative histochemical approach of Lowry et al (1956) — in which technique thin (6 μ) lyophilized tissue sections are exposed to the air for a few hours — in the rod outer segment layer of rabbit retina an activity of only 0.2 moles per kg dry weight per hour (MKH), while Bonting et al (1964 a) had determined in lyophilized rabbit outer segments an activity of 3.7 MKH. Preliminary experiments described in this chapter suggest that oxidation of unsaturated fatty acids present in the phospholipids in these preparations is the cause of this discrepancy. Phospholipids are an essential component of the Na-K ATPase system (Schatzmann, 1962; Roelofsen, Baadenhuyzen and Van Deenen, 1966; Wheeler and Whittam, 1970). It happens that rod outer segments contain highly unsaturated fatty acids in an unusually high proportion (Borggreven et al, 1970).

6.2. MATERIALS AND METHODS

6.2.1. *Tissue preparation*

Eyes of animals of different species were enucleated immediately after death and were kept on ice until dissection. The retina's were removed within one hour and homogenized in a tenfold volume of double distilled water. These homogenates were lyophilized in 0.2 ml aliquots at —20° C and stored at this temperature in vacuo.

For the separation of the different layers of the retina of Sepia officinalis the method of Hara and Hara (1965) was followed. The eyes were frozen directly after enucleating and kept at —20° C for 24 hours. After thawing the eyes, cornea, iris and lens were removed and the rest of the eyes were shaken in a neutral isotonic phosphate buffer (0.2 M, pH 7.0). In this way the rhabdomes and pigment granules were released from the rest of the retina. The remaining part of the retina, containing nuclei and nerve endings, could be removed from the eye cup by means of a spatula. Both retinal fractions, the "nuclear" and the "rhabdomal" layer were homogenised with double distilled water and centrifuged for 15 min. at 10.000 x g. This washing procedure was twice repeated to remove phosphate buffer. The procedure was carried out entirely at 4° C.

Cattle rod outer segments were prepared as described under 2.2.2., with the exception that all steps were carried out at 4° C. Frog rod outer segments were prepared according to the method of Duncan et al (1970) with a slight modification. Specimens of the common frog Rana temporaria were dark adapted for 1-3 hours under running tap water to remove superficial bacteria. After decapitation the retinae were extruded through a slit in the cornea. The outer segments were separated from the rest of the retina by shaking

for 2-3 min. in 8 ml of a modified Ringer solution (containing 100 mM NaCl, 3 mM KCl, 3 mM $MgCl_2$, and buffered to pH 7.6 with 10 mM Tris HCl) at 4° C and filtering over cotton gauze. The outer segment suspension was centrifuged for 5 min. at 4,000 x g at 4° C and the supernatant was discarded. The sediment was twice washed with double distilled water and centrifuged for 10 min. at 10,000 x g at 4° C. The resulting sediment was lyophilized at —20° C and stored in vacuo at this temperature.

6.2.2. *ATPase assay*

Na-K ATPase activity was determined according to the method of Bonting et al (1963). The amount of inorganic phosphate released upon incubation of tissue homogenates in various substrate media (Table XI) supplies measure

TABLE XI

COMPOSITION OF SUBSTRATE MEDIA USED FOR THE ASSAY OF Na-K ATPase

Medium Code	A	B	C	D	E
ATP	2	2	2	2	2
Mg^{2+}	1	1	1	1	1
Na^+	56	56	—	56	56
K^+	5	—	5	5	—
EDTA	0.1	0.1	0.1	0.1	0.1
Tris Histidine buffer pH 7.1	94	94	98	94	94
ouabaine	—	—	—	0.1	0.1

All concentrations are expressed in mmol per liter final concentration. The only other ion species present was chloride. In the experiments described in 6.3.3. and 6.3.4. the Mg^{2+} concentration was raised to 2 mM and the Tris-histidine buffer was replaced by an equimolar Tris buffer pH 7.5.

for the enzymatic activity. In medium A the sum of Mg activated and Na-K activated ATPase activities is determined. Since Na-K ATPase needs both sodium and potassium and is inhibited by ouabain, the amounts of inorganic phosphate released upon incubation of the homogenate in the media B (without K), C (without Na), D (with ouabain), and E (without K, with ouabain) represent the activity of Mg ATPase. Lyophilized tissue homogenate was weighed on a Cahn electrobalance and homogenized in double distilled water (0.1-0.3% w/v). Aliquots of 10 µl homogenate were added to 150 µl of the various substrate media. Three microtubes (30 x 4.7 mm) containing 10 µl aliquots were incubated for 1 hour at 37° C. Three other aliquots, serving as enzyme blanks, were immediately placed in ice and to each 30 µl

10% TCA was added. The incubated tubes received 30 µl 10% TCA after 1 hr. of incubation. After mixing the tube contents, 40 µl of the colour reagent was added and the tubes were centrifuged (16,000 x g for 2 min.) to remove protein.

The colour reagent was prepared by dissolving 400 mg ferrous sulphate in 5 ml of a 1% ammonium molybdate solution in 1.15 N H_2SO_4. It was used within two hours after its preparation. The resulting colour was read between 2 and 120 min. after addition of the colour reagent in a Zeiss PMQ II spectrophotometer fitted with a microcuvette attachment. Care was taken to keep the time elapsing between addition of the colour reagent and the reading of the optical density about the same for all tubes in order to exclude an effect of the non-enzymatic hydrolysis of ATP by molybdate (Weil-Malherbe and Green, 1951). Phosphate standards were prepared by adding 20 and 40 µl of an 50 mM KH_2PO_4 solution to 5 ml medium A. To 10 µl aliquots of these standards 30 µl 10% TCA and 40 µl colour reagent were added. Aliquots of 10 µl of medium A alone were taken as reagent blanks.

Enzyme activities were calculated from the optical densities — after correction for the reagent blanks — by relating the corrected densities to those of the standards. Enzyme activity was expressed in moles inorganic phosphate released per kg dry weight of tissue per hour (MKH).

6.2.3. *Fatty acid analysis*

The lipid fraction from a weighed amount of rod outer segment preparation was obtained by threefold extraction with chloroform-methanol (2 : 1). After evaporation of the solvent, the lipid residue was dissolved in a known volume of benzene-ethanol (4 : 1) and was stored at —20° C under nitrogen.

Preparation of fatty acid methylesters for gas liquid chromatography was carried out with boron trifluoride according to the method of Morrison and Smith (1964). The solvent was removed by blowing nitrogen over the lipid extract and 0.5 ml hexane and 1 ml 10% BF_3 in methanol were added. The mixture was placed in a closed tube under nitrogen and the tube was heated in a boiling water bath for 15′. After cooling, 4 ml pentane and 2 ml water were added. The water layer was removed and the pentane layer containing the methylesters of the fatty acids was stored under nitrogen at —20° C.

Fatty acid separation was carried out over a column of 10 or 15% diethyleneglycolsuccinate on Gas Chrom P (Applied Science) at 180° C.

6.3. RESULTS AND DISCUSSION

6.3.1. *Occurrence and distribution of Na-K ATPase in the retina of Sepia officinalis*

Table XII lists the relative activities of the retina of Sepia officinalis in the various substrate media. In media B to E the average activity was only 15% of that in medium A, which means that at this pH (7.1) and Mg concentration (1 mM) 85% of the total ATPase activity consisted of Na-K activated ATPase activity. Since the activities in media B to E were about equal, the enzyme distribution pattern was determined by media A (complete) and E (without K, with 10^{-4}M ouabain) only.

By means of the freezing and shaking procedure developed by Hara and Hara (1965) the rhabdomes and the pigment granules were separated from the rest of the retina. In Fig. 28 histological sections of an untreated retina and of a retina, treated in this way, are shown. In the latter retina only the nuclei and the nerve endings are left. The enzyme activities of the rhabdome fraction, of the nuclear layer and of the whole retina are listed in Table XIII. The activities of Na-K ATPase and of Mg ATPase were only about half of those in the nuclear layer, but there was still a considerable activity of Na-K ATPase (2.67 MKH; S.E.: 0.08; 4 detns.) in the rhabdome layer.

On the basis of histological observations as well as of dry weight determinations we can conclude that the rhabdome layer represents about two thirds of the total volume and weight of the retina. By multiplying the enzyme activities by their relative weights and adding the results one would expect to find the same activity as in the whole Sepia retina. The results in Table XIII show however that the sum is somewhat lower. This may be due to a loss of enzyme activity during the freezing and thawing procedure, since the complete retina was directly homogenized and lyophilised.

TABLE XII

ATPase ACTIVITY OF THE RETINA OF SEPIA OFFICINALIS, OBTAINED IN DIFFERENT INCUBATION MEDIA

Medium	ACTIVITY	
	absolute (M.K.H.)	relative (%)
A	4.35 ± 0.32	100
B	0.72 ± 0.15	16.6 ± 3.4
C	0.58 ± 0.09	13.3 ± 2.0
D	0.65 ± 0.15	15.0 ± 3.4
E	0.63 ± 0.10	14.5 ± 2.3
AVERAGE B-E	0.64 ± 0.05	14.7 ± 1.2

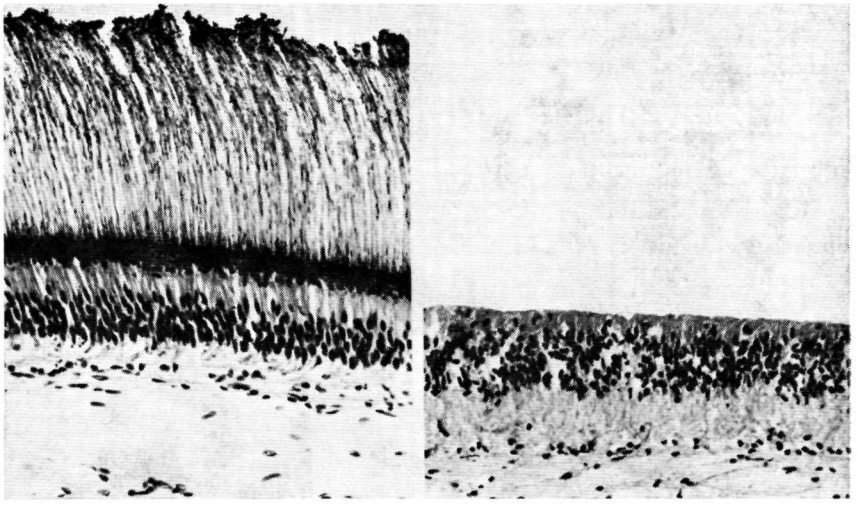


Fig. 28 Cross section of the retina of Sepia officinalis. Left the untreated retina; right a retina treated according to Hara and Hara (1965), consisting of nuclei and nerve fibers (called nuclear layer) and devoid of the rhabdomal layer. x 200

6.3.2. *Properties of Na-K ATPase and Mg ATPase in the retina of Sepia officinalis*

Fig. 29 shows the pH-activity curves for Mg ATPase and Na-K ATPase in this retina. The activities were determined by varying the pH of the buffer in media A and E. Above pH 7.5 a Tris buffer (0.5 M) and below pH 7.5 a Tris-histidine buffer (0.5 M) was used. The optima are 8.4 and 7.1 respectively, which is slightly lower than in most other tissues. In the stria vascularis of the inner ear Kuijpers and Bonting (1969) found pH maxima of 8.7 and

TABLE XIII

ACTIVITY AND DISTRIBUTION OF Na-K ATPase AND Mg ATPase IN DIFFERENT LAYERS OF THE RETINA OF SEPIA OFFICINALIS

	Na-K ATPase (M.K.H.)	Mg ATPase (M.K.H.)
Whole retina	4.35 ± 0.32 (6)	0.85 ± 0.11 (6)
Rhabdome layer	2.67 ± 0.08 (4)	0.31 ± 0.12 (4)
Nuclear layer	5.77 ± 0.29 (4)	1.01 ± 0.09 (4)
Recovery *	3.70 (85%)	0.54 (64%)

* The recovery was calculated by multiplying the activity in the two layers by their relative weights (two thirds for the rhabdome layer and one third for the nuclear layer) and by adding the resulting weighted activities. In parentheses on this line the recovery is expressed as the percentage of the activity for the whole retina.

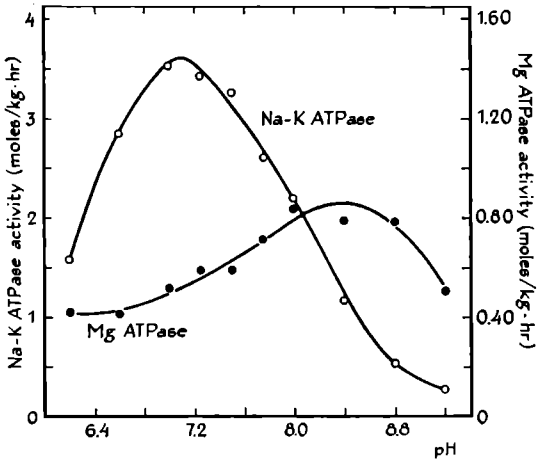


Fig. 29 Effect on pH on Na-K ATPase activity (-o-o-o-) and Mg ATPase activity (-●-●-●-) in homogenates of the sepia retina. The pH dependence was measured in media (A) complete and E (no K, + 10^{-4}M ouabain) prepared with Tris-Histidine buffers in the pH range of 6.2-7.5 and with Tris buffers in the range 7.5-9.2. The Mg ATPase activity was measured in medium E, while the Na-K ATPase activity was calculated from the difference in activities in media A and E.

7.3 respectively. The same values were found by Bakkeren and Bonting (1968) in rat liver. The pH-optimum of Na-K ATPase in marine animals is generally a few tenths of a pH unit lower than in terrestrial animals. Skou (1957, 1960) found in crab nerve a pH-optimum of 7.2, which value was also observed by Bonting et al (1964 b) in the salt gland of the herring gull. In the rectal gland of the spiny dog fish Bonting (1966) found a pH-optimum of only 7.0. Since we were primarily interested in the Na-K ATPase system, we have used a pH of 7.1 in the investigation of the distribution and absolute activity (see 6.3.1.), as well as of the properties of the enzyme.

In Fig. 30 the Na-activation curve for Na-K activated ATPase is given. This curve was obtained by adding to medium C increasing amounts of NaCl (0-300 mM). The concentration of potassium was kept constant (5 mM) in this procedure. Maximal activation was obtained at 50 mM. Half-activation was reached at a sodium concentration of 10 mM. This value is in good agreement with the reported values of other authors, e.g. 10 mM in rabbit pancreas (Ridderstap and Bonting, 1969) and 11.7 in the rectal gland of the spiny dog fish (Bonting, 1966).

Fig. 31 shows the activation of Na-K ATPase by potassium. For this experiment increasing amounts of KCl (0-30 mM) were added to medium B. The sodium concentration was kept constant at 58 mM. Maximal activation

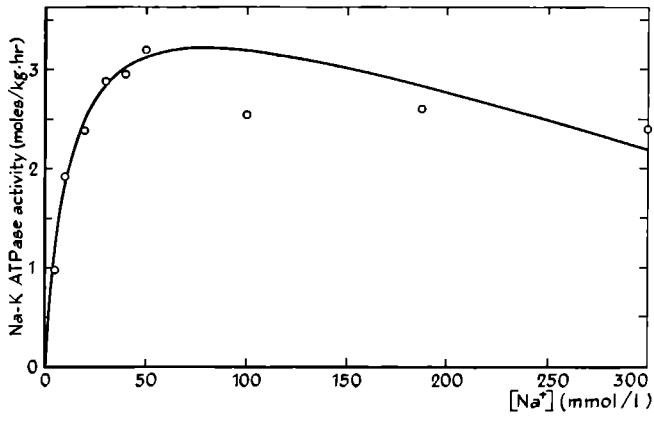


Fig. 30 Effect of Na concentration on Na-K ATPase activity in homogenates of the sepia retina. Increasing amounts of NaCl were added to medium C (no Na), while the K concentration was kept at 5 mM. Medium E (no K, + 10^{-4} ouabain) gave the Mg ATPase activity.

was obtained by 5 mM potassium. Half-maximal activation was reached at 0.9 mM potassium. This value is also in good agreement with values from other tissues, e.g. 0.9 mM in the stria vascularis of guinea pig (Kuijpers and Bonting, 1969) and rat liver (Bakkeren and Bonting, 1968) and 0.8 mM in rabbit pancreas (Ridderstap and Bonting, 1969).

Since we have used the isotope ^{86}Rb for ion movement studies in this retina (chapter 3) we were interested whether this cation could also activate the enzyme system in the presence of sodium. This had previously been found by Bonting et al (1963) in the rabbit lens and by Bakkeren and Bonting (1968) in rat liver. Fig. 31 shows that there is no observable difference between the activation curves for potassium and rubidium. Half-maximal activation was reached in both cases at a cation concentration of 0.9 mM.

Some authors (Bakkeren and Bonting, 1968; Ridderstap and Bonting, 1969) observed that sodium and potassium concentrations far above the optimal values lower the enzymatic activity. Bonting (1970) explained this by assuming that the cation present in high concentration displaces the other cation from its binding site. The sodium and potassium activation curves for Na-K ATPase in the retina of Sepia officinalis, however, show this effect to a much smaller extent. This could be due to the fact that in marine invertebrates the cation concentrations normally are much higher than in terrestrial animals. This lack of inhibition was also found in the rectal gland of the spiny dog fish (Bonting, 1966).

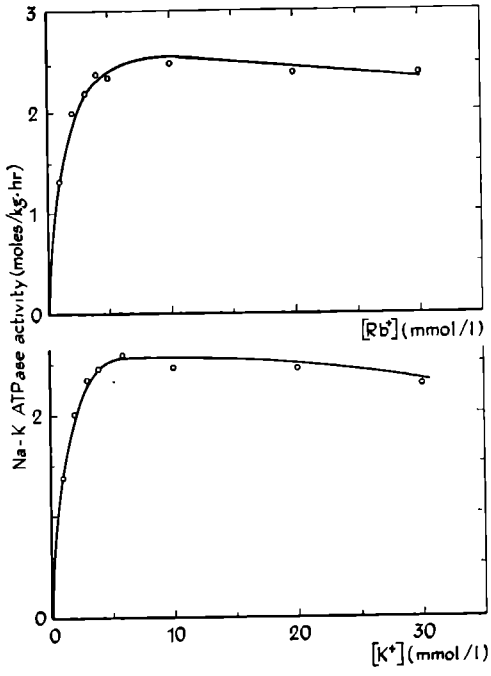


Fig. 31 Effect of Rb (upper curve) and K (lower curve) concentrations on Na-K ATPase activity in homogenates of the sepia retina. Increasing amounts of RbCl and KCl respectively, were added to medium B (no K), keeping the Na concentration at 63 mM. Medium E (no K, + 10^{-4}M ouabain) gave the Mg ATPase activity.

Fig. 32 shows the Mg activation curves of Na-K ATPase. The activities were determined after adding increasing amounts of $MgCl_2$ (0-6 mM) to the media A and E, while the ATP concentration was kept constant at 2 mM. In the absence of magnesium the activity of both Na-K ATPase and Mg ATPase were practically zero. Mg ATPase was maximally activated at about 4 mM and Na-K ATPase at a concentration of 2 mM. The optimal Na-K ATPase activity is thus obtained at a Mg : ATP ratio of 1 : 1, as was also found in other tissues, like the salt gland of the herring gull (Bonting et al, 1964 b) and the heart muscle of the guinea pig (Portius and Repke, 1967).

Fig. 33 shows the inhibition curve for ouabain. The total ATPase activity was measured in medium A, to which increasing amounts of ouabain (10^{-12} to 10^{-2}M) had been added. Ouabain concentrations below 10^{-9}M had no effect. From 10^{-8}-10^{-5}M ouabain an increasing inhibition of Na-K ATPase occured. At an ouabain concentration of 10^{-4}M or higher the inhibition of Na-K ATPase was complete and the activity measured was due to Mg

ATPase. The negative log of the ouabain concentration causing 50% inhibition of the Na-K ATPase system (pI_{50}) was 7.1. This high value, compared to the value of 3.9 in rat liver (Bakkeren and Bonting, 1968), of 4 in Escherichia coli (Hafkenscheid and Bonting, 1968), of 5.4 in rabbit pancreas (Ridderstap and Bonting, 1969), and of 6.8 in the rectal gland of the spiny dog fish (Bonting, 1966), indicates that Na-K ATPase in the retina of Sepia officinalis is highly sensitive to ouabain. The stimulation of Na-K ATPase activity by very low ouabain concentration (10^{-8}-10^{-9}M) observed in several other tissues, like heart muscle (Lee and Yu, 1963), chicken liver (Palmer and Nechay, 1964) and rabbit pancreas (Ridderstap and Bonting, 1969), was not observed in the Sepia retina.

Since the body temperature of the Sepia is that of the seawater, which was for our specimens 22° C, we determined the enzyme activity also at this temperature. The Na-K ATPase activity was 37% and the Mg ATPase activity was 33% of the activities at 37° C, yielding Q_{10} values of 1.9 for Na-K ATPase and of 2.2 for Mg ATPase. Bonting et al (1963) determined a value of 2.4 for Na-K ATPase in rabbit lens, while Lolley and Hess (1969) reported a value of 2.0 for undefined ATPase activity in frog rod outer segments.

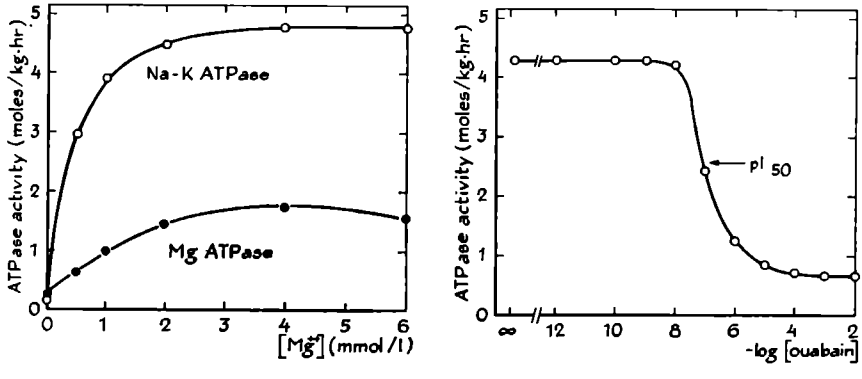


Fig. 32 Effect of Mg concentration on Na-K ATPase activity (-o-o-o-) and Mg ATPase (-●-●-●-) activity in homogenates of the sepia retina. Activities were measured in the media A (complete) and E (no K, + 10^{-4}M ouabain) to which $MgCl_2$ was added in increasing amounts. Medium E gave Mg ATPase activity, while the difference in activity between activities in media A and E gave Na-K ATPase activity.

Fig. 33 Effect of ouabain on ATPase activity in homogenates of the sepia retina. The ATPase activity was determined in medium A containing various amounts of ouabain (10^{-12}M-10^{-2}M). The pI_{50} is the negative logarithm of the molar ouabain concentration causing 50% of maximal inhibition.

6.3.3. *Calculation of pump capacity of the retina of Sepia officinalis*

The properties of the Na-K ATPase system in the retina of Sepia officinalis are generally the same as in other tissues. Moreover, we found that ouabain reduces the cation gradients (section 3.3.2.) as well as inhibits the enzyme. It is therefore reasonable to conclude that the function of the enzyme is to maintain the cation gradients across membranes in which the enzyme is localized. In the rhabdomes of the retina of Sepia officinalis we found an Na-K ATPase activity of 2.67 MKH at 37° C (Table XIII), or of 0.99 MKH at 22° C which was their body temperature. Since the Na-K ATPase system transports 3 moles of Na (or K) in one direction per mole ATP hydrolyzed, this means 2.97 moles of cation per kg dry wt. tissue per hr. can be pumped at 22° C or 0.74 moles per kg cell water per hr. (20% dry weight). The total amount of exchangeable potassium present intracellulary in the retina is 50 mmole per kg cell water. This means that the cation pump capacity is sufficient to accumulate the entire exchangeable potassium fraction in 4 min. Since the half time for the passive efflux of ^{86}Rb from the sepia retina in darkness is 34 min. (see 3.3.4.), the capacity of the pump is far above (15 x) the value required to maintain the normal cation gradient in darkness.

The high activity of this enzyme must be primarily required to re-establish the cation gradients after stimulation of the cell by light. In the in vitro experiments described in 3.3.4. there is a 6.4% loss of the intracellular ^{86}Rb upon illumination. When the pump works at full capacity it can re-establish the original cation composition in 0.064 x 240 sec. = 15 sec. At the lowest light intensity used in our experiments we calculated (section 3.2.7.) that each microvillus received 42 photons in the 30 second illumination period or one photon every 0.7 sec. The time between two successive photons is then considerably faster than the recovery time for the cation gradients. It is therefore not surprising that at higher light intensities no further leakage than 6.4% could be observed (Table II) and a kind of saturation phenomenon seems to occur at all light intensities used in our experiments.

6.3.4. *Na-K ATPase in retina and rod outer segments of various species*

The presence of Na-K ATPase in the retina of other species was also investigated. The retinal homogenates were incubated in media A and E for one hour at 37° C. The Na-K ATPase activities were calculated from the difference in activity between the two media. The activity of Mg ATPase was determined from that in medium E. The results are summarized in Table XIV. In all types of retina a high Na-K ATPase activity was found. The values for cattle, rabbit, frog and pig do not differ significantly from those previously reported (Bonting et al, 1964 a; Frank and Goldsmith, 1965, 1967). Values

TABLE XIV

ACTIVITY OF Na-K ATPase AND Mg ATPase IN RETINAE OF VARIOUS SPECIES

RETINA	Na-K ATPase (M.K.H.)	Mg ATPase (M.K.H.)
DOG	2.8 ± 0.36 (7)	1.53 ± 0.24 (7)
RABBIT	3.5 ± 0.29 (3)	2.78 ± 0.54 (3)
SHEEP	4.1 ± 0.26 (2)	2.90 ± 0.18 (2)
CATTLE	4.8 ± 0.31 (3)	1.76 ± 0.40 (3)
MONKEY	4.0 ± 0.27 (3)	2.41 ± 0.62 (3)
PIG	5.3 ± 0.43 (2)	2.68 ± 0.16 (2)
RAT	4.9 ± 0.60 (11)	1.80 ± 0.20 (11)
FROG	4.3 (1)	2.87 (1)
SEPIA	4.3 ± 0.32 (6)	0.85 ± 0.11 (6)

for Na-K ATPase in dog, sheep, monkey, rat and sepia retina have not been previously reported. When the Na-K ATPase was determined in isolated rod outer segments of cattle and frog, values considerably lower than those reported by Bonting et al (1964 a) were found (3.1 ± 0.50, 7 detns vs. 6.2 ± 0.27 for cattle; 0.51 ± 0.17, 3 detns vs. 2.9 ± 0.24 for frog). This may be due either to the different isolation technique used by us (gradient centrifugation vs. sucrose density centrifugation) or to the longer lyophilizing periods used by us. In one cattle rod outer segment preparation that was very shortly lyophilized about the same value was found as reported by Bonting et al (1964 a).

6.3.5. *Effect of air exposure on Na-K ATPase of cattle rod outer segments*

In the course of this investigation we observed that the activity of both Na-K ATPase and Mg ATPase in the Sepia rhabdomes and also in the isolated rod outer segments was lower when the preparation was not stored in vacuo. This had never been found for homogenates of other tissues in various investigations in our laboratory. In addition Matschinsky (personal communication), who determined the activity of Na-K ATPase in various layers of the rabbit retina by microdissection technique, found in the rod outer segment layer an activity of only 0.2 MKH against the value of 3.7 MKH in isolated rabbit rod outer segments (Bonting et al, 1964 a). The low results of Matschinsky do not appear to be due to an error in assay technique, since his values for the cochlea agree with those obtained by Kuijpers and Bonting (1969) on material dissected in the wet state at 0° C. Neither can his low results for the outer segment layer be due to poor penetration of the substrate to the particulate enzyme system, because addition of a deter-

gent (1% lubrol) to the assay medium did not increase the observed activity. This suggests that the loss of enzyme activity occurs during microdissection procedure. In this procedure thin (6 μ) lyophilized tissue sections and the fragments dissected from them are exposed to air for a few hours at room temperature.

We have therefore investigated the influence of air on the enzyme activity by exposing lyophilized cattle rod outer segments, rat brain and rat kidney to air for varying lengths of time. Thereafter, the preparations were homogenized and incubated in the normal way and the enzyme activity determined. The results are given in Table XV. While in rat brain and rat kidney no large decrease in activity was observed, there was a marked decrease (more than 90%) in the Na-K ATPase and the Mg ATPase activities of cattle rod outer segments. This indicates that ATPase in cattle rod outer segments is peculiar in being quite sensitive to air exposure.

It appears that phospholipids are essential components of the Na-K ATPase system. Incubation of various tissue preparations (erythrocytes, liver plasma membranes, and brain) with phospholipase C (Schatzmann, 1962; Roelofsen et al, 1966; Emmelot and Bos, 1968), phospholipase A (Tatibana, 1963; Ohnishi and Kawamura, 1964; Roelofsen et al, 1966) or detergents (Tanaka and Abood, 1964; Tanaka and Strickland, 1965; Wheeler and Whittam, 1970) leads to a loss of Na-K ATPase activity. Schatzmann (1962) and Roelofsen et al (1966) reported a similar loss in Mg ATPase activity, but other authors (Tatibana, 1963; Ohnishi and Kawamura, 1964), did not confirm this. Incubation of these preparations with phospholipids, especially

TABLE XV

EFFECTS OF AIR EXPOSURE ON ATPase ACTIVITIES AND FATTY ACID COMPOSITION IN LYOPHILIZED CATTLE ROD OUTER SEGMENTS, RAT BRAIN AND RAT KIDNEY

	Duration of air exposure in hrs					
	0		2.5		5	
	mole/kg/hr	%	mole/kg/hr	%	mole/kg/hr	%
Rod outer segments						
Na-K ATPase	5.0	100	1.66	33	0.48	9.5
Mg ATPase	3.2	100	1.14	35	0.24	7.4
C22:6 acid	0.61 *	100	0.34 *	56	0.12 *	20
Brain						
Na-K ATPase	12.9	100	—	—	10.3	80
Mg ATPase	4.2	100	—	—	4.1	98
Kidney						
Na-K ATPase	5.7	100	—	—	5.2	92
Mg ATPase	4.5	100	—	—	4.4	98

* ratio of C22:6 acid to sum of saturated fatty acids.

phosphatidylserine (Fenster and Copenhaver, 1967; Roelofsen, 1968; Wheeler and Whittam, 1970) led to a partial recovery of the Na-K ATPase activity. Borggreven et al (1970) found that 75% of the total lipid content of cattle rod outer segments consists of phospholipids, and that the highly unsaturated fatty acid docosahexaenoic acid (C 22 : 6) constitutes 35% of all the fatty acids. Both the phospholipids and the Na-K ATPase and Mg ATPase system in rod outer segments are part of the membranes of these structures, hence it seems reasonable to assume that the C 22 : 6 fatty acid is also the dominant fatty acid in the phospholipids associated with the rod outer segment ATPase system. Since this fatty acid is quite oxidizable, we determined its concentration in cattle rod outer segment preparations exposed to air for varying lengths of time. The results in Table XV indicate that the ratio of C 22 : 6 to total saturated fatty acids (C 14 : 0, C 16 : 0, C 18 : 0) decreased very markedly (80%) upon exposure to air at room temperature. This suggests that oxidation of the C 22 : 6 fatty acid might be causing the decrease in ATPase activities observed upon exposure of outer segment preparations to air.

6.4. SUMMARY

The properties, distribution and activity of Na-K ATPase in the retina of Sepia officinalis have been determined. The properties of the enzyme were found to be quite similar to those of other tissues. The activity in the rhabdome layer is considerably lower than in the nuclear layer, but is still high (2.67 moles per kg dry weight per hour) compared to many other tissues. The activity of the enzyme is sufficient to maintain the ionic gradient in the dark and upon stimulation at low light intensities.

The Na-K ATPase activity was determined in retina from various species and in all cases a high activity was found. Exposure to air lowered the activity in cattle rod outer segment very considerably ($> 90\%$), but not in rat kidney and rat brain. There was a parallel decrease (80%) in the level of the highly unsaturated fatty acid C 22 : 6 in outer segments. This suggests that the loss of enzyme activity is due to the oxidation of the unsaturated fatty acids in the phospholipids associated with the Na-K ATPase system.

A MODEL FOR THE VISUAL EXCITATION MECHANISM

7.1. INTRODUCTION

According to the aldimine cation channel hypothesis (1.3.5.) the visual excitation mechanism can be briefly characterized as follows. Illumination leads to the cis-trans isomerization of the retinaldehyde group in the rhodopsin complex. One of the ensuing dark reactions leads to an increase in the permeability of the photoreceptor membrane and the resulting ion flux and/or membrane potential change stimulates the synapse with the bipolar cell. In the regeneration phase the original cation permeability of the photoreceptor membrane and the cation gradients across it are restored.

In this concluding chapter this hypothesis is reformulated and extended in the light of the results from our investigations and from recent publications by other authors.

7.2. CHEMICAL REACTIONS LEADING TO THE INCREASE IN PERMEABILITY

Most studies of the chemical events in the photolytic cycle, which may lead to an increase in membranes permeability have been studied in vertebrates, especially in cattle retina. In 1.2.9. we described the evidence favouring the assumption that in vertebrates the transition metarhodopsin I → metarhodopsin II triggers visual excitation. The half-time for this transition is of the same order as that for excitation (about 1 msec.). Of all steps in photolysis this one shows by far the largest thermodynamic and spectral changes. This step is the first one which requires water and it involves the uptake of one proton per molecule rhodopsin. The behaviour towards reagents in aqueous solution suggests conformational changes leading to a shift of the aldimine bond from a hydrophobic to a hydrophilic environment.

Bonting and Bangham (1967) have suggested that in this step retinaldehyde shifts from its original binding site inside the membrane to an amino group on the surface of the membrane. Poincelot et al (1969) were able to obtain by extraction of dry rhodopsin or metarhodopsin I with anhydrous acid methanol followed by reduction by sodium borohydride retinyl-phosphatidylethanolamine in high yield. When metarhodopsin II was treated in the same way they found retinaldehyde bound to an ε-amino group of lysine, confirming earlier observations of Akhtar et al (1965) and Bownds and Wald

(1965). This indicates that in the metarhodopsin I → metarhodopsin II transition a transiminisation of retinaldehyde from phosphatidylethanolamine to lysine would occur. Akhtar and Hirtenstein (1969) confirmed this conclusion by denaturating rhodopsin with trichloroacetic acid and reducing the product with sodiumborohydride. In the resulting product retinaldehyde was bound to phosphatidylethanolamine.

The binding of retinaldehyde to phosphatidylethanolamine in rhodopsin would also explain in a large part the red shift — difference between the maximum in absorption of retinylidene imines (360 nm) and rhodopsin (500 nm) — of the visual pigment. Daemen and Bonting (1969 a) showed that the phosphate group of this phospholipid could protonate the nitrogen of the imine link with retinaldehyde in anhydrous circumstances, which would resemble the hydrophobic conditions prevailing inside the photoreceptor membrane. Protonation of an aldimine leads to a spectral shift from 360 to 440 nm (Fig. 19) and reduces the difference between the maxima of rhodopsin and a retinylidene imine from 140 to 60 nm. Such a protonation had already been suggested by Morton and Pitt (1955) and Erickson and Blatz (1968) and strongly supported by experiments of Hubbard (1969). However, the strongly acidic group required as a protonating agent had never been found before. This investigation of Daemen and Bonting (1969 a) also supports the idea of a transiminisation reaction during photolysis of rhodopsin, since it is known that protonated aldimines are highly reactive towards nucleophilic agents like amines (Jencks, 1964). Daemen and Bonting (1970) were indeed able to show that in an organic aprotic solvent transiminisation of retinaldehyde occurs from phosphatidylethanolamine to dodecylamine (used as an analogue for the lysine present in opsin) but not in the opposite direction.

For this transiminisation mechanism the presence of at least one molecule of phosphatidylethanolamine per molecule of retinaldehyde in pure and intact rhodopsin is essential. In rod outer segment preparations about 80 phospholipid molecules are present of which about 35 are phosphatidylethanolamine. However, Heller (1968) was able to extract with cetyltrimethylammomium bromide a spectrally intact rhodopsin preparation which contained less than 2% phospholipids. In our laboratory Borggreven (unpublished observations) has been able to remove 90-95% of the phospholipids by treatment with phospholipase C without affecting the photolytic property of rhodopsin, leading to the conclusion that no more than 4 molecules of phospholipid are essential for this property. An even more extreme conclusion was reached by Hall and Bacharach (1970) from experiments in which visual pigment was isolated and purified according to Heller (1968)

from frogs previously injected with ^{32}P. They contend that no phospholipids at all are essential for the photolytic property of rhodopsin. However, implicit in their calculations is the unproven preposition that the turnover of all molecules of phosphatidylethanolamine is equal, regardless of their binding condition. Evidence of differences in binding condition for phosphatidylethanolamine in rod outer segments was found by Borggreven et al (1970). Thus, notwithstanding the impressive evidence for a transiminization reaction in the metarhodopsin I → metarhodopsin II step, the most essential requirement for this reaction, namely that at least one molecule of phosphatidylethanolamine is necessary for the photolytic property of rhodopsin, has still not be proven.

A further point in the excitation mechanism is the question how transiminization would lead to an increase in cation permeability. As described in 1.3.5., Bonting and Bangham (1967) observed an increase in cation permeability of isolated cattle rod outer segments upon illumination as well as upon addition of retinaldehyde. This led them to the suggestion that aldimine formation between retinaldehyde and a normally protonated amino group on the surface of the photoreceptor membranes would lead to a loss of protonation and thus to a more negative surface charge, which in turn would result in an increased cation permeability of the membrane. Supporting this conclusion is the observation by Daemen and Bonting (1968) that retinol or retinoic acid did not give the effect on cation permeability noticed with retinaldehyde. Subsequently, Duncan (unpublished observations) in our laboratory made the same observation in tracer studies with isolated frog rod outer segments.

Studies with artificial phospholipid micelles (liposomes) shed some further light on this mechanism. Addition of retinaldehyde to liposomes consisting of phosphatidylethanolamine greatly increased their cation permeability, but no such effect occurred with liposomes consisting of phosphatidylcholine (Bonting and Bangham, 1967; Daemen and Bonting, 1969 b). It was also shown that aldimine formation between phosphatidylethanolamine and retinaldehyde took place, accompanied by an increased negativity in surface charge of the liposomes. Supporting the essential role of the blocking of an amino group are the observations that benzaldehyde and the amino group reagent dinitrofluorobenzene (DNFB), but not retinol, retinoic acid, benzylalcohol and benzoic acid, have the same effect on cation permeability of the phosphatidylethanolamine micelles (Daemen and Bonting, 1969 b). Previously, Bangham et al (1965) had observed that the induction of a negative surface charge in lecithin liposomes by addition of anionic detergents led to a marked increase in cation permeability. Berg et al (1965) showed

that addition of DNFB to erythrocytes led also to a striking increase in cation permeability and that the dinitrophenyl group is attracted to the ε-amino group of lysine present in the membrane proteins. These findings show that a transiminization reaction leading to induction of a negative surface charge (as of lysine in the metarhodopsin I → metarhodopsin II step) could increase the cation permeability of the photoreceptor membrane.

In the monolayer experiments described in chapter 2 we showed that illumination of a rhodopsin monolayer leads to an increase in film pressure which effect could also be obtained upon penetration of retinaldehyde into these monolayers. Experiments with phospholipid monolayers proved that an extra increase in film pressure only occurred under circumstances under which aldimine formation took place: penetration of an aldehyde into an amino group-containing phospholipid monolayer at a subphase pH above 6.5. This indicates that aldimine formation in the phospholipid monolayers changes the surface properties of the monolayer and that a similar process occurs in the rhodopsin monolayer, which serves as a model for a rod outer segment membrane.

Relatively less is known about the biochemical aspects of the photolytic cycle in invertebrates. As described in 1.2.10., there are strong indications that in the cephalopods visual excitation happens in the step lumirhodopsin → acid metarhodopsin. However, it is not known whether in this step also a transiminization of retinaldehyde occurs. Our experiments with the Sepia retina, in which an increased cation leakage is observed upon addition of retinaldehyde and DNFB (Table III), support a basically similar excitation mechanism in invertebrates.

7.3. IONIC MOVEMENTS AND VISUAL EXCITATION

7.3.1. *Invertebrates*

There is abundant evidence that in invertebrate photoreceptor cells, like those of limulus (Kikuchi et al, 1962; Millechia and Mauro, 1969 a, b), crayfish (Eguchi, 1965), honey bee (Fulpius and Baumann, 1969), barnacle (Brown et al, 1969) and squid (Hagins et al, 1962; Hagins, 1965), there is a negative intracellular potential of about —50 mV. This potential is, like in nerve cells, mainly caused by the permeability of the photoreceptor membrane to potassium, as shown by the dependence of the potential of the external potassium concentration (Millechia and Mauro, 1969 a). The cation concentrations determined by us in the retina of Sepia officinalis (3.3.1.) are in agreement with this conclusion.

Illumination causes in all invertebrates studied a depolarization of the membrane, accompanied by a decrease in resistance of the photoreceptor

membrane (Fuortes, 1959; Rushton, 1959). In Limulus photoreceptor Smith et al (1968) found that the decrease in resistance could also be obtained upon applying ouabain. They explained this by assuming that part of the resting potential was caused by an ouabain-sensitive electrogenic pump, and that illumination would decrease the electrogenicity of the pump leading to depolarization. However, they did not take into account that the rhabdomeres have a very high surface to volume ratio compared to a structure like the squid axon, which means that inhibition of the sodium pump could lead to a fast and marked change in cation concentrations and thus to depolarization of the membrane (Duncan and Bonting, 1969). Our experiments, in which the application of ouabain to isolated Sepia retina caused strong inhibition of the ^{86}Rb uptake (26 mM vs. 50 mM in normal ASW; Fig. 14, Table I) support this possibility. Moreover, Millechia and Mauro (1969 b) measured the depolarization current with the voltage clamp technique and found that the theory of Smith et al (1968) would require the ouabain-sensitive potential to amount to 2-3 V. and to reverse its sign of an externally applied voltage of +15 mV. At this potential however the emf would be independent of the stimulating light and would not vary in time. Since this appears to be improbable, they reject the theory of Smith et al (1968). A direct effect of illumination on the permeability for cations in invertebrate photoreceptor cells was shown by our experiments with Sepia retina (3.3.4.) where we observed an increase in the efflux rate of ^{86}Rb, accompanied by a marked decrease in the rubidium concentration.

Our experiments with ouabain show two more phenomena. In darkness the efflux rate of ^{86}Rb in the presence of ouabain was twice as high as in artificial seawater without ouabain and the permeability increase upon illumination was considerably decreased in the presence of ouabain. Both effects of ouabain can be explained by a decrease in membrane potential caused by the inhibition of the sodium pump and the resulting changes in cation composition. We also established the presence in the rhabdomes of the Sepia of Na-K ATPase, the enzyme system responsible for active cation transport (Table XIII). The cation pump thus appears to play an important role in the establishment and restoration of the cation gradients necessary for visual excitation.

7.3.2. *Vertebrates*

An increased potassium efflux upon illumination was observed in frog retina by Sekoguti (1960 a, b). Buckser and Diamond (1966) reported an increased uptake of ^{22}Na by frog retina after illumination. Bonting and Bangham (1967) used isolated cattle rod outer segments suspended in a

sucrose medium and found that upon illumination over a wide range of intensities a relative decrease in the potassium and an equivalent increase in the sodium concentration occurred. Incubation of isolated frog outer segments in frog Ringer with trace amounts of ^{42}K or ^{86}Rb led to an increased isotope efflux upon illumination, while after incubation with ^{22}Na the opposite effect occurred (Duncan et al, 1969).

It was suggested by Bonting and Bangham (1967) that the intrasaccular space would be high in potassium and the extrasaccular space high in sodium. Upon illumination the permeability of the sac membrane would increase, leading to a flow of ions according to their gradients. This hypothesis was confirmed by Duncan et al (1970) in studies with isolated frog outer segments. The initial overall concentrations of sodium and potassium in these outer segments were about equal, but incubation in a frog Ringer's solution in which sodium was replaced by choline caused a much larger decrease in the concentration of sodium than in that of potassium. Calculations on the basis of these observations indicate that the intrasaccular sodium and potassium concentrations were 33 and 87 mM and the extrasaccular concentrations 101 and 19 mM, respectively, against extracellular concentrations of 117 and 3 mM, respectively.

Although the light-stimulated ion movements in photoreceptor cells of vertebrates are in the same direction as those in invertebrates, it is much more difficult to correlate the ion movements in the former with electrophysiological observations in view of the presence of two membranes: the rod sac membrane and the outer membrane. The α-wave of the electroretinogram originates from the photoreceptor cells (Tomita et al, 1960; Brown and Watanabe, 1962 a, b; Arden and Brown, 1965) and is sensitive to the extracellular ionic concentrations (Hamasaki, 1963, 1964; Hanawa et al, 1967). Application of ouabain on the receptor surface of the frog retina for only seven minutes led to complete disappearance of the α-wave (Frank and Goldsmith, 1967). Silmann et al (1969 a, b) were able to record pure receptor potentials from the photoreceptor cells by suppressing other light-induced potential changes by means of sodium aspartate. They found that the amplitude of the receptor potential increased linearly with the logarithm of the external sodium concentration and decreased proportionally to the logarithm of the external potassium concentration. The potential was completely abolished in seven minutes when ouabain (10^{-4}M) was applied to the external medium. When ouabain was added to a sodium-free medium, the receptor potential also disappeared, but it could be re-established by changing to a medium with normal sodium concentration, even if this contained ouabain. These experiments indicate that the effect of ouabain is due to a change in

ionic concentrations and not to an inhibition of an electrogenic pump.

The experiments described in the preceding paragraph all refer to light induced potential changes, measured with electrodes placed outside the photoreceptor cells. True intracellular recordings of membrane potentials and resistance changes were only obtained in large vertebrate photoreceptors like those of mud puppy, gecko, and carp (Bortoff, 1964; Tomita, 1964; Tomita et al, 1967; Toyoda et al, 1969). The result obtained with these experiments was that the outer membrane of the vertebrate photoreceptor cell hyperpolarizes upon illumination and that its resistance increases. Still another approach was used by Penn and Hagins (1969). They placed microelectrodes extracellularly along the photoreceptor cell in isolated rat retina. From the potential differences between their electrodes they concluded that in the absence of light a dark current is flowing from the inner segment to the outer segment. Illumination led to a decrease in the dark current, which they called a photocurrent. Both dark durrent and photocurrent were rapidly abolished by 1 mM KCN and by ouabain (Yoshikami and Hagins, 1970).

There is an apparent contradiction between the electrophysiological experiments, which suggest a permeability decrease upon illumination, and the experiments on ion movements, which suggest a light-induced permeability increase. Duncan et al (1970) have offered an explanation for this contradiction. They assume that the primary ion movements take place across the rod sac membrane, where the visual pigment molecules are localized, whereas the intracellular recordings are obtained across the outer membrane of the photoreceptor cell. Hagins et al (1970) have indicated that the outer membrane of the outer segment has a low resistance and a high sodium permeability compared to the plasma membrane of the inner segment. This would explain the high sodium concentration of the extrasaccular space calculated by Duncan et al (1970). The latter authors assume that the light-induced permeability increase in the rod sac membranes would lead to a temporary decrease of the extrasaccular sodium concentration. Thus the sodium gradient between the extrasaccular space and the inner segment space would decrease and this would lead to lowering of the dark current observed by Penn and Hagins (1969). In isolated outer segments this would cause a secondary efflux of potassium too and an influx of sodium from the surrounding medium as observed in tracer experiments (Duncan et al, 1970). The inhibition of the dark current by cyanide would be due to inhibition of the production of ATP for the sodium pump on the inner segment membrane, which is essential to keep the dark current of sodium ions going. The fast effect of ouabain observed by Yoshikami and Hagins (1970) can likewise be explained by an inhibition of the sodium pump of the inner segment. This model can also

explain the hyperpolarization of the outer segment plasma membrane, observed by Toyoda et al (1969). The sudden release of potassium ions from the rod sac to the extrasaccular space would increase the potassium gradient across the plasma membrane, leading to a hyperpolarization of this membrane. If this explanation of the visual mechanism in vertebrate rods is correct, it would imply that the primary effect of light in vertebrate and invertebrate photoreceptor cells is an increase in cation permeability of the visual pigment bearing membrane.

7.4. REGENERATION

Assuming that light causes an increase in cation permeability of the membranes containing the visual pigment molecules, which leads to stimulation of the synapse with the bipolar or nerve cell, then regeneration of the photoreceptor cell would involve at least two processes. The photoreceptor membrane must return to its original low permeability for cations, and secondly the original cation gradients must be restored.

In the preceding section (7.2.) we have reviewed the evidence that removal of a proton from the ε-amino group of lysine located at the outside of the membrane due to transiminization of the retinaldehyde chromophore leads to the increase in cation permeability of the photoreceptor membrane. The only process required for re-establishing the original low cation permeability is the removal of the retinaldehyde from this amino group. In chapter 5 we have shown that retinoldehydrogenase from cattle rod outer segments can reduce retinylidene imines to free retinol without previous formation of free retinaldehyde. Assuming that this enzyme works in vivo at its maximal velocity, we could calculate (see 5.4.3.) that at rod saturation level, where each rod sac receives one photon per 0.23 seconds (Aguilar and Stiles, 1954) this reduction is possible in 0.00063 sec. This indicates that this process is not rate-limiting and is therefore not responsible for the rod saturation phenomenon. On the other hand, this process is so fast that the period of high cation permeability of the photoreceptor membrane lasts only for such a brief period that complete leakage of ions cannot take place. In experiments with isolated retina (Matthews et al, 1963; Baumann, 1967, 1968; Cone and Brown, 1969) the formation time of retinol is of the order of minutes. In these experiments, however, nearly all the rhodopsin present is bleached. Since per gram rod outer segment 4.9 mmoles of retinaldehyde per kg dry weight rod outer segments are present (Borggreven et al, 1970), and since we found a maximal activity of 600 mmoles retinaldehyde converted per kg dry weight rod outer segments, the minimal time for a complete reduction is 30 sec. This means that sufficient enzymatic activity is present to account

for the observed rate of conversion of metarhodopsin II to retinol in the isolated retina.

For the invertebrates we do not know the chemical reaction leading to the increased cation permeability. As a consequence it is also impossible at this moment to indicate the reaction which would restore the original low cation permeability. Moreover, in cephalopods alkaline metarhodopsin appears to be the end product of the visual cycle, while no retinoldehydrogenase activity could be observed in this retina (Wald, 1960). It appears therefore, that in cephalopods another mechanism occurs, which may involve the retinochrome pigment (see 1.2.12.).

The second process in the regeneration phase must be the restoration of the cation gradients. This process would involve the enzyme Na-K ATPase, the presence of which has been demonstrated by us in the retinae of a large number of species (6.3.4.). Specifically, its presence in the outer segments of cattle and frog has been shown (Bonting et al, 1964 a). Bonting and Bangham (1967) have calculated that the capacity of the Na-K ATPase system in cattle rods is sufficient to explain the regeneration of the cation gradient at the light level where in man rod saturation occurs. At this level, where each human rod sac receives one quantum per 0.23 sec. (Aguilar and Stiles, 1954, assuming a number of 1000 sacs per rod), the recovery of the cation gradient in a single rod sac would take 0.55 sec. At higher light intensities the capacity of the cation pump would be insufficient to restore the cation gradients, and the rate of stimulation of the bipolar cells does not further increase.

For invertebrates it is unknown whether above certain light levels a saturation of the light response occurs. However, in section 6.3.3. we calculated that in Sepia the pump capacity suffices to restore the cation gradients up to a quantum incidence rate per microvillus of one photon per 15 seconds. This indicates that here also a saturation phenomenon must occur.

The model for the mechanisms of visual excitation and regeneration described and investigated in this thesis has received considerable support from our studies and from other recent investigations. However, further work in this field will be necessary in order to determine whether this theory is correct as stated here or whether it needs further revision.

In the study of the mechanism of visual excitation the key problem is how the absorption of a single photon by a visual pigment molecule in the outer segment of the visual photoreceptor can lead to a stimulation of the synapse at the other end of the photoreceptor cell. To explain this phenomenon, Bonting and Bangham (1967) have proposed a biochemical model for the visual excitation mechanism. This hypothesis can be summarized as follows: illumination of the visual pigment rhodopsin, located in the photoreceptor membranes, causes a chemical conversion in which the chromophoric group retinaldehyde shifts from its original binding site to an ε-amino group of lysine, which is part of the rhodopsin complex, but is located on the membrane surface. This reaction makes the surface charge of the photoreceptor membrane more negative. This local negative surface charge causes an increased permeability for cations. The resulting flow of ions leads to a stimulation of the synapse. For the restoration of the cation gradients after stimulation the enzyme Na-K activated ATPase located in the photoreceptor membrane is responsible.

The investigations described in this thesis are based on this "aldimine cation channel hypothesis". In the first chapter the anatomy of the photoreceptor system in vertebrates and invertebrates is briefly described. Our present knowledge about the visual pigments and their photolytic conversion is reviewed inasfar as it contributes to a better understanding of the experiments described in this thesis. After a brief description of the various types of hypotheses put forth to explain the visual excitation mechanism, the aldimine cation channel hypothesis, which can be considered as a more detailed version of the ionic mechanism, is described in detail and the aim of our investigation is formulated.

One of the observations which brought Bonting and Bangham (1967) to their model, is the increase in film pressure upon illumination of a rhodopsin monolayer. Penetration of retinaldehyde into this monolayer gave a similar increase in film pressure. They suggested that both phenomena could be explained by aldimine formation between the aldehyde group of retinaldehyde and amino groups in the visual pigment monolayer. We have repeated and confirmed these experiments (chapter 2) and have investigated the aldimine hypothesis by penetrating phospholipid monolayers with retinaldehyde and retinol. We found that penetration of retinaldehyde in a phosphatidylethanolamine monolayer even at high initial film pressure caused an increase in film pressure, which was not observed in phosphatidylcholine monolayers. On the other hand, with retinol no difference in penetration

behavior existed for the two phospholipids. That aldimine formation is responsible for the preferential penetration of retinaldehyde in phosphatidylethanolamine monolayers was shown by spectrophotometric observations of the monolayer material and by studies of the dependence of the effect on the pH of the subphase.

In chapter 3 we have described investigations of the dark movements of radioactive rubidium and the influence of light on these movements in the retina of Sepia officinalis. This species was studied since the visual pigment of cephalopods has a different photolytic sequence than that of vertebrates, and since in invertebrates the ion movements can be better correlated with the results from electrophysiological observations. We have calculated the intracellular sodium and potassium concentrations by flamephotometric determinations of these cations before and after incubation of the retina in artificial seawater in which sodium had been replaced by choline. Rubidium, which behaves like potassium in cation transport, was taken up by this retina against its concentration gradient and this uptake was markedly inhibited by ouabain. The efflux kinetics revealed a fast-exchanging extracellular compartment ($t\frac{1}{2}$ = 1.5 min.) and a slowly exchanging intracellular compartment ($t\frac{1}{2}$ = 34 min.). The efflux rate of ^{86}Rb was enhanced 1.7-4.8 times upon illumination, leading to a simultaneous 6% decrease in the rubidium content. The presence of ouabain in the incubation and efflux media increased the dark efflux rate, and markedly reduced the effect of illumination upon the efflux rate. Addition of retinaldehyde gave also an increase in the dark efflux rate. These experiments indicate that in the invertebrate retina a light-induced permeability increase occurs and that this is probably the basis of the visual excitation mechanism.

One aspect of the aldimine cation channel hypothesis which needed further study is the way in which the original low cation permeability is restored after illumination. We have investigated whether the retinoldehydrogenase present in rod outer segments could also convert retinaldehyde bound in an aldimine to free retinol and so uncover the amino group to which retinaldehyde is being bound in the step leading to the increased cation permeability. For this investigation a method of preparing stable aldimines at neutral pH without a large excess of amine was required (chapter 4). After developing a spectroscopic technique for the determination of aldimines in aqueous solution the equilibrium constants for the formation and the protonation of these aldimines were determined. We found that the apparent equilibrium constant for the aldimine formation was dependent on the chain-length of the amine, probably due to the fact that the reaction takes place at the micellar lipid-water interface. With long-chain amines it was possible to

prepare aldimines of retinaldehyde in good yield at neutral pH and with a relatively small excess of amine.

Chapter 5 describes how in the presence of NADPH retinoldehydrogenase can convert these aldimines almost as rapidly as free retinaldehyde to retinol. Calculation showed that the observed conversion rates were too high to explain the aldimine conversion as a reduction of free retinaldehyde resulting from non-enzymatic hydrolysis of aldimine. Horse liver alcoholdehydrogenase, which does reduce free retinaldehyde, was unable to convert aldimines of retinaldehyde. Another type of retinoldehydrogenase was found in the rest of the retina, which in contrast to the rod outer segment preparation shows no preference for NADPH but works at nearly equal rates with NADH and NADPH. These findings suggest that the retinoldehydrogenase of rod outer segments can by such a reductive hydrolysis remove the retinaldehyde from its link to the ε-aminogroup of lysine and thereby restore the original low cation permeability of the rod outer segment membrane.

The enzyme Na-K ATPase is responsible for the restoration of the cation gradients after light stimulation. In chapter 6 we have investigated the properties, distribution and activity of Na-K ATPase in the retina of Sepia officinalis. The properties of this enzyme were found to be quite similar to those of the same enzyme present in other tissues. The activity in the rhabdome layer, though lower than in the nuclear layer, is high (2.67 moles per kg dry weight per hour) compared to many other tissues. The activity of the enzyme is large enough to maintain the cation gradients in the dark and at low light intensities. In addition the Na-K ATPase activity in retina's from various species was determined and in all cases a high activity was found. The enzyme in outer segments differed in one respect from that in other tissues. Exposure to air for a few hours lowered the activity in cattle rod outer segments very considerably (90% in 5 hrs.), but not in rat kidney and brain. There was a parallel decrease (80% in 5 hrs.) in the level of the highly unsaturated fatty acid C 22 : 6 in cattle rod outer segments. This suggest that the loss of enzyme activity in outer segments upon exposure to air is due to oxidation of unsaturated fatty acids present in the phospholipids associated with the Na-K ATPase system.

In chapter 7 we have reformulated and extended the aldimine cation channel hypothesis in the light of these and other recent investigations. Generally speaking our observations lend further support to this hypothesis. Various hitherto unexplained phenomena in the visual excitation mechanism were elucidated. Further investigations will still be required in order to determine whether this theory is correct in its present form or needs further revision.

Bij de bestudering van het mechanisme van de visuele excitatie gaat het om de beantwoording van de vraag, hoe de absorptie van een foton door een visueel pigment molecuul in het buitensegment van de fotoreceptor cel kan leiden tot stimulatie van de synaps, gelegen aan het andere eind van deze cel. Ter verklaring van dit fenomeen hebben Bonting en Bangham (1967) een biochemisch model geponeerd. Deze hypothese komt, kort samengevat, op het volgende neer: belichting van het visuele pigment rhodopsine, gelegen in de fotoreceptor membranen, veroorzaakt een chemische omzetting, waarbij de chromofore groep retinaldehyde verschuift van zijn oorspronkelijke bindingsplaats naar een ε-amino groep van lysine, dat ook deel uitmaakt van het rhodopsine complex, maar aan het membraanoppervlak gelegen is. Door deze reactie wordt de oppervlaktelading van het fotoreceptormembraan meer negatief. Deze plaatselijke negative lading veroorzaakt een toename in de permeabiliteit van het membraan voor kationen. Daardoor onstaat een ionenstroom welke aanleiding geeft tot stimulatie van de synaps. Het enzym Na-K ATPase, dat ook aanwezig is in het fotoreceptormembraan, is dan verantwoordelijk voor het herstel van de gradienten voor kationen.

De onderzoekingen, beschreven in deze dissertatie, hebben deze "aldimine kationen porie hypothese" als basis. In het eerste hoofdstuk wordt allereerst een kort overzicht gegeven van de anatomie van het fotoreceptorsysteem in vertebraten en invertebraten. Vervolgens wordt onze huidige kennis over de visuele pigmenten en hun fotolytische omzetting beschreven, voorzover dit kan bijdragen tot een beter begrip van de experimenten beschreven in dit proefschrift. Na een korte uiteenzetting van de verschillende soorten hypotheses, die geponeerd zijn om het visuele excitatie mechanisme te verklaren, wordt de aldimine kationen porie hypothese, die kan worden beschouwd als een meer gedetailleerde versie van de ionaire hypothese, uitgebreid beschreven en het doel van ons onderzoek uiteengezet.

Een van de waarnemingen, die Bonting en Bangham (1967) er toe brachten om hun model te poneren, was de toename in filmdruk bij belichting van een monolaag van rhodopsine. Penetratie van retinaldehyde in deze monolaag gaf een analoge toename in filmdruk. Zij poneerden dat dit verschijnsel kon worden verklaard door aldimine vorming tussen de aldehyde groep van retinaldehyde en aminogroepen in de rhodopsine monolaag. Wij hebben deze experimenten herhaald en bevestigd (hoofdstuk 2) en hebben de hypothese dat aldiminevorming dit verschijnsel veroorzaakte onderzocht door monolagen van fosfolipiden te penetreren met retinaldehyde en retinol. Wij vonden, dat penetratie van retinaldehyde in een fosfatidylethanolamine mono-

laag zelfs bij een hoge begindruk een stijging in de filmdruk veroorzaakte. Bij gebruik van een fosfatidylcholine monolaag werd geen stijging in de filmdruk waargenomen. Aan de andere kant werd bij penetratie van retinol geen verschil in het penetratiegedrag waargenomen tussen de twee fosfolipiden. Dat aldimine vorming de extra penetratie tussen retinaldehyde en fosfatidylethanolamine veroorzaakt werd aangetoond door spectroscopische observaties van het monolaag materiaal en door bestudering van de afhankelijkheid van dit effect van de pH van de subfase.

In hoofdstuk 3 hebben wij onderzoekingen beschreven over de bewegingen van radioactief rubidium in de retina van Sepia officinalis in het donker en in het licht. Deze diersoort werd bestudeerd omdat het visuele pigment van cefalopoden een andere fotolytische cyclus heeft en omdat in invertebraten de ionenbewegingen beter gecorreleerd kunnen worden met de resultaten van electrofysiologische waarnemingen. De intracellulaire kalium en natrium concentraties werden door ons bepaald door vlamfotometrische bepalingen van deze kationen voor en na incubatie van de retina in kunstmatig zeewater waarin natrium was vervangen door choline. Rubidium, dat zich in het kationen transport vrijwel identiek gedraagt als kalium, werd door de retina opgenomen tegen een concentratiegradient in en deze opname werd grotendeels geblokkeerd door ouabaine. De effluxkinetiek leidde tot de conclusie dat de retina een extracellulaire ruimte bevat, waaruit de isotoop uitgewisseld werd met een halfwaarde tijd van $1\frac{1}{2}$ min., en dat er een intracellulaire ruimte is, van waaruit de isotoop werd uitgewisseld met een halfwaarde tijd van 34 min. Belichting leidde tot een 1.7-4.8 voudige toename in de snelheidsconstante van de ^{86}Rb efflux en had een afname van 6% in het isotoop gehalte tot gevolg. Bij aanwezigheid van ouabaine in de opname- en uitstroommedia was de snelheidsconstante van de efflux verhoogd, terwijl het effect van belichting op deze constante aanmerkelijk geringer was. Deze experimenten tonen aan dat in de invertebratenretina licht een aanzienlijke stijging in de kationenpermeabiliteit induceert, die waarschijnlijk de basis is van het visuele excitatiemechanisme.

Een aspect van de aldimine kationen porie hypothese dat verder onderzoek verdiende is de wijze waarop de oorspronkelijke lage kationenpermeabiliteit na de belichting wordt hersteld. Wij hebben onderzocht of het in buitensegmenten aanwezige enzym retinoldehydrogenase retinaldehyde, gebonden in de aldimine vorm, zou kunnen omzetten in retinol. Hierdoor zou de aminogroep waaraan retinaldehyde wordt gebonden in de stap die leidt tot stijging van de kationenpermeabiliteit van het membraan, weer worden vrijgemaakt. Eerste vereiste voor dit onderzoek was een methode om stabiele aldimines bij neutrale pH zonder grote overmaat amine te bereiden (hoofd-

stuk 4). Na ontwikkeling van een spectroscopische techniek ter bepaling van aldimines in waterige oplossing, werden de evenwichtsconstanten voor de vorming en de protonering van deze aldimines bepaald. De gevonden evenwichtsconstante voor de vorming van het aldimine bleek afhankelijk te zijn van de ketenlengte van het gebruikte amine, waarschijnlijk ten gevolge van het feit, dat de reactie plaatsvindt aan het micellaire lipide-water grensvlak. Met langketenige amines bleek het mogelijk aldimines van retinaldehyde bij neutrale pH en met een relatief kleine overmaat amine in goede opbrengst te bereiden.

Hoofdstuk 5 beschrijft hoe retinoldehydrogenase deze aldimines in aanwezigheid van NADPH bijna even snel als vrij retinaldehyde kon omzetten in retinol. Berekeningen bewezen dat de waargenomen omzettingssnelheden veel te hoog waren om de aldimine omzetting te kunnen verklaren als een reductie van vrij retinaldehyde, dat gevormd wordt door niet-enzymatische hydrolyse van het aldimine. Alcoholdehydrogenase van paardelever dat wel vrij retinaldehyde reduceerde, was niet in staat om deze aldimines om te zetten. In de rest van de retina werd een ander retinoldehydrogenase aangetoond dat in tegenstelling tot het staafjespreparaat geen voorkeur vertoont voor NADPH, maar met vrijwel gelijke snelheid werkt met NADH en NADPH. Deze resultaten maken het aannemelijk dat de retinoldehydrogenase in de buitensegmenten door een reductieve hydrolyse retinaldehyde kan verwijderen uit zijn binding met de ε-amino groep van lysine, waardoor de oorspronkelijke lage kationenpermeabiliteit van het fotoreceptormembraan hersteld wordt.

Het enzym Na-K ATPase is verantwoordelijk voor het herstel van de kationengradienten na stimulatie door licht. Eigenschappen, verdeling en activiteit van het Na-K ATPase systeem in de retina van Sepia officinalis werden beschreven in hoofdstuk 6. De eigenschappen van dit enzym vertoonden een goede overeenstemming met die van hetzelfde enzym in andere weefsels. De activiteit in de rhabdomenlaag is, hoewel kleiner dan in de kernlaag, hoog (2.67 mol ATP gesplitst per uur per kg drooggewicht) vergeleken met vele andere weefsels. De activiteit van het enzym is voldoende om de kationengradienten in het donker zowel als bij lage lichtintensiteiten in stand te houden. Bovendien werd de Na-K ATPase activiteit in de retina's van verschillende species bepaald en in alle gevallen werd een hoge activiteit gevonden. Het enzym in de buitensegmenten verschilt in een opzicht met dat in andere weefsels. De activiteit in buitensegmenten loopt sterk terug (90% in 5 uur) bij blootstelling van het drooggevroren materiaal aan de lucht, doch dit gebeurt niet met preparaten van rattenier en rattehersenen. Tegelijk met de verlaging van enzymactiviteit was er een afname (80% in

5 uur) in het niveau van het sterk onverzadigde vetzuur C 22 : 6 in buitensegmenten. Dit zou kunnen betekenen dat het verlies van enzym activiteit door blootstelling aan de lucht wordt veroorzaakt door oxidatie van onverzadigde vetzuren, aanwezig in de fosfolipiden die geassocieerd zijn met het Na-K ATPase systeem.

In het laatste hoofdstuk hebben wij de aldimine kationen porie hypothese opnieuw geformuleerd en uitgebreid in verband met deze en andere recente onderzoekingen. Samenvattend kunnen wij zeggen, dat onze waarnemingen deze hypothese verder ondersteunen en verschillende tot nu toe nog niet verklaarde verschijnselen bij de visuele excitatie kunnen verklaren. Nadere onderzoekingen zijn echter noodzakelijk om te bepalen of deze theorie in zijn huidige vorm juist is of dat verdere herziening nodig is.

Abrahamson, E. W., and Erhardt, F., (1964).Protein configuration changes in the photobleaching of rhodopsin Federation Proc , *23,* 384.
Abrahamson, E W , and Ostroy, S E , (1967). The photochemical and macromolecular aspects of vision Progr. Biophys. Mol. Biol., *17,* 179.
Adams, R G , and Hagins, W. A., (1960). The ionic composition of squid photoreceptors. Biol Bull , *119,* 300
Aguilar, M , and Stiles, W S , (1954) Saturation of the rod mechanism of the retina at nigh levels of stimulation Optica Acta, *1,* 59.
Akhtar, M , and Hirtenstein, M D , (1969) Chemistry of the active site of rhodopsin. Biochem J., *115,* 607.
Akhtar, M , Blosse, P. T., and Dewhurst, P B , (1965) The reduction of rhodopsin derivatives Life Sci., *4,* 1221
Albrecht, G , (1957). Terminal amino acids of rhodopsin. J Biol. Chem , *229,* 477.
Andrews, J. S , and Futterman, S , (1964) Metabolism of the retina V. The role of microsomes in vitamin A esterification in the visual cycle J. Biol Chem , *239,* 4073.
Arden, G. B., and Brown, K. T., (1965) Some properties of components of the cat electroretinogram revealed by local recording under oil J. Physiol (London), *176,* 429.
Arden, G B , and Ikeda, H , (1968) The minimum latency of the α-wave J Physiol. (London), *176,* 429.
Baker, P F , Hodgkin, A L , and Meves, H., (1964) The effect of diluting the internal solution on the electrical properties of the squid giant axon J Physiol , (London) *170,* 541.
Bakkeren, J A J M., and Bonting, S L., (1968) Studies on Na-K acivated ATPaseXX.
Bakkeren, J A. J. M , and Bonting, S L , (1968), Studies on Na-K activated ATPase XX. Properties of Na-K-activated ATPase in rat liver Biochim. Biophys Acta, *150,* 460.
Ball, S , Goodwin, T. W., and Morton, R. A , (1948) The preparation of retinene$_1$-vitamin A aldehyde Biochem J , *42,* 516
Bangham, A D , Standish, M. M., and Watkins, J. C., (1965) Diffusion of lipid bilayer membranes separating two aqueous phases, determination of membrane thickness. J Mol Biol , *13,* 238.
Baumann, C , (1966) Der Einfluss von Metarhodopsin auf die Sehpurpurbleichung in der isolierten Netzhaut Vision Res., *6, 5*
Baumann, C., (1967) Sehpurpurbleichung und Stabchenfunktion in der isolierten Froschnetzhaut Plugers Archiv, *298,* 44.
Baumann, C., (1968) Dark reactions following bleaching of rhodopsin in the isolated frog retina Pflugers Archiv, *300,* R 100.
Becker, B , an Cotlier, E , (1965) The efflux of ^{86}rubidium from the rabbit lens. Invest. Ophthalmol , *4,* 117.
Berg, H. C , Diamond, J M . and Marfey, P. S., (1965). Erythrocyte membrane chemical modification. Science, *150,* 64
Bligh, E G , and Dyer, W. J , (1958). A rapid method of total lipid extraction and purification Can J. Biochem Physiol , *37,* 911.
Bliss, A. F , (1948). The mechanism of retinal vitamin A formation. J Biol Chem , *172,* 165.
Bliss, A F , (1949) Reversible enzymic reduction of retinene to vitamin A Biol. Bull , *97,* 221
Bliss, A. F , (1951 a) The equilibrium between Vitamin A alcohol and aldehyde in the presence of alcoholdehydrogenase Arch. Biochem Biophys., *31,* 197
Bliss, A F , (1951 b). Properties of the pigment layer factor in the regeneration of rhodopsin J Biol. Chem., *193,* 525.
Boll, F , (1877) Zur Anatomie und Physiologie der Retina Monatsber Kgl Akad. Wiss Berlin, p. 783.
Bonting, S. L., (1966). Studies on Na-K activated adenosine triphosphatase. XV. The

rectal gland of elasmobranchs Comp Biochem Physiol , *17*, 953
Bonting, S. L , (1969) The mechanism of the visual process Curr Topics Bioenerg., *3*, 351
Bonting, S L , (1970) Sodium-potassium activated adenosinetriphosphatase and cation transport In "Membrane, metabolism and ion transport" (E E Bittar ed)
Bonting, S L , and Bangham, A D , (1967) On the biochemical mechanism of the visual process Exp Eye Res , *6*, 400
Bonting, S L , Simon, K A , and Hawkins, N M , (1961) Studies on Na-K activated adenosinetriphosphatase I Quantitative distribution in several tissues of the cat Arch Biochem Biophys , *95*, 416
Bonting, S L , Caravaggio, L L , and Hawkins, N M , (1963) Studies on Na-K activated adenosinetriphosphatase VI Its role in cation transport in the lens of cat, calf and rabbit Arch Biochem Biophys , *101*, 47
Bonting, S L , Caravaggio, L L , and Canady, M R , (1964 a) Studies on Na-K activated adenosinetriphosphatase X Occurrence in retinal rods and relation to rhodopsin Exp Eye Res , *3*, 47
Bonting, S L , Caravaggio, L L , Canady, M R , and Hawkins, N M , (1964 b) Studies on Na-K activated adenosinetriphosphatase XI The salt gland of the herring gull Arch Biochem Biophys , *106*, 49
Borggreven, J M P M , Daemen, F J M , and Bonting, S L , (1970) Biochemical aspects of the visual process VI The lipid composition of native and hexane extracted cattle rod outer segments Biochim Biophys Acta, *202*, 374
Bortoff, A , (1964) Localization of slow potential responses in the Necturnus retina Vision Res , *4*, 627
Bownds, D , and Wald, G , (1965) Reaction of the rhodopsin chromophore with sodium borohydride Nature, *205*, 254
Bridges, C D B , (1962 a) Studies on the flash photolysis of visual pigments III Interpretation of the slow thermal reactions following flash irradiation of frog rhodopsin solutions Vision Res , *2*, 201
Bridges, C D B , (1962 b) Studies on the flash photolysis of visual pigments IV Dark reactions following the flash irradiation of frog rhodopsin in suspensions of isolated photoreceptors Vision Res , *2*, 215
Bridges, C D B , (1967) Spectroscopic properties of porphyropsins Vision Res , *7*, 349
Briggs, G E , Hope, A B , and Robertson, R N , (1961) Electrolytes and Plant cells (Blackwell, Oxford)
Brown, P K , and Brown, P S , (1958) Visual pigments of the octopus and cuttlefish Nature, *182*, 1288
Brown, K T , and Watanabe, K , (1962 a) Isolation and identification of a receptor potential from the pure cone fovea of the monkey retina Nature, *193*, 958
Brown, K T , and Watanabe, K , (1962 b) Rod receptor potential from the retina of the night monkey Nature, *196*, 547
Brown, H M , Meech, R W , Koike, H , and Hagiwara, S , (1969) Current-Voltage relations during illumination photoreceptor membrane of a barnacle Science, *166*, 240
Buckser, S , and Diamond, H , (1966) Increase in sodium concentration of the isolated frog retina after light stimulation Biochem Biophys Res Commun , *23*, 240
Caldwell, P C , and Keynes, R D , (1960) The permeability of the squid giant axon to radioactive potassium and chloride ions J Physiol (London), *154*, 177
Cohen, A I , (1961) The fine structure of the extrafoveal receptors of the rhesus monkey Exp Eye Res , *1*, 128
Cohen, A I , (1963) Vertebrate retinal cells and their organization Biol Rev Cambridge Phil Soc , *38*, 427
Cohen, A I , (1960) New evidence supporting the linkage to extracellular space of outer segment saccules of frog cones but not rods J Cell Biol , *37*, 424

Cole, K. S., and Curtis, H. J., (1939). Electric impedance of squid giant axon during activity. J. Gen. Physiol., *22*, 649.
Collins, F. D., Love, R. M., and Morton, R. A., (1952). Studies in rhodopsin 4. Preparation of rhodopsin. Biochem. J., *51*, 292.
Collins, F. D., Green, J. N., and Morton, R. A., (1953). Studies in rhodopsin 6. Regeneration of rhodopsin. Biochem. J., *53*, 152.
Collins, F. D., Green, J. N., and Morton, R. A., (1954). Studies in rhodopsin 7. Regeneration of rhodopsin by comminuted ox retina. Biochem. J., *56*, 493.
Cone, R. A., and Brown, P. K., (1969). Spontaneous regeneration of rhodopsin in the isolated rat retina. Nature, *221*, 818.
Cordes, E. H., and Jencks, W. P., (1963). The mechanism of hydrolysis of Schiff bases derived from aliphatic amines. J. Am. Chem. Soc., *85*, 2843.
Crescitelli, F., (1967). Extraction of visual pigments with certain alkyl phenoxy polyethoxy ethanol surface active compounds. Vision Res., *7*, 685.
Crescitelli, F., and Dartnall, H. J. A., (1953). Human visual purple. Nature, *172*, 195.
Crescitelli, F., Mommaerts, W. F. H. M., and Shaw, T. I., (1966). Circular dichroism of visual pigments in the visible and ultraviolet spectral regions. Proc. Natl. Acad. Sci. U.S., *56*, 1729.
Daemen, F. J. M., (1967). A convenient synthesis of phosphatidylethanolamines. Chem. Phys. Lipids, *1*, 476.
Daemen, F. J. M., and Bonting, S. L., (1968). Biochemical aspects of the visual process. III. Specificity of the retinaldehyde effect on cation movements in rod outer segments. Biochim. Biophys. Acta, *163*, 212.
Daemen, F. J. M., and Bonting, S. L., (1969 a). Internal protonation in retinylidene phosphatidylethanolamine and the red shift in rhodopsin. Nature, *222*, 879.
Daemen, F. J. M., and Bonting, S. L., (1969 b). Biochemical aspects of the visual mechanism IV. Aldehydes and cation permeability of artificial phospholipid micelles. Biochim. Biophys. Acta, *183*, 90.
Daemen, F. J. M., and Bonting, S. L., (1970). Transiminization of retinaldehyde imines and rhodopsin photolysis. Federation Proc., *29*, 393 Abs.
Dartnall, H. J. A., and Goodeve, C. F., (1937). Scotopic luminosity curve and absorption spectrum of visual purple. Nature, *139*, 409.
Dainty, J., and House, C. R., (1966). Unstirred layers in frog skin. J. Physiol. (London), *182*, 66.
deRobertis, E., and Lasansky, A., (1961). Ultrastructure and chemical organization of photoreceptors. In "The structure of the eye" G. K. Smelser ed., p. 29.
Dewey, M. M., Davis, P. K., Blasie, J. K., and Barr, L., (1969). Localization of rhodopsin antibody in the retina of the frog. J. Mol. Biol., *39*, 395.
Donner, K. O., and Reuter, T., (1967). Dark adaptation processes in the rhodopsin rods of the frogs retina. Vision Res., *7*, 17.
Donner, K. O., and Reuter, T., (1969). The photoproducts of rhodopsin in the isolated retina of the frog. Vision Res. *9*, 815.
Dowling, J. E., (1960). Chemistry of visual adaptation in the rat. Nature, *188*, 114.
Dowling, J. E., (1967). The organization of vertebrate visual receptors. In "Molecular organization and Biological function" (J. M. Allen ed.) Harper and Row, New York, p. 186.
Duncan, G., (1969 a). Relative permeabilities of the lens membranes to sodium and potassium. Exp. Eye Res., *8*, 315.
Duncan, G., (1969 b). Kinetics of potassium movement across amphibian lens membranes. Exp. Eye Res., *8*, 413.
Duncan, G., and Bonting, S. L., (1969). Photoreception in Limulus: Role of an electrogenic sodium pump? Science, *164*, 1188.

Duncan, G., Daemen, F. J. M., and Bonting, S. L., (1969). Light stimulated ion movements in frog rod outer segments. Fed. Proc., *28*, 331.
Duncan, G., Bonting, S. L., and Daemen, F. J. M., (1970). Light stimulated ion movements in frog outer segments. to be published.
Eakin, R. M., (1965). Evolution of photoreceptors. Cold Spring Harbor Symp. Quant. Biol., *30*, 363.
Eguchi, E., (1965). Rhabdom structure and receptor potential in single crayfish retinular cells. J. Cell. Comp. Physiol., *66*, 411.
Eichberg, J., and Hess, H. H., (1967). The lipid composition of frog retinal rod outer segments. Experientia, *23*, 993.
Emmelot, P., and Bos, C. J., (1968). Studies on plasma membranes. V. On the lipid dependance of some phosphohydrolases of isolated rat liver plasma membranes. Biochim. Biophys. Acta, *150*, 341.
Erickson, J. O., and Blatz, P. E., (1968). N-retinylidene-1-amino-2-propanol. A Schiff base analog for rhodopsin. Vision Res., *8*, 1367.
Falk, G., and Fatt, P., (1963). Photoconductive changes in a suspension of rod outer segments. J. Physiol. (London), *167*, 36 P.
Falk, G., and Fatt, P., (1966 a). Rapid hydrogen ion uptake of rod outer segments and rhodopsin solutions on illumination. J. Physiol. (London), *183*, 211.
Falk, G., and Fatt, P., (1966 b). Components of conductive change in rod outer segments. J. Physiol. (London), *186*, 104 P.
Farrar, K. R., Hamlet, J. C., Henbest, H. B., and Jones, E. R. H., (1952). Studies in the polyene series. XLIII. The structure and synthesis of vitamin A_2 and related compounds. J. Chem. Soc. (London), p. 2657.
Fenster, L. J., and Copenhaver, Jr, J. H., (1967). Phosphatidylserine requirement of Na-K activated ATPase from rat kidney and brain. Biochim. Biophys. Acta, *137*, 406.
Fernández-Morán, H., (1961). The fine structure of vertebrate and invertebrate photoreceptors as revealed by low-temperature electron microscopy. In "Structure of the eye" (G. K. Smelser, ed.), Academic Press, New York, p. 521.
Frank, R. N., and Goldsmith, T. H., (1965). Adenosine triphosphatase activity in the rod outer segments of the pig's retina. Arch. Biochem. Biophys., *110*, 517.
Frank, R. N., and Goldsmith, T. H., (1967). Effects of cardiac glycosides on electrical activity in the isolated retina of the frog. J. Gen. Physiol., *50*, 1585.
Fulpius, B., and Baumann, F., (1969). Effects of sodium, potassium and calcium ions on slow and spike potentials in single photoreceptor cells. J. Gen. Physiol., *53*, 541.
Fuortes, M. G. F., (1959). Initiation of impulses in visual cells of Limulus. J. Physiol. (London), *148*, 14.
Futterman, S., (1963). Metabolism of the retina III. The role of reduced triphosphopyridine nucleotide in the visual cycle. J. Biol. Chem., *238*, 1145.
Futterman, S., (1965), Stoichiometry of retinal vitamin A metabolism during light adaptation. In "Biochemistry of the retina" (C. N. Graymore, ed.), Academic Press, New York, p. 16.
Futterman, S., and Andrews, J. S., (1964). Metabolism of the retina IV. The composition of vitamin A ester synthesized by the retina. J. Biol. Chem., *239*, 81.
Futterman, S., and Saslaw, L. D., (1961). The estimation of vitamin A aldehyde with thiobarbituric acid. J. Biol. Chem., *236*, 1652.
Fyfe, W. S., (1955). Complex ion formation. Part III. The entropies of reaction of the silver and hydrogen ions with some aliphatic amines. J. Chem. Soc. (London), 1347.
Gaines, Jr, G. L., (1966). Insoluble monolayers at liquid-gas interfaces. Interscience New York, p. 59.
Garvin, J. E., and Karnovsky, M. L., (1956). The titration of some phosphatides and related compounds in a non-aqueous medium. J. Biol. Chem., *221*, 211.
Hafkenscheid, J. C. M., and Bonting, S. L., (1968). Studies on Na-K activated ade-

nosinetriphosphatase XIX. Occurrence and properties of a Na-K activated ATPase in Escherichia Coli. Biochim. Biophys. Acta, *151*, 204.
Hagins, W. A., (1956). Flash photolysis of rhodopsin in the retina. Nature, *177*, 989.
Hagins, W. A., (1965). Electrical signs of information flow in photoreceptors. Cold Spring Harbor Symp. Quant. Biol., *30*, 403.
Hagins, W. A., and Adams, R. G., (1960). Movement of ^{24}Na and ^{42}K in the squid retina. Biol. Bull., *119*, 316.
Hagins, W. A., and Jennings, W. H., (1959). Radiationless migration of electronic excitation in retinal rods. Discussions Faraday Soc., *27*, 180.
Hagins, W. A., Zonana, H. V., and Adams, R. G., (1962). Local membrane current in the outer segments of squid photoreceptors. Nature, *194*, 844.
Hagins, W. A., Penn, R. D., and Yoshikami, S., (1970). Dark current and photocurrent in retinal rods. Biophys. J., in press.
Hall, M. O., and Bacharach, A. D. E., (1970). Linkage of retinal to opsin and absence of phospholipids in purified frog visual pigment$_{520}$. Nature, *225*, 637.
Hamasaki, D. I., (1963). The effect of sodium ion concentration on the electroretinogram of the isolated retina of the frog. J. Physiol. (London), *167*, 156.
Hamasaki, D. I., (1964). The electroretinogram after application of various substances to the isolated retina. J. Physiol. (London), *173*, 449.
Hanawa, I., Kuge, K., and Matsumara, K., (1967). Effects of some common ions on the transretinal D.C. potential and the electroretinogram of the isolated frog retina. Japan. J. Physiol., *17*, 1.
Hara, T., and Hara, R., (1965). New photosensitive pigment found in the retina of the squid Ommastrephes. Nature, *206*, 1331.
Hara, T., and Hara, R., (1967). Rhodopsin and retinochrome in the squid retina. Nature, *214*, 573.
Hara, T., and Hara, R., (1968). Regeneration of squid retinochrome. Nature, *219*, 450.
Hara, T., and Hara, R., and Takeuchi, J., (1967). Vision in octopus and squid. Nature, *214*, 572.
Hartley, J. S., and Roe, J. W., (1940). Ionic concentrations at interfaces. Trans. Faraday Soc., *36*, 101.
Hecht, S., and Williams, R. E., (1922). The visibility of monochromatic radiation and the absorption spectrum of visual purple. J. Gen. Physiol., *5*, 1.
Hecht, S., Shlaer, S., and Pirenne, M. H., (1942). Energy, quanta and vision. J. Gen. Physiol., *25*, 819.
Heller, J., (1968). Structure of visual pigments I. Purification, molecular weight, and composition of bovine visual pigment$_{500}$. Biochemistry, *7*, 2906.
Hodgkin, A. L., and Katz, B., (1949). The effects of sodium and potassium on the electrical activity of the giant axon of the squid. J. Physiol. (London), *108*, 37.
Hoeir, C. W., McCorkle, M. R., and Ralston, A. W., (1943). Studies on high molecular weight aliphatic amines and their salts. X. Ionization constants of primary and symmetrical secondary amines in aqueous solution. J. Am. Chem. Soc., *65*, 328.
Hubbard, R., (1956). Retinene isomerase. J. Gen. Physiol., *39*, 935.
Hubbard, R., (1958). The bleaching of rhodopsin by light and by heat. Nature, *181*, 1126.
Hubbard, R., (1959). The thermal stability of rhodopsin and opsin. J. Gen. Physiol., *42*, 259.
Hubbard, R., (1969). Absorption spectrum of rhodopsin: 500 nm absorption band. Nature, *221*, 432.
Hubbard, R., and Dowling, J. E., (1962). Formation and utilization of 11-cis vitamin A by the eye tissues during light and dark adaptation. Nature, *193*, 341.
Hubbard, R., and Kropf, A., (1958). The action of light on rhodopsin. Proc. Natl. Acad. Sci. U.S., *44*, 130.

Hubbard, R., and St. George, R. C. C., (1958). The rhodopsin system of the squid. J. Gen. Physiol., *41*, 501.
Hubbard, R., and Wald, G., (1951). Cis-trans isomers of vitamin A and retinene in the rhodopsin system. Proc. Natl. Acad. Sci. U.S., *37*, 69.
Hubbard, R., and Wald, G., (1952). Cis-trans isomers of vitamin A and retinene in the rhodopsin system. J. Gen. Physiol., *36*, 269.
Jencks, W. P., (1964). Mechanism and catalysis of simple carbonyl group reactions. Prog. Phys. Org. Chem., *2*, 63.
Jenkins, W. T., and Sizer, I. W., (1957). Glutamic aspartic transaminase. J. Am. Chem. Soc., *79*, 2655.
Katz, B., and Miledi, R., (1967). A study of synaptic transmission in the absence of nerve impulses. J. Physiol. (London), *192*, 407.
Kikuchi, R., Naito, K., and Tanaka, I., (1962). Effect of sodium and potassium ions on the electrical activity of single cells in the lateral eye of the horse shoe crab. J. Physiol. (London), *161*, 319.
Kimuzuka, H., and Koketsu, K., (1964). Ion transport through cell membrane. J. Theoret. Biol., *6*, 290.
Kito, Y., and Takezaki, M., (1966). Optical rotation of irradiated rhodopsin solution. Nature, *211*, 197.
Koen, A. L., and Shaw, C. R., (1966). Retinol and alcoholdehydrogenases in retina and liver. Biochim. Biophys. Acta, *128*, 48.
König, A., (1894). Über den menschlichen Sehpurpur und seine Bedeutung für das Sehen. Sitzber. Kgl. Preuss. Akad. Wiss. Berlin, *30*, 577.
Köttgen, E., and Abelsdorff, G., (1896). Absorption und Zersetzung des Sehpurpurs bei den Wirbeltieren. Z. Psychol. Physiol. Sinnesorg., *12*, 161.
Kornberg, A., (1950). Enzymic synthesis of triphosphopyridine nucleotide. J. Biol. Chem., *182*, 805.
Krinsky, N. I., (1958 a). The lipropotein nature of rhodopsin. Arch. Ophtalmol., *60*, 688.
Krinsky, N. I., (1958 b). The enzymatic esterification of vitamin A. J. Biol. Chem., *232*, 881.
Kropf, A., Brown, P. K., and Hubbard, R., (1959). Action of light on visual pigments. Nature, *183*, 442.
Kühne, W., (1878). Ueber den Sehpurpur. Untersuch. Physiol. Inst. Univ. Heidelberg, *1*, 1.
Kuijpers, W., and Bonting, S. L., (1969). Studies on Na-K activated ATPase. XXIV. Localization and properties of ATPase in the inner ear of the guinea pig. Biochim. Biophys. Acta, *173*, 477.
Lee, K. S., and Yu, D. H., (1963). A study of the sodium and potassium activated adenosinetriphosphatase activity of heart microsomal fraction. Biochem. Pharmacol., *12*, 1253.
Liebman, P. A., (1962). In situ microspectrophotometric studies on the visual pigments of single retinal rods. Biophys. J., *2*, 161.
Liebman, P. A., and Entine, G., (1967). Cyanopsin, a visual pigment of retinal origin. Nature, *216*, 501.
Lolley, R. N., and Hess, H. H., (1969). The retinal rod outer segment of the frog. Detachment isolation, phosphorous fractions and enzyme activity. J. Cell. Physiol., *73*, 9.
Lowry, O. H., Roberts, N. R., and Lewis, C., (1956). The quantitative histochemistry of the retina. J. Biol. Chem., *220*, 879.
Lythgoe, R. J., and Quilliam, J. P., (1938). The thermal decomposition of visual purple. J. Physiol. (London), *93*, 24.
Mc Connell, D. G., (1965). The isolation of retinal outer segment fragments. J. Cell. Biol., *27*, 459.

Mc Connell, D. G., and Scarpelli, D. G., (1963). Rhodopsin: an enzyme. Science, *139*, 848.
Maeda, Y., and Isemura, T., (1967). Monolayer films of retinal and the effects of light on them. Nature, *215*, 765.
Marks, W. B., Dobelle, W. H., and Mac Nichol, E. F., (1964). Visual pigments of single primate cones. Science, *143*, 1181.
Matthews, R. G., Hubbard, R., Brown, P. K., and Wald, G., (1963). Tautomeric forms of metarhodopsin. J. Gen. Physiol., *47*, 215.
Millecchia, R., Bradbury, J., and Mauro, A., (1966) Simple photoreceptors in Limulus polyphemus. Science, *154*, 1199.
Millecchia, R., and Mauro, A., (1969 a). The ventral photoreceptor cells of Limulus II. The basic photoresponse. J. Gen. Physiol., *54*, 310.
Millecchia, R., and Mauro, A., (1969 b). The ventral photoreceptor cells of Limulus III. A voltage clamp study. J. Gen. Physiol., *54*, 331.
Moody, M. F., (1964). Photoreceptor organelles in animals. Biol. Rev., *39*, 43.
Radding, C. H., and Wald, G., (1958). The action of enzymes on rhodopsin. J. Gen.
Morrison, W. R., and Smith, L. M., (1964). Preparation of fatty acid methylesters and dimethylacetals from lipids with boronfluoride-methanol. J. Lipid Res., *5*, 600.
Morton, R. A., and Pitt, G. A. J., (1955). Studies on rhodopsin. 9. pH and hydrolysis of indicator yellow. Biochem. J., *59*, 128.
Morton, R. A., and Pitt, G. A. J., (1957). Visual pigments. Fortschr. Chem. Org. Naturstoffe, *14*, 244.
Muherjee, P., and Bonerjee, K., (1964). A study of the surface pH of micelles using solubilized indicator dyes. J. Phys. Chem., *68*, 3567.
Onishi, T., and Kowamura, H., (1964). Rôle des phoshatides dans l'adinosine triphosphatase sensitive à l'ouabaine localisée dans les membranes d'erythrocyte. J. Biochem., *56*, 377.
Oroshnik, W. P., Brown, P. K., Hubbard, R., and Wald, G., (1956). Hindered cis isomers of vitamin A and retinene: the structure of the neo-b isomer. Proc. Natl. Acad. Sci. U.S., *42*, 578.
Physiol., *42*, 371.
Ostroy, S. E., Erhardt, F., and Abrahamson, E. W., (1966). Protein configuration changes in the photolysis of rhodopsin. II. The sequence of intermediates in thermal decay of cattle metarhodopsin in vitro. Biochim. Biophys. Acta, *112*, 265.
Palmer, R. F., and Nechay, B. R., (1964). Biphasic renal effects of ouabain in the chicken: correlation with a microsomal Na+-K+-stimulated ATP-ase. J. Pharmacol. Exptl. Therap., *146*, 92.
Pangborn, M. C., (1951). A simplified purification of lecithin. J. Biol. Chem., *188*, 471.
Penn, R. D., and Hagins, W. A., (1969). Signal transmission along retinal rods and the origin of the electroretinographic α-wave. Nature, *223*, 201.
Pitt, G. A. J., Collins, F. D., Morton, R. A., and Stok, P., (1955). Studies on rhodopsin. 8. Retinylidene methylamine, an indicator yellow analogue. Biochem. J., *59*, 122.
Poincelot, R. P., and Zull, J. E., (1969). Phospholipid composition and extractibility of light- and dark-adapted bovine retinal rod outer segments. Vision Res., *9*, 647.
Poincelot, R. P., Millar, P. G., Kimbel, R. L. jr., and Abrahamson, E. W., (1969). Visual pigments. Lipid to protein chromophore transfer in visual pigment photolysis. Nature, *221*, 256.
Portius, H. J., and Repke, K., (1967). Properties and function of the Na-K-activated, Mg-dependent ATP phosphohydrolase system of heart muscle. Acta Biol. Med. German., *19*, 907.
Post, R. L., Merrit, C. R., Kinsolving, C. R., and Albright, C. D., (1960). Membrane ATP-ase as a participant in the active transport of Na and K in the human erythrocyte. J. Biol. Chem, *235*, 1796

Pratt, D. C., Livingston, R., and Grellmann, K. H., (1964). Flash photolysis of rod particle suspensions. Photochem. Photobiol., *3*, 121.
Radding, C. M., and Wald, G., (1956 a). Acid-base properties of rhodopsin and opsin. J. Gen. Physiol., *39*, 909.
Radding, C. H., and Wald, G., (1956 b). The stability of rhodopsin and opsin effects of pH and aging. J. Gen. Physiol., *39*, 923.
Reuter, T., (1966). The synthesis of photosensitive pigments in the rods of the frog retina. Vision Res., *6*, 15.
Ridderstap, A. S., and Bonting, S. L., (1969). Na+-K+ activated ATPase and pancreatic secretion in the dog. (Studies on Na-K activated adenosinetriphosphatase XXII). Amer. J. Physiol., *216*, 547.
Robinson, R. A., and Kiang, A. K., (1956). The spectrophotometric determination of the ionization constants of a dibasic acid. Trans. Faraday Soc., *52*, 327.
Roelofsen, B., (1968). Some studies on the extractability of lipids and the ATP-ase activity of the erythrocyte membrane. Thesis, University Utrecht.
Roelofsen, B., Baadenhuysen, H., and van Deenen, L. L. M., (1966). Effects of organic solvents on the adenosine triphosphatase activity of erythrocyte ghost. Nature, *212*, 379.
Rosenberg, B., (1958). Photoconductivity and the visual receptor process. J. Opt. Soc. Am., *48*, 581.
Rosenberg, B., (1959). Photoconduction and cis-trans isomerism in 6-carotene. J. Chem. Phys., *31*, 238.
Rosenberg, B., and Harder, H. C., (1967). Semiconduction and photoconduction activation energies of the retinals. Photochem. Photobiol., *6*, 629.
Rosenberg, B., Orlando, R. A., and Orlando, I. M., (1961). Photoconduction and semiconduction in dried receptors of sheep eyes. Arch. Biochem. Biophys., *93*, 395.
Rushton, W. A. H., (1957). Blue light and the regeneration of human rhodopsin in situ. J. Gen. Physiol., *41*, 419.
Rushton, W. A. H., (1959). A theoretical treatment of Fuortes's observations upon excentric cell activity in Limulus. J. Physiol. (London), *148*, 29.
Schatzmann, H. J., (1962). Lipoprotein nature of red cell adenosine triphosphatase. Nature, *196*, 677.
Schick, M. J., (1966). Micelle formation in mixtures of nonionic and cationic detergents. J. Am. Oil Chem. Soc., *43*, 681.
Sekoguti, Y., (1960 a). The changes in the K-level of retina. Ann. Rept. Sci. Works, Fac. Sci. Osaka Univ., *8*, 75.
Sekoguti, Y., (1960 b). The effects of illumination on the K-loss from the retinal tissue. Ann. Rept. Sci. Works, Fac. Sci. Osaka Univ., *8*, 83.
Sekoguti, Y., (1960 c). On the ATPase activities in the retina and the rod outer segments. J. Cell. Comp. Physiol., *56*, 126.
Shichi, H., Lewis, M. S., Irreverre, F., and Stone, A. L., (1969). Biochemistry of visual pigments I. Purification and properties of bovine rhodopsin. J. Biol. Chem., *244*, 529.
Shields, J. E., Dinovo, E. C., Henriksen, R. A., Kimbel, R. L., and Millar, P. G., (1967). The purification and amino acid composition of bovine rhodopsin. Biochim. Biophys. Acta, *147*, 238.
Silmann, H. J., Ito, H., and Tomita, T., (1969 a). Studies on the mass receptor potential of the isolated frog retina I. General proporties of the response. Vision Res., *9*, 1435.
Silmann, H. J., Ito, H., and Tomita, T., (1969 b). Studies on the mass receptor potential of the isolated frog retina II. On the basis of the ionic mechanism. Vision Res., *9*, 1443.
Sjöstrand, F. S., (1961). Electron microscopy of the retina. In "The structure of the Eye" (G. K. Smelser, ed.). Academic Press, New York, p. 1.
Sjodin, R. A., and Mullins, L. J., (1967). Tracer and nontracer potassium fluxes in squid giant axons and the effects of changes in external potassium concentration and membrane potential. J. Gen. Physiol., *50*, 533.

Skou, J C , (1957). The influence of some cations on ATPase from peripheral nerve Biochim Biophys Acta, *23,* 394
Skou, J C , (1960) Further investigations on a Mg-Na activated ATPase possibly related to the active, linked transport of Na and K across the nerve membrane Biochim Biophys Acta, *42,* 6.
Smith, T G., Stell, W K , Brown, J E , Freeman, J A , and Murray, G C , (1968). A role for the sodium pump in photoreception in Limulus Science, *162,* 454
Stieve, H , (1965) Interpretation of the generator potential in terms of ionic processes Cold Spring Harbor Symp Quant Biol , *30,* 451
Tanaka, R , and Abood, L G , (1964) Phospholipid requirement of Na^+K^+ activated adenosine triphosphatase from rat brain Arch Biochem Biophys , *108,* 47
Tanaka, R , and Strickland, K P , (1965) Role of phospholipid in the activation of Na-K-activated ATPase of beef brain Arch. Biochem. Biophys , *111,* 583
Tansley, K , (1931) The regeneration of visual purple, its relation to dark adaptation and night blindness J Physiol (London), *71,* 442
Tatibana, M , (1963) Effect of snake venom upon membrane adenosine triphosphatase of human erythrocyte. J. Biochem , *53,* 260.
Tolbert, O C , (1968) Isolation of retinal bovine retinoldehydrogenase isozymes Invest Ophthalmol , *7,* 120
Tomita, T , (1964) Mechanisms subserving color coding in the vertebrate retina IOPAB International Biophys. Meeting, Paris-Orsay, Abstr., II CIII
Tomita, T , Murakami, M , and Hashimoto, Y , (1960) On the R membrane in the frog's eye. its localization and relation to the retinal action potential J Gen. Physiol , *43,* Pt 2, 81
Tomita, T , Kanedo, A , Murakami, M , and Pautler, E. M , (1967). Spectral response curves of single cones in the carp. Vision Res., *7,* 519.
Toyoda, J , Nosaki, H., and Tomita, T., (1969). Light induced resistance changes in single photoreceptors of Necturnus and Gekko Vision Res., *9,* 453
Traube, J., (1891) Ueber die Capillaritatsconstanten organischer Stoffe in wasserigen Losungen. Ann *265,* 27.
Truijllo-Cenóz, O., (1965) Some aspects of the structural organization of the arthropod eye Cold Spring Harbor Symp. Quant. Biol , *30,* 371.
Trurnit, H J., and Colmano, G , (1959). Chloroplast studies. I Absorption spectra of chlorophyll monolayers at liquid interfaces Biochim Biophys Acta, *31,* 434.
Van Zwieten, P. A., (1968). The use of ^{86}rubidium for the determination of changes in membrane permeability in guinea pig atrial tissue. Pflugers Archiv, *303,* 81.
Wald, G., (1935). Carotenoids and the visual cycle J. Gen Physiol., *19,* 351.
Wald, G., (1936). Pigments of the retina. I. The bullfrog J. Gen. Physiol., *19,* 781
Wald, G , (1937 a) Visual purple system in fresh-water fishes. Nature, *139,* 1017.
Wald, G , (1937 b). Photo-labile pigments of the chicken retina. Natura, *140,* 545.
Wald, G., (1938) On rhodopsin in solution. J. Gen Physiol , *21,* 795.
Wald, G., (1939). The porphyropsin visual system J. Gen Physiol., *22,* 775
Wald, G , (1950) The interconversion of the retinenes and vitamins A in vitro. Biochim Biophys. Acta, *4,* 215.
Wald, G , (1960). The distribution and evolution of visual systems. In "Comp. Biochemistry" Academic Press, New York, p 311
Wald, G , and Brown, P K., (1952) The role of sulfhydryl groups in the bleaching and synthesis of rhodopsin. J Gen. Physiol., *35,* 797
Wald, G., and Brown, P K , (1958) Human rhodopsin. Science, *127,* 222.
Wald, G , and Brown, P K , (1964) Visual pigments in single rods and cones of the human retina. Science, *144,* 45.
Wald, G , and Hubbard, R , (1949) The reduction of retinene$_1$ to vitamin A_1 in vitro. J Gen Physiol , *32,* 367.

Wald, G., Durrell, J., and St. George, R. C. C., (1950). The light reaction in the bleaching of rhodopsin. Science, *111*, 179.
Wald, G., Brown, P. K., and Smith, P. H., (1953). Cyanopsin, a new pigment of cone vision. Science, *118*, 505.
Wald, G., Brown, P. K., and Smith, P. H., (1955). Iodopsin. J. Gen. Physiol., *38*, 623.
Wald, G., Brown, P. K., and Gibbons, I. R., (1963). The problem of visual excitation. J. Opt. Soc. Am., *53*, 20.
Weil-Malherbe, H., and Green, R. H., (1951). The catalytic effect of molybdate on the hydrolysis of organic phosphate bonds. Biochem. J., *49*, 286.
Wheeler, K. P., and Whittam, R., (1970). ATP-ase activity of the sodium pump needs phosphatidylserine. Nature, *225*, 449.
Yamamoto, T., Tasaki, K., Sugawara, Y., and Tononsaki, A., (1965). Fine structure of the octopus retina. J. Cell Biol., *25*, 345.
Yoshikami, S., and Hagins, W. A., (1970). Ionic basis of dark current and photocurrent of retinal rods. Biophys. J., in press.
Yoshizawa, T., and Wald, G., (1963). Pre-lumirhodopsin and the bleaching of visual pigments. Nature, *197*, 1279.
Yoshizawa, T., and Wald, G., (1964). Transformations of squid rhodopsin at low temperatures. Nature, *201*, 340.
Young, J. Z., (1962 a). The retina of cephalopods and its degeneration after optic nerve section. Phil. Trans., *B 245*, 1.
Young, J. Z., (1962 b). The optic lobes of octopus vulgaris. Phil. Trans., *B 245*, 20.
Young, R. W., (1967). The renewal of photoreceptor cell outer segments. J. Cell Biol., *33*, 61.
Young, R. W., (1969). A difference between rods and cones in the renewal of outer segment protein. Invest. Ophthalmol., *8*, 222.
Zachman, R. D., and Olsen, J. A., (1961). A comparison of retinene reductase and alcohol dehydrogenase of rat liver. J. Biol. Chem., *236*, 2309.
Zonana, H. V., (1961). Fine structure of the squid retina. Bull. Johns Hopkins Hosp. *109*, 185.

ABBREVIATIONS

A.S.W.	artificial seawater
ATP	adenosinetriphosphate
ATPase	adenosinetriphosphatase
cpm	counts per minute
DNFB	2,4-dinitro-fluoro-benzene
EDTA	ethylenediaminetetraacetate
MKH	moles converted per kg dry weight per hour
NADH	nicotinamide dinucleotide reduced form
NADPH	nicotinamine dinucleotide phosphate reduced form
PC	phosphatidylcholine
PE	phosphatidylethanolamine
ROS	rod outer segments
S.E.	standard error of the mean
TCA	trichloroacetic acid
Tris	tris-(hydroxymethyl)-aminomethane

STELLINGEN

I

De door Morill en Reiss gevonden afname in binding tussen calcium en RNA bij hoge concentraties van het kation wordt door hen ten onrechte toegeschreven aan een verandering in electrostatische interacties.

Morill, G. A. and Reiss, M. J., Biochim. Biophys. Acta, *179*, 43 (1969).

II

Het is onwaarschijnlijk, dat het door Bonati en Ugo gevonden signaal voor de N-methyleenproton resonantie in S-ethyl N,N-diethyldithiocarbamaat te wijten is aan koppeling met ^{14}N.

Bonati, F. and Ugo, R., J. Organmetal. Chem., *10*, 257 (1967).

III

Aan de door Plack en Pritchard gegeven evenwichtsconstanten van bepaalde retinylidene imines kan weinig waarde worden toegekend.

Plack, P. A. and Pritchard, D. J., Biochem. J., *115*, 927 (1969).

IV

De door Lodish beschreven experimenten bewijzen onvoldoende dat na translatie van f_2 RNA in het celvrije systeem van Bacillus stearothermophillus compleet rijpingseiwit is gesynthetiseerd.

Lodish, H. F., Nature, *224*, 867 (1969).

V

Het door Müller en Crothers geponeerde bindingsmodel tussen actinomycine en DNA sluit niet uit dat de naast het guanine gelegen purine of pyrimidine base mede verantwoordelijk is voor de binding.

Müller, W. and Crothers, D. M., J. Mol. Biol., *35*, 251 (1968).

VI

De door Panos gesuggereerde correlatie tussen celwand remming bij bacteriën en een vermindering in het gehalte aan cis-vaceenzuur of cyclopropaanvetzuren in de membraan lipiden geldt niet voor alle vormen van celwand remming.

Panos, C., Ann. N. Y. Acad. Sci., *143*, 152 (1967).

VII

Er zijn aanwijzigingen om te veronderstellen dat het Antihemofilie-globuline meer lipid bestanddelen bevat, dan tot nu toe wordt aangenomen.

Veder, H. A., Nature, *209*, 202 (1966).
Paulssen, M. M. P. Wouterlood, A. C. M. G. B. and Scheffers, H. L. M. A., Thrombos. Diathes. haemorrh. (Stuttg.), *22*, 577 (1969).

VIII

De hypothese van Robert, Payrau en medewerkers, dat een verstoring in de biosynthese van structurele componenten door antilichamen of door beschadiging troebeling veroorzaakt in de cornea, kan op analoge wijze een verklaring geven voor pathologische verschijnselen in andere bindweefsels.

Robert, L. and Robert, B., in Biochemistry of the Eye, Symp. Tutzing Castle (Karger, Basel/New York) 1968, p. 20.
Payrau, P. in Biochemistry of the Eye, Symp. Tutzing Castle (Karger, Basel/New York, 1968) p. 1.

IX

De waarde die Heller aangeeft voor de molaire extinctie van rhodopsine is onjuist.

Heller, J., Biochemistry, *7*, 2906 (1968).
Wald, G. and Brown, P. K., J.Gen.Physiol., *37*, 189 (1953).
Shichi, H. Lewis, M. S. Irreverre, F. and Stone, A. L., J.Biol.Chem., *244*, 529 (1969).

X

Bij solubilizatie van membraan gebonden componenten wordt veelal te weinig rekening gehouden met de door de detergens teweeggebrachte veranderingen in de microomgeving van deze componenten.

XI

Aan de getalswaarde voor het percentage Na-K geactiveerde ATPase betrokken op het totaal ATPase moet geen andere dan practische betekenis worden toegekend.

XII

Het belang dat het in beperkte mate geven van onderwijs heeft voor de vorming en het werk van de wetenschappelijke onderzoeker, wordt veelal onderschat.

XIII

De instelling van een Teleac cursus Voetbal verdient de hoogste prioriteit.

J. J. H. H. M. de Pont
17 juni 1970