Following the demonstration of distinct intermediates in the thermal decay of rhodopsin, characterizable by their different spectra (Summary: WALD 26), the main question was how to interpret these spectral shifts in chemical terms and to elucidate the molecular mechanism of visual excitation. The findings of MATTHEWS et al. 18 constituted a major step in this direction. They found that in the whole scale of intermediates in rhodopsin photo-lysis there exists one reaction which runs down just within the latent period of the receptor and which is connected with a very remarkable spectral change. This transition from metarhodopsin I to metarhodopsin II is widely accepted as being the essential step initiating visual excitation (Summaries: l.c. 11, 5). Furthermore, this reaction is coupled with a rapid pH-change, which was first observed by WALD’s group 18, 23 and repeatedly confirmed by other groups later on l.c. 11, 20, 21.

Concerning the chemical significance of the metarhodopsin I—II reaction, two different hypotheses have been proposed in principle: the first, advanced by WALD and co-workers (Summary 26) assumes the retinene in rhodopsin to be coupled with the protein part of the molecule, the opsin. The metarhodopsin I—I reaction consequently appears as a conformational change of the protein-retinene compound. In the second hypothesis, however, the metarhodopsin I—I reaction is regarded as a lipid to protein transfer of the retinene, the latter being linked to phosphatidylethanolamine (PE) as a Schiff-base in the stages from rhodopsin to metarhodopsin I and to opsin in metarhodopsin II (POINCELOT et al. 52). The first hypothesis is favoured by papers by BOWNDS 6, KITO 17, and HUBBARD 15, the second by de PONT et al. 8, FUKAMI and FUKAMI 12, and DAEMEN and BONTING 5. In the most recent discussions these two hypotheses

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remain the salient point, one group of investigators finding no phospholipid at all in purified solutions of rhodopsin, others in contrast isolating a retinene-PE complex from rhodopsin. The aim of this paper is to use measurements of rapid pH-changes to provide additional evidence for one hypothesis or the other. The experiments were performed on isolated bovine rod outer segments (ROS). The measuring equipment was a rapid registering repetitive flash photometer with signal/noise improvement after Witt. Using pH-indicators, rapid pH-changes were measured optically in addition to direct registrations of the kinetics of the metarhodopsin I decay and the rise of metarhodopsin II.

Methods

Bovine eyes, enucleated directly after the death of the animal in the slaughterhouse were kept in the dark and cooled during one hour to about 0°C. The following operations were carried out in deep-red light (Osram 4563). All vessels and solutions were cooled to about 3°C. The retinæ of ca. 30 eyes were excised and suspended in about 2 ml of Ringer solution. They were ground in a mortar for 5 min. and poured through two layers of a nylon texture (Nytal 25 TI-35 from Henry Simon Ltd., Textiles Dept., Chedale Heath, Stockport, Chesire, G.B.). In a centrifuge tube 3 ml of this solution were layered on 3 ml of a sucrose solution (d = 1.140) respectively and centrifuged in a swinging bucket at 30,000 g for 12 minutes. The dense deep scarlet layer of ROS in the upper part of the tube was aspirated using a hypothermic needle (inner diameter = 0.8 mm, bent over 90° at the end). This sucrose-containing ROS solution was resuspended in relation 1:4 in a buffer-free salt solution (0.9% NaCl, 0.04% KCl, 0.025% CaCl₂ (w/v)) and centrifuged at 3,000 g for 10 minutes. The pellet was resuspended in 7 ml of the same salt solution and stored in samples á 0.2 ml under liquid N₂ in the dark following a rapid freezing procedure. Rhodopsin-determinations were performed according to McConnell. ROS counted using a blood corpuscle counting chamber. The ROS-preparations were contaminated to 10–30% by erythrocytes and nerve cells.

The measuring equipment was a rapid registering repetitive flash photometer after Witt. The flash (Osram X IE 200) had an intensity of about 7 Ws and a half-life of 10⁻⁵ sec. The photomultiplier (EMI 9558 AQ) was DC-coupled to an average-computer (Nuclear Data ND-801 Enhancetron 1024). The electrical bandwidth was 5 kHz. In each experiment 30 single counts were sampled and averaged. Measurements were performed using an (QS)-microcuvette (d = 1 mm, Φ = 23 mm, V = 0.5 ml). In each experiment 0.1 ml ROS-solution (2–5 x 10⁻⁵ M/L rhodopsin) and 0.4 ml salt solution with or without a pH-indicator was used. The measuring light, monochromized by a Bausch and Lomb monochromator (optical bandwidth: ±3 nm), was adjusted to λ = 485 nm (decay of metarhodopsin I), λ = 385 nm (rise of metarhodopsin II), λ = 555 nm (pH-change, phenolphthaleine), λ = 576 nm (pH-change, o-kresolred), λ = 595 nm (pH-change, bromcresolpurple, thymolphthaleine), λ = 618 nm (pH-change, bromcresolgreen). pH-measurements and titrations were performed using an Ingold glass electrode and a precision pH-meter (Knick). Additional details are given in the legends.

Parallelism of the rapid pH-change with the metarhodopsin I–II reaction

A correlation between the kinetics of the pH-changes and chemical reactions in rhodopsin-photolysis can be measured only if diffusion processes are excluded as rate-limiting factors. The decay of metarhodopsin I is influenced by damaging of the ROS-membranes to a very small extent only (own observations), whereas this does not apply in the case of the pH-changes: If the ROS-membranes are

Fig. 1. pH-change, measured with the indicator bromcresol-purple (3 x 10⁻⁵ M) at 595 nm: upwards-deflection indicates alkalization of the solution. Isolated ROS in a buffer-free salt solution (0.9% NaCl, 0.04% KCl, 0.025% CaCl₂ (w/v)) adjusted to pH 5.80 with N/100 HCl (rhodopsin conc. in cuvette: 6 x 10⁻⁴ M/L). Upper registration: intact ROS without additions, second registration: addition of 5 x 10⁻⁷ M CCCP, third registration: ROS washed in aqua dest. before measurement. T = 19°C.
kept intact by the use of isotonic solutions, the ΔpH-signal as measured for instance with the indicator bromcresolpurple (BCP), is delayed about 30-fold as compared with the metarhodopsin I—II signal (Fig. 1, top). The upwards-deflection of the ΔpH-signal indicates an alkalinization of the solution. Since the indicator predominates in the outer phase, the delay may result from the diffusion of protons across the plasma membrane as has been suggested by Von Sengbusch. Facilitation of this transport by addition of carbonylcyanid-m-chlorophenylhydrazone (CCCP) accelerates the kinetics of the ΔpH-signal (Fig. 1, middle). If the membranes of ROS are destroyed completely by an osmotic shock, the ΔpH-signal follows the course of the kinetics of the metarhodopsin I—II decay (Fig. 1, bottom). Since the metarhodopsin I—II reaction depends to a great extent on temperature, a comparison of the temperature-dependence of the ΔpH-signal with the metarhodopsin I—II kinetics is a good test for the parallelism of both. As shown in Table 1, there is only a very slight delay of the ΔpH-signal of osmotically damaged ROS as compared with the metarhodopsin I—II reaction, a fact which may be explained by diffusion between the indicator molecule and rhodopsin.

<table>
<thead>
<tr>
<th>Temperature:</th>
<th>Half-life of the metarhodopsin I—II rapid alkalization in reaction (385 nm)</th>
<th>Half-life of the metarhodopsin I—II rapid alkalization in osmotically damaged ROS (BCP)</th>
</tr>
</thead>
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<tr>
<td>7 °C</td>
<td>60 msec</td>
<td>84 msec</td>
</tr>
<tr>
<td>22 °C</td>
<td>6.0 msec</td>
<td>7.5 msec</td>
</tr>
<tr>
<td>31 °C</td>
<td>0.9 msec</td>
<td>2.0 msec</td>
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</tbody>
</table>

Table 1. Temperature-dependence of the kinetics at pH 5.9.

The rapid alkalization may have two reasons in principle: firstly there may be a transmembrane proton transport as observable across the thylakoid membrane, in photosynthesis for instance. The second possibility is the release or disappearance of an acid or base during the reaction. Since the pH-change can also be observed when the membranes are completely destroyed (by severe hypotonic osmotic shock or by detergents even in large amounts), a transmembrane transport can be rejected as an explanation.

In 1963 a chemical equilibrium between metarhodopsin I and metarhodopsin II was observed at low temperatures (2—3 °C) by Matthews et al., which was distinctly dependent on the pH (Fig. 2, left side). The extinction in relation to the pH was observed as a sigmoid curve (Fig. 2, right side), the turning-point of which was interpreted as the pK-value of the buffer released in this reaction. The pK-value was estimated by the authors at pH 6.4 and considered to indicate the imidazole group of histidine. Our measurements of the metarhodopsin II rise in dependence on pH at equilibrium temperature (2 °C) agree with these results in so far as they show a turning-point at pH 6.4 (Fig. 3). On the other hand, an additional positive absorption change is found in the alkaline region. The turning-point of this peak is about pH 10.5. This indicates participation of an alkaline buffer in the metarhodopsin I—II reaction.

The pH-dependence of the chemical equilibrium between metarhodopsin I and metarhodopsin II

![Fig. 2. Chemical equilibrium between metarhodopsin I (478 nm) and metarhodopsin II (380 nm) at 3.2 °C, redrawn after Matthews et al. Left: pH-dependence of the equilibrium, right: Extinction of metarhodopsin I as a function of pH. The circle indicates the turning-point at pH 6.4.](image)

![Fig. 3. Absorption change of metarhodopsin II, measured at 385 nm at 2 °C as a function of pH. ROS (rhodopsin-conc. in cuvette: 4 × 10⁻⁶ M/L) in 1.5 × 10⁻¹ M buffer-solution (McIlvaine/Phosphate/Glycine).](image)
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Direct titration experiments

If the turning-points of the curves shown above actually signify pK-values of buffers liberated during the reaction, they must be demonstrable also by direct titration. Solutions of isolated ROS were in each case divided into two equal parts. Titrations were performed in a N₂-atmosphere. One part was titrated in the dark at equilibrium-temperature (2 °C). The other part was titrated at 2 °C in normal day-light following an illumination of 5 min. with a 100 Watt lamp in a distance of 50 cm. In Fig. 4 (A and C) the upper curves show the results of the illuminated ROS, the lower ones those of the dark-samples (each curve shows one of three similar titrations). If one calculates the difference between the two titration-curves (B and D), the turning-points work out at pH 6.3 and 10.5. Furthermore, the titration experiments point to the possibility of estimating the pH-values on the basis of ΔpH measurements as a function of pH.

The pH-dependence of the rapid alkalization in the metarhodopsin I—II reaction

Since the indication-region of each pH-indicator is limited, it is necessary to use different overlapping indicators, if one intends to measure the pH-change as a function of pH in the whole pH-scale. If the measurements are normalized to maximal steepness of each indicator and the various indicators are adapted to each other by overlapping measurements, the complete pH-dependence of the rapid alkalization is revealed. In the measurements shown in Fig. 5, the indicators brom cresol green (pK = 4.7), brom cresol purple (pK = 6.1), o-kresol red (pK = 8.2), phenolphthaleine (pK = 9.3), and thymolphthaleine (pK = 10.0) were used. In the acid region the turning-point of the function is observed at about pH 6.1. In the alkaline the signal is only small and the turning-point appears at about pH 10.5. These values require some correction since the turning-points of the ΔpH-curve and the titration difference-curve are not identical. If this correction is made, the pK-values are about pH 6.3 and 10.6.

Discussion

Using three different methods: the metarhodopsin I—II absorption change at equilibrium-temperature, the direct titration at equilibrium-temperature and the ΔpH-method, two pK-values have been estimated, one at about pH 6.3 and one at about pH 10.5 (Table 2). This indicates that the rapid alkalization may not result from the release of the imidazole-group of histidine, since in this view the alkaline...
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Absorption Titration / pH-method
change at 2 °C 385 nm

<table>
<thead>
<tr>
<th>pK_1</th>
<th>pK_2</th>
<th>pK_1</th>
<th>pK_2</th>
<th>pK_1</th>
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<td>10.5</td>
<td>6.2</td>
<td>10.4</td>
<td>6.1-6.3</td>
<td>—</td>
</tr>
</tbody>
</table>

Table 2. Estimation of the pK-values of the buffers involved in the metarhodopsin I-II reaction.

The pK-value (10.5) is not explained. Titration of the phospholipid PE yields a pK-value of 10.4 concerning the amino-group of ethanolamine. Experiments with regard to the molecular meaning of the pK 6.3 are in progress and will be published in a subsequent paper.

Our results are quite compatible with the findings of Poincelot et al. that retinene shifts from PE to protein during the metarhodopsin I-II reaction. According to the concept of Poincelot et al. the polar region of PE is connected with retinene as a Schiff-base in rhodopsin until metarhodopsin I. This bond has been shown to be in a hydrophobic region of the molecule by Akhtar, Blosse and Dewhurst and by Bownds and Wald since it was not reducible by an aqueous solution of sodium borohydride (NaBH_4) in contrast to the bond in metarhodopsin II. PE is enclosed in a hydrophobic core in the stage of rhodopsin and all the intermediates until metarhodopsin I and released into the water phase in metarhodopsin II. If we assume that the PE-molecule is incorporated in the insulating lipid layer of the photoreceptor membrane, this effect may result in a remarkable conformation change of the membrane. This will be discussed in a subsequent paper.

I am extremely grateful to Prof. Dr. W. Kreutz for many stimulating discussions and for his valuable criticism, to Prof. Dr. H. T. Witt, who gave me the opportunity to work with the repetitive flash photometer, and to Miss G. J. E.-M. Zippan for drawing the Figures and preparing the ROS.