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Measurements of Fast Light-Induced Light-Scattering and -Absorption Changes in Outer Segments of Vertebrate Light Sensitive Rod Cells*

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Abstract. Flash-induced changes of light-absorption and of light-scattering of vertebrate rod outer segments (ROS) from frog and cattle in suspension were measured at 380 and 800 nm. The photometer used allows the observation of light intensity changes under well defined angles.

We studied the successive decrease of the signal amplitude in series of flashes. One flash bleaches about 1 % rhodopsin.

The following results are discussed:

1. The signal at 380 nm is a superposition of the absorption change caused by formation of metarhodopsin II and of a biphasic additional signal. The latter exists only for the initial range of bleaching (15 to 25 % rhodopsin).

2. At 800 nm three scattering signals are observed which are characterized by their successive amplitude decrease and time course:

N: A small signal with time course and successive amplitude decrease comparable to the metarhodopsin II absorption change, probably arising from a structural change within the disc membrane.

N_i: A slow signal, disappearing with the first flash, which may be understood as an outer membrane effect.

P: A biphasic signal with a successive decrease rate, by a factor of 10 to 20 higher than that of the metarhodopsin II signal. The two kinetically different components are separated by variation of the observation angle. Two regions of different extension appear to change structurally with different time course. "*P*" may reflect an influence of the light-induced transmitter release on disc shape and/or mass.

Key words: Flash Photolysis — Light Scattering — Vertebrate Photoreceptor — Structural Changes.

Introduction

The rod photoreceptor is characterized by its own ability to adaptation (Grabowski *et al.*, 1973) and its high sensitivity. To explain this, a first amplification and regulation step is necessary, which is preconnected in series to the regulation of the diffusion current through the outer membrane. This first step can be thought of as something like a "chemical photomultiplier", realized by coupled

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enzymes (Wald, 1974), by a cooperative behaviour of disc membrane transmitter-binding sites or by a disc pore mechanism. As early as in 1954, G. Wald with his compartment concept provided a first idea how such a primary amplification can be imagined. Hypotheses going into greater detail were deduced mainly on the basis of measurements at the level of the released transmitter and of the outer membrane potential characteristics (summarized by H. Stieve, 1974).

In our investigations—the results obtained up to now being discussed in this paper—we look for further indications elucidating the intracellular events involved in primary amplification and adaptation. Thereby we observe flash-induced light-absorption and light-scattering signals using a flash photometer described in principle by Rüppel and Witt (1969) and modified by us, as described later on.

The main parameter used is the successive decrease of the amplitude of such signals in series of flashes.

Rhodopsin bleaching in vivo reaches in vertebrate rod outer segments at a maximum 50% (Barlow, 1973). The latency period of the photoreceptor cell is in the order of milliseconds (Arden, 1969). To get enough resolution in these ranges with relatively small flash energies we had to carry out our experiments on isolated rod outer segments (ROS).

Experimental Procedure

1. The *measuring beam* has a cross section of cylindrical symmetry. The Bausch & Lomb monochromator we used is therefore equipped with entrance and exit holes instead of slits. The divergence of the measuring beam ("parallelity") is variable via the angle of detection (Fig. 1) adjusted within $2° \leq \Delta\theta \leq 10°$ by a diaphragm in the focus plane of the lens 2.

For measurements with angles $\theta \geqq 0$ we used the photometer shown in Fig. 2. The scattered light is detected with circular mounted light-guides picking out equivalent scattering angles. All optics are replaced by a selffocusing ball-shaped cuvette. The angle of detection is given by the light-guide diameter and therefore fixed at about 2°. Each light-guide detects the light scattered within a certain direction; this light is parallel within the cuvette with an accuracy of 2°. Because of the detection of 20 equivalent light beams the S/N ratio is enhanced by a factor of $\sqrt{20}$.

Red or blue Schott glass filters (not shown in the figures) cut off the green light of the flash.

The measuring beam was switched off between the flash groups (see below).

2. The *flashlight* of an Osram XIE 200 flash tube with a flash duration of 20 μsec is filtered by a combination of two Schott glass filters (BG 18, OG 495) and one broad band interference filter supplied by Dr. Hugo Anders KG, Diendorf, West Germany. The maximum transmittance is at 520 nm and the band width is 35 nm. Excitation light was the same in all experiments. Only 10% of the quanta of the flash are absorbed by the sample; this ensures that no inhomogeneity of the excitation in the sample occurs. In fact, nearly no influence of the efficient sample thickness or concentration on the excitation can be observed. The flashlight is focused in the case of the cylindrical-shaped cuvette by cylindrical optics; the flash

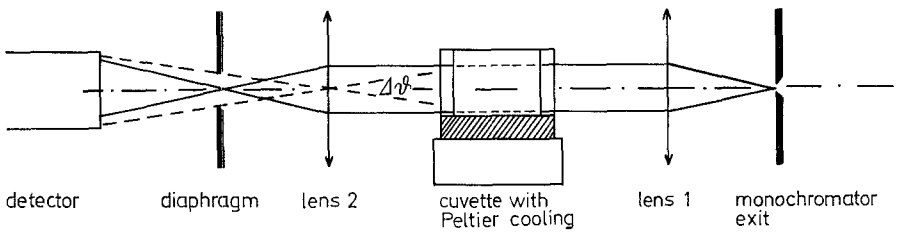

Fig. 1. Measuring beam for the observation angle $\theta = 0$ Monochromator exit: 1.2 mm ⌀ hole (corresponding to 9 nm bandwidth). The angle of detection $\Delta\theta$ defines the "parallelity" of the light beam; it is adjusted with the diaphragm in the focus plane of lens 2

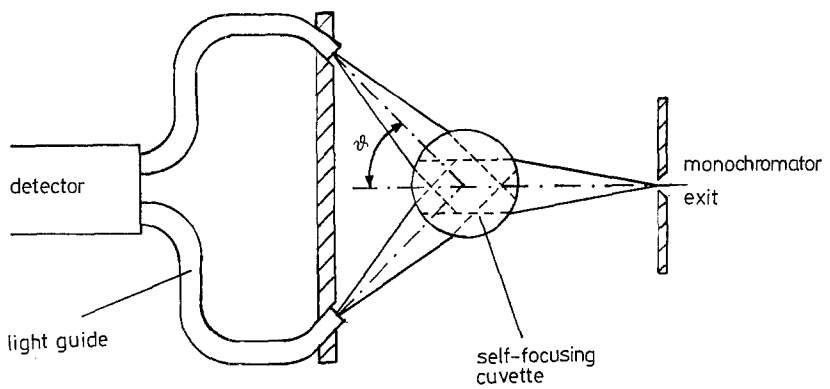

Fig. 2. Device for the measurement with observation angles $\theta \geq 0$. Only two light guides are drawn instead of 20; their entrances are all at the focus of the cuvette and enclose the angle θ with the optical axis. $\Delta\theta$ is given by the entrance diameter and therefore fixed at 2°

intensity was measured for the single flash registrations. For larger deviations a correction was made.

3. *Temperature* was stabilized at 20,0° C for all experiments with a Peltierelement and an electronic adjustment. The cuvette is made of a massive block of aluminium to avoid temperature differences influencing the reaction time.

4. To avoid *mechanical perturbations,* the photometer is mounted on a heavy sandwich of stone plates and polyester resting on air-filled rubber tires.

5. *ROS were prepared* under dim red light by the following methods:

a) Frog (method after Dulson, 1974).

Retinae were excised and ROS separated by gently shaking in a ringer solution of the following composition [after Sickel (1974)]

NaCl 80 mM, KCl 2 mM, $CaCl_2$ 0.15 mM, $MgCl_2$ 0.1 mM, Na-phosphate-buffer (pH = 7.0) 15 mM (Osmolarity: 210 mosmol). The pH-value was controlled with a micro-glass-electrode during some measurements. It remained constant within 0.02 pH-unities.

After passing an 8-fold layer of gauze the suspension was measured. The ROS from 1 to 2 eyes were suspended in 6 ml of Ringer for one sample. The relative

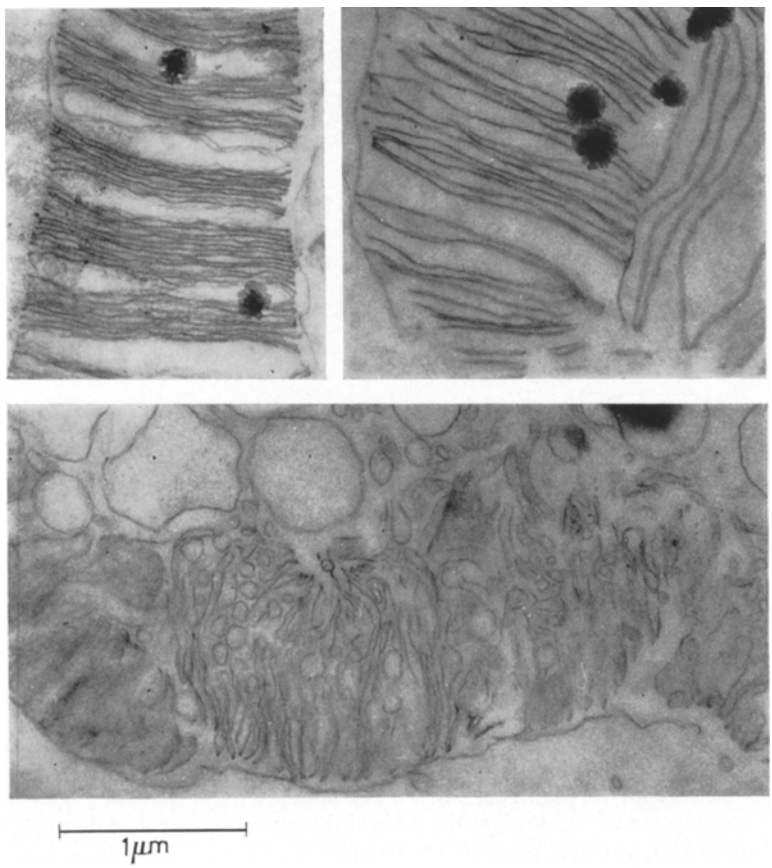

Fig. 3. Electronmicroscopic pictures by K. Goebel. ROS were fixed with osmium tetroxide, embedded and cut following the usual procedure; double staining with uranylacetate and lead-citrate. *Above:* ROS after freezing in liquid propane: the outer membrane is injured, discs are loosely stacked, somewhere dense stacked groups are found. *Below:* ROS after 5 min hyperosmotic shock with aqua dest.: some discs are no longer intact, smaller vesicles are formed, all vesicles are swollen

concentrations of the suspensions were measured by light scattering and by determination of rhodopsin. One fraction of each preparation was observed in the phase contrast microscope prior to and after the measurements. Each measurement lasted about 45 min after sacrificing the animals.

b) Bovine.

Bovine eyes were collected in a dark container, usually about 3 min after slaughtering. Sometimes the eyes were obtained immediately after slaughtering; this, however, did not change the results received.

In addition to the above, bovine ROS were purified by a sucrose flotation procedure and washed and stored in Ringer solution of the following composition (S. Stange, 1973).

NaCl 120 mM, KCl 2 mM, $CaCl_2$ 0.15 mM, $MgCl_2$ 0.1 mM, Na-phosphate-buffer (pH = 7.0) 15 mM (Osmolarity: 290 mosmol).

The usual rhodopsin content after this procedure was 4 to 5×10^{-5} M; when filling the cuvette, 100 µl (for $\lambda_m = 380$ nm) or 200 µl (for $\lambda_m = 800$ nm) were diluted in 6 ml ringer solution (cuvette Fig. 1) and in 50 ml (cuvette Fig. 2).

Some signals can also be obtained with samples which were stored in small portions of 100 or 200 µl in liquid nitrogen. The freezing of these samples was carried out by dipping the single portions in liquid propane to avoid Leidenfrost's phenomenon and thus accelerate the freezing.

The ROS we obtained appeared in the electron microscope as shown in Fig. 3.

Some samples were prepared with an osmotic shock treatment as follows: 200 µl ROS from the density gradient in isotonic ringer solution were diluted in 3 ml aqua dest.; after 5 min the same amount of double concentrated Ringer-solution was added, to reconstitute the original ionic milieu for the measurement.

6. *Signal detection and storage*

Light intensity changes were detected with an EMI 9558 QB photomultiplier (S20 characteristics). The preamplified voltage signals were filtered through a 0.5 kHz – 3 dB frequency filter into a Nicolet 1072 signal averager equipped with an SD-71B (12 bit) AD-converter. The storage of the averager can be divided in 4 groups, each containing 256 addresses. In order to detect long-time structural changes and recovery effects during the measurements of an amplitude decrease curve, overlapping groups of flashes were applied reproducing the measurements: signals evoked by the flashes of one group were averaged; the time delay between the groups was 2 min, the time between the flashes was 1 sec.

Averaging of signals belonging to one step in the decrease curve, but obtained from several samples, was sometimes used to enhance the S/N ratio.

7. The *orientation change* of the *ROS* in suspension may be too slow to reconstitute a random distribution of orientations between the flashes. An orientation effect may, therefore, be expected for the ROS containing highly ordered chromophores. This was checked by varying the time between the flashes and also by gently stirring some samples between the flashes. The results obtained were not essentially changed by these procedures.

8. *Error bars* shown in the figures correspond to the error due to noise and signal perturbations.

Results and Discussion

I. Measurements at $\lambda_m = 380$ *nm*

There are several reasons to believe that, at least in vertebrates, the meta-rhodopsin I-II reaction (MI, MII) transmits the stimulus to the surroundings of the rhodopsin molecule (Stieve, 1974). Therefore, we reinvestigated this reaction in the initial phase of bleaching with flash energies bleaching only 1% of the unbleached rhodopsin. Fig. 4 shows a typical first signal obtained at $\lambda_m = 380$ nm from bovine ROS.

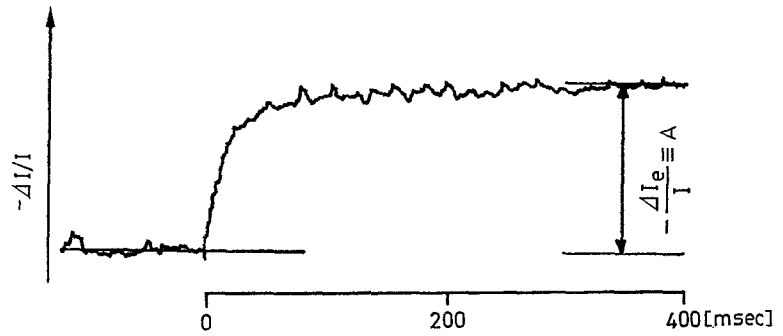

Fig. 4. Bovine ROS suspension: Total light intensity change $-\frac{\Delta I(t)}{I} \equiv A(t)$ in the measuring beam. Measuring wavelength $\lambda_m = 380$ nm ($\Delta\lambda = 9$ nm). Observation angle $\theta = 0$ ($\Delta\theta = 2°$). pH = 7.0; $T = 20°$ C. The flash was applied at the zero point of the time scale. As shown in the figure, the signal amplitude $A \equiv -\frac{\Delta I_e}{I}$ is defined as the asymptotical value ($t \to \infty$) of the light intensity change. $\frac{\Delta Ie}{I} = 2 \times 10^{-3}$; the signal corresponds to 1% rhodopsin bleaching

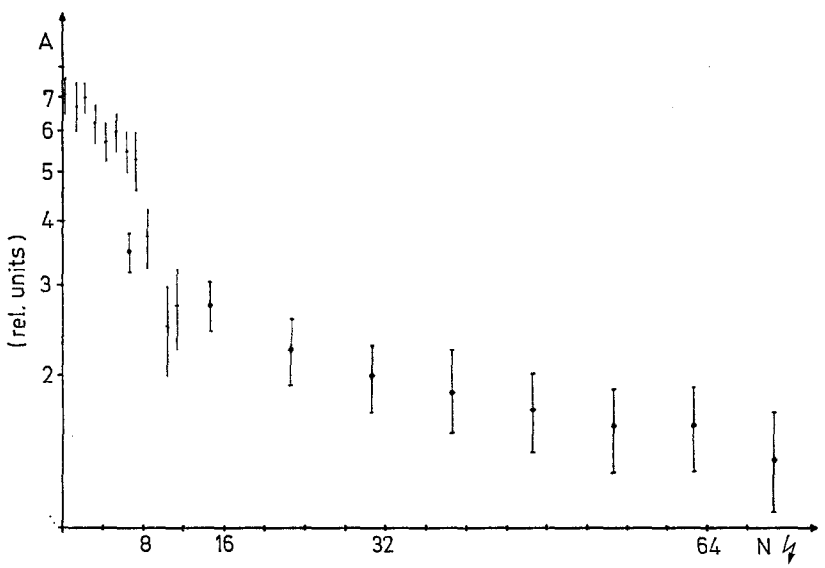

Fig. 5. ROS suspension from the dark adapted frog: Decrease of the signal amplitude A (defined in Fig. 4), measured at 380 nm, in a series of flashes. Observation angle $\theta = 0$. pH = 7.0; $T = 20°$ C. $N\lightning$: number of the exciting and simultaneously 1% rhodopsin bleaching flashes

1. The Amplitude Decrease

We applied a series of flashes on ROS suspension, each evoking a signal as shown in Fig. 4 and, at the same time, bleaching the ROS a further step. The measured intensity changes (Fig. 4) as a function of the flash number are shown in Fig. 5. This measurement was done with ROS from the dark adapted frog.

Within the first 20 flashes a deviation from the linear slope of the amplitude decrease curve is observed. As shown later, a similar decrease is observed with bovine ROS.

This effect is not dependent on time, *i.e.* on the flash groups described above. Only a small amplitude recovery effect is sometimes observed between the flash groups which does not influence the main slope of the decrease curve. We carried out the same experiments with frogs which were illuminated 2 hrs with the light of four 100 W bulbs at a distance of about 40 cm. They were then prepared immediately in the dark in the same manner as the dark adapted ones. An initial signal smaller by a factor of 6 to 10 than that seen for the dark adapted ones was then obtained. For the first 4 flashes a decrease rate smaller than 2% was observed. In the case of the light adapted frog, the decrease, in its initial phase, seems to behave similar as with the dark adapted one, superceding flash Nr.'s 15 to 20.

2. The Reason for the Nonlinear Decrease

Three mechanisms causing the observed amplitude decrease in the case of the dark adapted frog ROS can be taken into consideration:

1. In the initial phase, one quantum gives rise to the formation of more than one MII molecule.

2. There is an effect on the absorption coefficient or on the band position of MII.

3. There is a light-induced event within the ROS, generating a superimposed signal.

The first possibility, at first favoured by us (Kreutz *et al.*, 1974), is ruled out now by the following experiments with bovine ROS.

A fresh ROS-suspension is divided into 7 fractions. 0, 5, 10, 20, 50 and 100 flashes were applied to six fractions respectively. Each fraction was treated with Na-desoxycholate after flash illumination to disintegrate the disc-membranes. The concentration of rhodopsin was then determined by measuring its absorption spectrum. The rhodopsin content of the different illuminated samples was plotted against the flashes applied (Fig. 6a). The flash-induced signal of the seventh fraction is measured at 380 nm as described above (Fig. 6b).

The rhodopsin content (a) and the 380 nm-signal decrease (b) are parallel and linear for larger bleaching than 15% of the original rhodopsin. For smaller bleaching values, the deviation from the linear slope only occurs in the 380 nm amplitude decrease curve.

An absolute determination of the bleached rhodopsin content is difficult because of calibration problems. As a matter of fact, the comparison of our initial signal at 380 nm for dark and light adapted frogs yields a bleached rhodopsin content of at least 25% prior to sacrifice.

The comparison of the amplitude deviation of the 380 nm-signal from the linear slope, in the case of bovine and frog ROS, leads us to estimate that ca. 3 to 7% of the rhodopsin is bleached in our animals. We also found a seasonal dependence for this deviation, which is within this range.

Effects as mentioned in point 2 must be kept in mind, since illumination causes changes of the molecular order, especially in the rhodopsin membrane surroundings, as can be taken from ESR spin label experiments carried out in our laboratory (F. Siebert, 1975). But measurements of the difference spectrum rhodopsin-

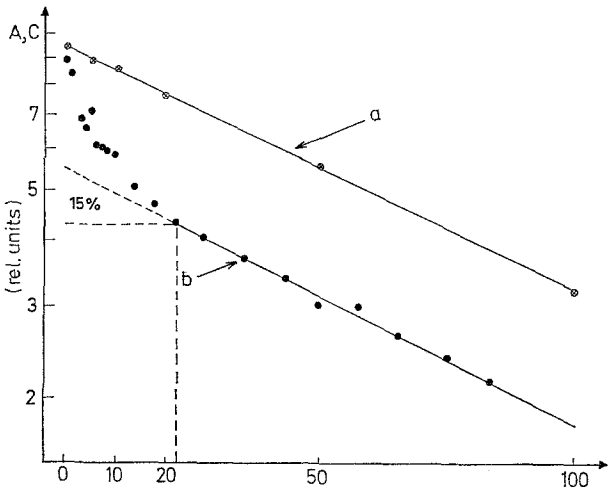

Fig. 6. Bovine ROS suspension [same preparation in a) and b)]: a) Decrease of the unbleached rhodopsin content C measured with the integral absorption of rhodopsin, b) decrease of the signal amplitude A (defined in Fig. 4), measured at 380 nm, observation angle $\theta = 0$, both in a series of flashes. pH = 7.0; $T = 20°$ C. $N\not{\iota}$: number of the 1 % rhodopsin bleaching and in the case of b) simultaneously exciting flashes

metarhodopsin II, which we performed as a function of bleaching, do not allow the assumption of such an effect.

Thus it follows, that the explanation of the amplitude deviation due to superimposed signals, as suggested in point 3, is plausible. The following consideration of the signal time course as a function of bleaching fits well to this supposition.

3. The Time Course as a Function of Bleaching

In Fig. 7, an evaluation in the logarithmic scale is made for the signals in the bleaching series. The signal turns out to be of higher order in a range embracing about the first 20 % of rhodopsin bleaching.

The admixture of slower kinetics (40 to 70 msec) decreases parallel to the approximation of the signal amplitudes to the linear amplitude decrease curve. The amplitude curve is not only caused by this admixture but the additional amplitudes also consist of kinetics with about 15 msec and of slower kinetics. The contradiction between earlier data (for a summary see Abrahamson [6]) is perhaps solved by this measurement. In order to come closer to an understanding of the described effects their physical character should be clarified first. Is there another chemical product appearing only in the initial phase of bleaching and thus a superimposed absorption change, or is there a structural change causing a change in scattered light or are both possibilities realized as a consequence of the same event?

The above mentioned difference spectra as a function of bleaching cannot, as yet, be fully interpreted to gain a clear answer. Measurements of the amplitude deviation with variation of the parallelity of the measuring beam (Fig. 1) seem to

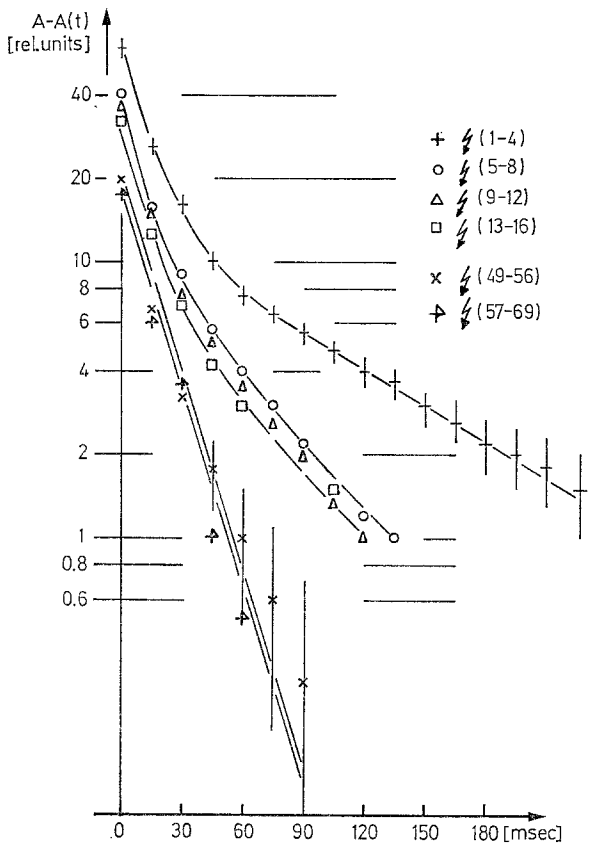

Fig. 7. Bovine ROS suspension: Time course analysis of signals $A(t)$, measured at 380 nm. Observation angle $\theta = 0$. Parameter: illumination, *i.e.* flash number N in a series of flashes. $\lightning(N_i - N_j)$ denotes an averaged signal, evoked by a group of flashes. For definition of the ordinate $A - A(t)$ and the abszissa (time after flash), see Fig. 4. pH = 7.0; $T = 20°$ C

show that the effect is unchanged with variation of $\Delta\theta$ ($2° \leq \Delta\theta \leq 10°$). The problem arises in any case, that the interesting effect is only superimposed on a larger basic absorption change. However, we found signals on the red end of the visible spectrum whose amplitude decrease can be correlated to the observations described up to now.

II. Measurements at $\lambda_m = 700$ to 800 nm

In the 700 to 800 nm range of the spectrum, no indication of a chromophoric absorption could be found with our bovine ROS. Near infrared overtones from molecular vibrations, whose fundamentals are situated in the infrared (4000 to 700 cm^{-1}), are very weak in this range. Therefore, the whole intensity change measured within a certain angle of detection $\Delta\theta$ and under an observation angle θ (cf. Fig. 2) is expected to be due to light scattered in this direction. Fig. 8 shows how such a change might be imagined as caused by a transition of the ROS from one state into another.

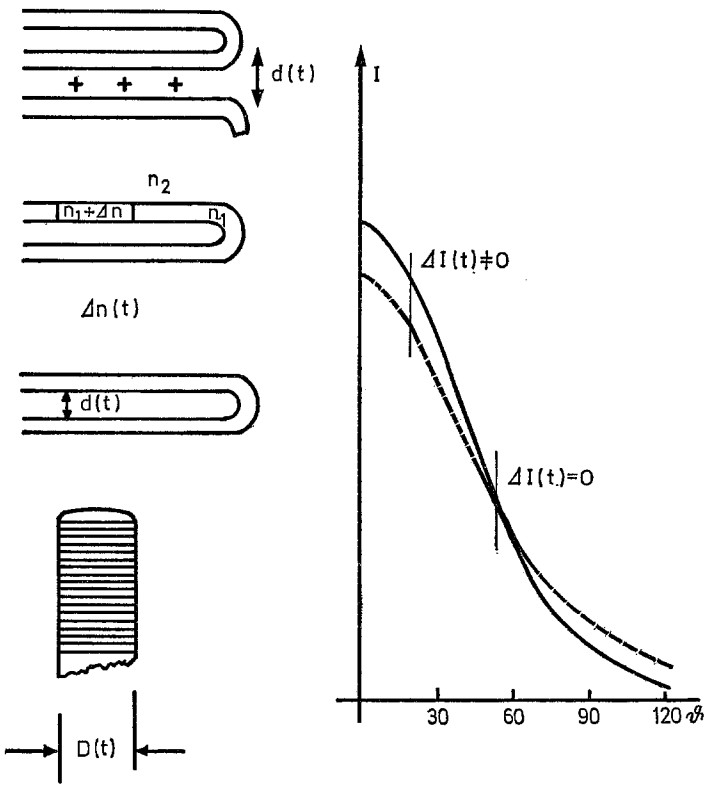

Fig. 8. *Left:* Schematic picture of some morphological and structural changes within the ROS which are able to cause changes in scattered light. *Right:* The static light scattering curve $I(\theta)$ (solid line) corresponds to the state of the system prior to light stimulation; after changes as shown on the left the scattering curve may be represented by the dotted line. Positive and negative light intensity changes $\Delta I(t)$ are observed, dependent on the observation angle θ

All the measurements discussed in this chapter were obtained from bovine ROS, easily available in large amounts. With frog ROS, something like the large signal P (see below) was observed as well. For the frog, further effects were not investigated. There are perhaps interesting differences due to the different morphology of the frog disc.

In order to get sufficient scattered light intensity, we had to put up with concentrations of our suspensions where single scattering no longer exists. $\theta = 0$ transmission of the measuring beam with the device shown in Fig. 2 is about 30%. Therefore, an interpretation of our experiments in structural terms will be even more difficult as in the case of static light scattering experiments. Complementary experiments with high transmission and small angles θ are in progress.

1. General Observations

The observed intensity change (apparent absorbance change) $-\Delta I(t)/I$ at 800 nm is shown in Fig. 9. Starting from a large positive value at flash 1, the signal decreases to zero in only 17 flashes. It goes to a smaller negative maximum at flash numbers 30 to 40. At first glance this behaviour seems rather strange, but there is a simple explanation: one positive (P) and one negative (N) signal may be caused by independent light induced events decreasing with different rates.

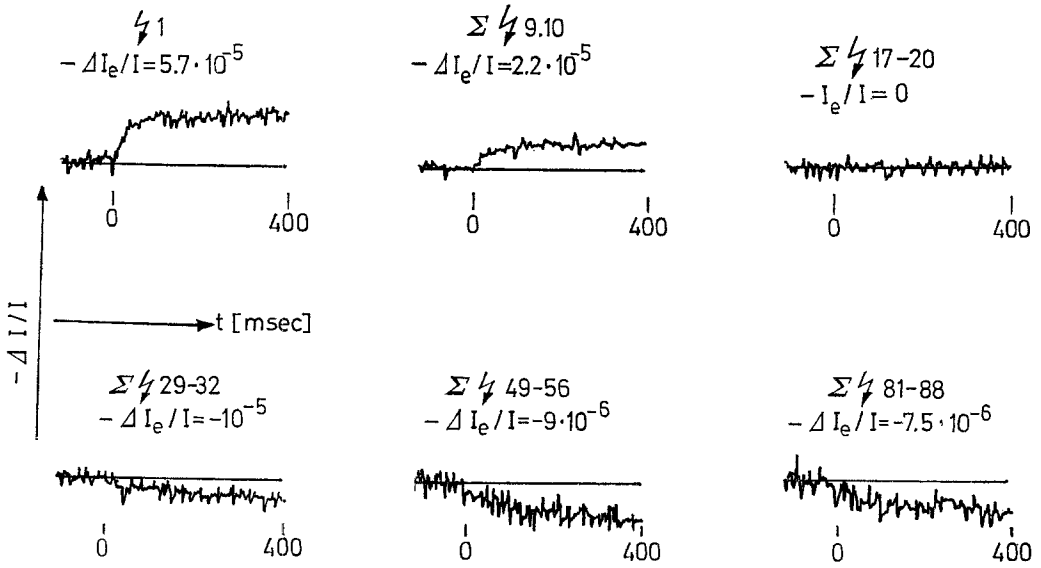

Fig. 9. Bovine ROS suspension: Total light intensity change $-\frac{\Delta I(t)}{I} \equiv A(t)$ in the measuring beam. Measuring wavelength $\lambda_m = 800$ nm ($\Delta\lambda = 25$ nm). Observation angle $\theta = 0$ ($\Delta\theta = 2°$). pH = 7.0; $T = 20°$ C. The flash was applied at the zero point of the time scale. Amplitudes are defined as in Fig. 4 and refer to a single flash bleaching 1 % rhodopsin. $\Sigma\lightning N_i - N_j$ denotes an averaged signal, evoked by a group of flashes

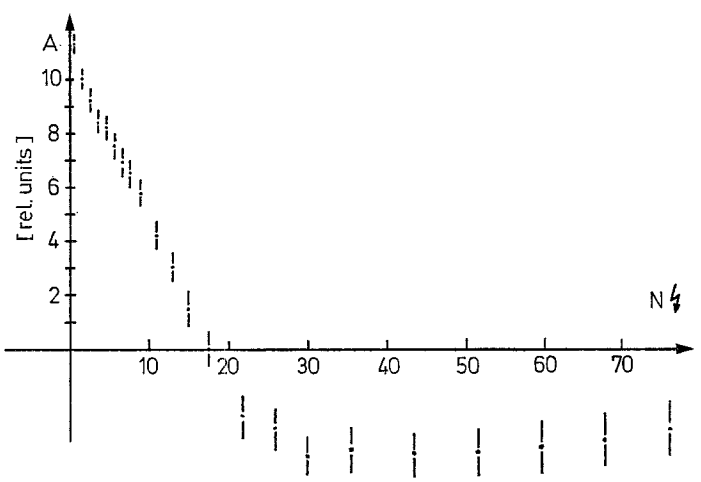

Fig. 10. Bovine ROS suspension: Course of the signal amplitude A (defined in Fig. 4 and Fig. 9), measured at 800 nm, in a series of flashes. Observation angle $\theta = 0$ ($\Delta\theta = 2°$). pH = 7.0; $T = 20°$ C. $N\lightning$: number of the exciting and simultaneously 1 % rhodopsin bleaching flashes

The intensity change $\Delta I(t)$ asymptotically approaches a new equilibrium state $I + \Delta I$ after about 200 msec (at 20° C). The signal amplitudes $\left(-\frac{\Delta I}{I}\right)$ are shown as a function of the flash number in Fig. 10. Since the signals P and N are different

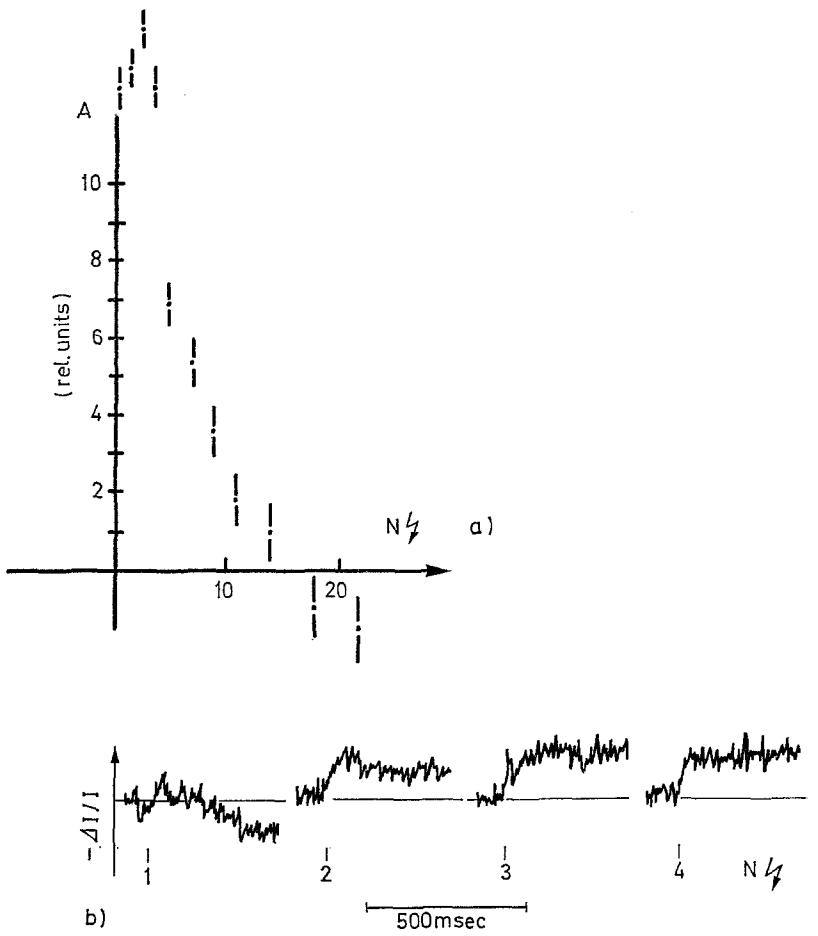

Fig. 11. Bovine ROS suspension (very fresh): a) Course of the signal amplitude A (defined in Fig. 4 and Fig. 9), measured at 800 nm, in a series of flashes. b) The first 4 signals from the same suspension, but diluted by a factor of 5; detection is sufficient to recognize that the first amplitude is negative. Observation angle $\theta = 0$ ($\Delta\theta = 2°$). pH = 7.0; $T = 20°$ C

in amplitude and decrease rate, they can be separated. The decrease rate can be followed up separately in ranges where the respective signals predominate.

As far as can be detected, N decreases parallel to the 380-signal for larger flash-numbers where the latter represents the MII production. Decrease rates of P and N differ by a factor of 10 to 20. For the best preparations, where the outer membrane of the ROS appears intact in the electron microscopic picture, there arises another signal in addition to P and N which disappears already with the first 3 flashes. It supercedes the amplitude P for very dilute suspensions, as shown in Fig. 11a, but it is also observed for our usual concentrations (Fig. 11b). This slow initial signal will be denoted by the symbol N_i.

To obtain more knowledge about the processes within the ROS causing the observed signals, at least three different possibilities exist:

1. Variation of the ionic milieu, as it is suggested that ionic effects could be the origin of the observed signals.

2. Stepwise destruction of the outer membrane and the discs, in the hope of finding a connection between these organelles and the obtained effects.

3. Variation of physical parameters like θ, λ_m, and concentration of the suspension to get a better separation of the effects, and thus elucidate the physical underlying effects (absorption-, scattering-, turbidity-change). Naturally, points 1 to 3 are dependent on each other. For example, by variation of the ionic milieu, structures can be destroyed. Concerning point 1, mainly the variation of Ca^{2+}, Na^+ and H^+ is of interest.

Results obtained with variations as described in point 2 do not suffice for an anambiguous interpretation, as far as carried out by us up to now. We treated the ROS with osmotic shock as described in the experimental procedure.

The variation of physical parameters (point 3) offers the advantage that no influence on the sample is to be taken into account, except the self-destruction of the ROS. We measured the θ-dependence of the amplitude and time course of the component P. Single frozen samples from the same preparation were thawed immediately prior to the measurement.

2. The scattering signals

The small signal N. This signal can be described as follows:

1. Its amplitude decreases in the flash series like the MII-signal for flash numbers larger than 60, where N is separated.

2. The range of τ is 20 to 40 msec; the signal is detected with S/N ratio of only about 5 (Fig. 9) for a group of 8 flashes. Therefore, the kinetics cannot be measured exactly.

3. N is not influenced by osmotic shock treatment.

4. N is negative for observation angles 0° to 3°; at about $\theta = 3°$, N is zero and becomes positive for larger observation angles. At $\theta = 45°$, N is no longer detectable.

The signal N seems to be caused by structural changes within the disc membrane connected directly with the MI→ MII transition. This is suggested by its change in sign at an angle θ very near to $\theta = 0$ (point 4) which corresponds to a turbidity change. Not only its comparability in amplitude decrease with MII, but also its resistance against osmotic shock treatment suggests that it is caused by membrane-inherent processes not influenced by the inter-discal hydrous milieu.

There seems to be a connection with the birefringence work done by Liebman *et al.* (1974). These authors excluded any scattering effect, but our experimental conditions are very different in the case where N is observed.

McConnell (1975) measured the increase and/or decrease of the integral scattered light with illumination extended over minutes. Adding up our signals, the integral scattering effect as a function of illumination cannot be connected with the results of McConnell; however, we also observed superimposed long-time scattering effects as a function of the total illumination caused by our flashes.

The initial negative signal N_i. The features of this signal are:

1. A high percentage of the ROS outer membrane must be intact in order to obtain this signal as suggested by electron microscopic pictures.

2. It is negative in sign and observed only for the first flash; any appearance with the second flash is believed to be an artefact caused by the above discussed orientation effect.

3. The half mean time is about 200 msec; the order of the time course is not detectable.

The conditions necessary for the signal to occur suggest that there is an effect which causes a swelling of the whole ROS. According to the observations of Korenbrot and Cone (1972), the outer membrane must be expected to reduce its sodium permeability to zero with only one flash of the intensity applied. The 200 msec time delay is in an order of magnitude which is expected for diffusion processes of this kind.

A still existing current through the outer membrane, as discussed for isolated ROS, too (Zuckerman, 1973), is very improbable in our preparations. Diffusion of osmotically active solutes from the disc into the cytoplasma, and at the same time, only with the first flash, blocking of permeation of equilibrating agents through the outer membrane, should be a sufficient condition. Since there are up to 70 msec kinetics for the P-signal, possibly caused by the effect of such solutes onto the disc-thickness (see below), the simple addition of a diffusion through the cytoplasma could in any case lead to a 200 msec process as observed via the swelling of the whole ROS.

The rapidly bleaching signal P. The main features are:

1. The rate of decrease is higher by a factor 10 to 20 than that of the MII signal, depending on preparation (for instance, seasonal dependence); it is, however, identical for one and the same preparation.

2. For $\theta = 0$ a mixture of positive 15 to 20 and 40 to 70 msec kinetic components is observed; the kinetics are separable by variation of the observation angle (see point 6).

3. P is influenced by osmotic shock treatment as follows:

a) Osmotic shock with aqua dest. for 5 min and succeeding measurement in a very diluted ionic milieu: P is no longer detected.

b) Osmotic shock as above and reconstitution of the isotonic Ringer milieu: P is again detected with the same amplitude but slowed down by a factor of about 2.

c) Osmotic shock and reconstitution of a double hypotonic Ringer of the same composition: P is increased by a factor of 1.5.

4. P is not influenced by freezing of the ROS. In these preparations most of the discs are still stacked, but with larger and more variable interdisc spaces as seen in the electron microscopic picture (Fig. 3).

5. In isotonic sucrose the total light scattering of the ROS reduces strongly. P decreases drastically in amplitude to about 10% of the value which is reached in isotonic Ringer solution. However, if the sucrose solution contains more than 3 mM and for more than 30 mM NaCl, total light scattering and signal P are unchanged with respect to the isotonic Ringer solution.

6. With increasing Ca^{2+} concentration P decreases to minimally 30% of its original value.

7. The θ-dependence of the kinetic components, constituting P, is given in Fig. 12. The faster and slower components are not very well detected, but their

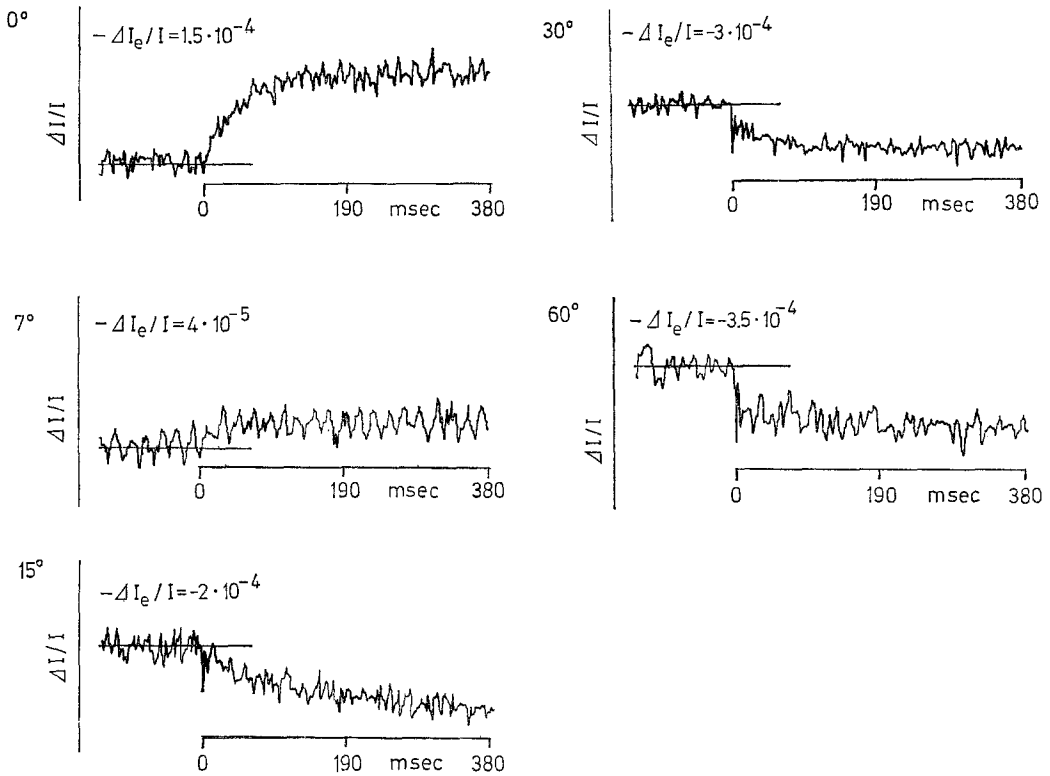

Fig. 12. Bovine ROS suspension: Total light intensity change $-\frac{\Delta I(t)}{I} \equiv A(t)$ as a function of the observation angle θ in the initial phase of bleaching. Measuring wavelength $\lambda_m = 800$ nm ($\Delta\lambda = 25$ nm). The measuring device is shown in Fig. 2. Transmission at $\theta = 0$: 30 %; $\Delta\theta = 2°$. pH = 7.0; $T = 20°$ C. A slower (40 to 70 msec) and a faster (15 to 20 msec) kinetic component may be distinguished, both changing sign at about 7°. For large angles, the faster component predominates more and more

different behaviour with variation of θ is distinguished. For angles $\theta > 15°$, both components are negative.

As follows from point 4, P is neither influenced by destruction of the outer membrane nor by merely breaking up of the regular stacking of the discs. Therefore, it can at least be deduced that an effect especially on the discs underlies the signal P. The sign of P would agree with the disc shrinking effect measured by Chabre and Cavaggioni (1975).

It has, however, not been proved that the effects causing the signal P are the disc shrinkage or, at least, that they are the shrinkage alone. As yet we are quite generally not able to determine to which extent disc-form changes and/or changes in the effective mass and refraction index enter into the difference scattering spectrum of $P(\Delta I/I$ as a function of $\theta)$.

This information is additionally needed for an unambiguous interpretation of the osmotic shock experiments as well. Nevertheless, regarding the findings

described in point 3, the following statements can be made: With increasing ionic strength of the reconstituted Ringer, P is initially increased (3c), but it is zero for very low ionic strength (3a). Thus, a more complicated dependence of the signal producing disc features on large variations of the ionic strength is to be deduced, than would correspond to a pure osmolarity behaviour of the unchanged membrane.

A basis for an understanding of the latter is given by Heller *et al.* (1971) and by Cohen (1971).

The intricate occurrences during variation of the ionic strength may be due to an influence on the discshape, *e.g.* via ions on the disc-cytoplasmic edge, or to direct influence on a signal-producing process. This cannot yet be determined.

However, the slowing down of the whole signal (3b) favours the possibility that an ionic process is one of the events responsible for the signal, since such a pronounced influence on the signal time course can be imagined most easily via diffusion processes.

Signal P consists of two kinetics; because of their different θ-dependences it is suggested that there are two independent changes on structures of different dimensions. The common decrease behaviour of the two components (expressed by their common name P) shows, however, that there is one basic event causing both changes. Because of the decrease rate, it cannot be the metarhodopsin I$\rightarrow$ II transition itself, but must be some ensuing process triggered by the decay of rhodopsin.

The high rate of the amplitude decrease represents a hint that the suggested structural changes could be involved in the regulating and transmitting mechanism of the ROS.

As far as the concrete mechanism which causes the shrinkage and/or related effects on the disc is concerned, the transmitter release could act as an agent influencing the disc thickness osmotically, via effects of the ionic electrical field or effects on the hydration shell of the molecules constituting the membrane. In this case, the ratio of transmitter molecules per rhodopsin bleached would be determined by the ratio of the decrease rates of P and MII.

A comparison of the amplitude deviation effect, measured at 380 nm, and of the signal P with respect to their kinetics and amplitude decrease reveals a parallel behaviour. There should be, therefore, at least one and the same origin for both effects.

More evidence must certainly be collected befor it can be ascertained that the high observed decrease rates of our signals are not merely the product of some physiologically irrelevant structural breakdown. However, their reproducibility, their sole dependence on light and a certain inherent order therein, let us hope that these and further similar studies are of physiological significance.

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