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Comparative Kinetic Light-Scattering and -Absorption Photometry

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Abstract. A recent development of kinetic light-scattering and -absorption photometry is described.

Essential points are:

1) In the scattering experiment, amplitude resolution of $2 \cdot 10^{-5}$ (single flash) by application of a differential detector, stability of the same magnitude due to optical compensation, high intensity at the detector due to special optics for the scattered light and semiconductor sources.

2) In the absorption measurement, elimination of scattering contributions by the dual wavelength-method and by high aperture optics.

3) Simultaneous measurement of absorption and scattering. The application of the method is described in using signals from isolated bovine rod outer segments. A reliable procedure is described by the use of which the originally measured light-scattering effects can be split up into single signals.

The method allows comparative kinetic analysis of absorption and scattering signals. The possible causal connections between pigment and membrane structure processes can be selected.

Key words: Light-scattering – Flash photometry – Kinetics – Visual transduction – Biomembranes

Introduction

Membrane-bound processes play an important role in the present considerations on visual transduction (e.g., Liebman and Pugh 1979). There are mainly two arguments to claim such processes, possibly in addition to that of the widely accepted concept of a cytoplasmic transmitter:

1) The electrical response, terminating the transduction chain in rods, reflects in its kinetics more than one rate determining process (Penn and Hagins

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1972). None of these can be attributed to transmitter diffusion since quantum responses are uniform in size and kinetics (Baylor et al. 1979).

2) An amplification of $2-3 \cdot 10^5$ is achieved in the dark adapted rod receptor. From a comparison with technical amplifying devices it is suggested that such high degrees of amplification are realized best by several stages of moderate amplification.

Membrane-bound processes can be indicated by changes in the structure of the membranes.

Light-induced changes of the physical state of the disc have been investigated by a number of authors (Falk and Fatt 1968, 1973a, b; Mason et al. 1974; McConnell 1975; Asai et al. 1975, 1977; Norisuye and Yu 1977; Wey and Cone 1978; Harary et al. 1978). In some of our investigations, we described fast light-scattering effects within the rod receptor (Kreutz et al. 1974; Hofmann et al. 1976). An essential part of further work has been dedicated to the so-called P-signal. This effect was shown to be due to a size or shape effect of the disk vesicles (Uhl et al. 1977).

The amplitude of the P-signal shows a characteristic dependence on the amount of bleached pigment. The saturation of the P-amplitude with light intensity has also been observed similarlily in the admittance changes studied by Fatt and Falk (1977). It is remarkable that this relation resembles the Rushton-Dowling equation of dark adaptation. Therefore, the P-signal seemed to be worth a more extended investigation.

To perform these investigations it was necessary to develop specially a flash photometric method, which allows the sensitive measurement of scattering signals and a simultaneous recording of absorption changes.

The method presented in this paper is mainly addressed to the study of rod outer segment suspensions but it may also serve for investigating kinetic light-scattering problems in general. The simultaneous measurement of absorption changes will not be required for all applications.

General Considerations About Measurement of Kinetic Light-Scattering

Kinetic light-scattering photometry is an instationary relaxation method. The reader is referred to general descriptions of such methods (e.g., Rüppel 1978; Eigen and de Mayer 1973). If a flash is used as a source for exciting scattering signals, the method can be regarded as being derived from flash photometry which was described first by Norrish and Porter (1949; see: Porter 1963).

In Fig. 1, the light-scattering flash method is explained using the analogy between the photochemical state of a system – represented by its absorption spectrum – and its structural state – represented by its scattering curve. Transitions between different states are indicated in both cases by changes in the monitoring light intensity. By measuring the transitions for a complete set of values of the monitoring wavelengths resp. scattering angles, photochemical resp. structural information is obtained. In analogy to difference spectra, difference scattering curves can be defined.

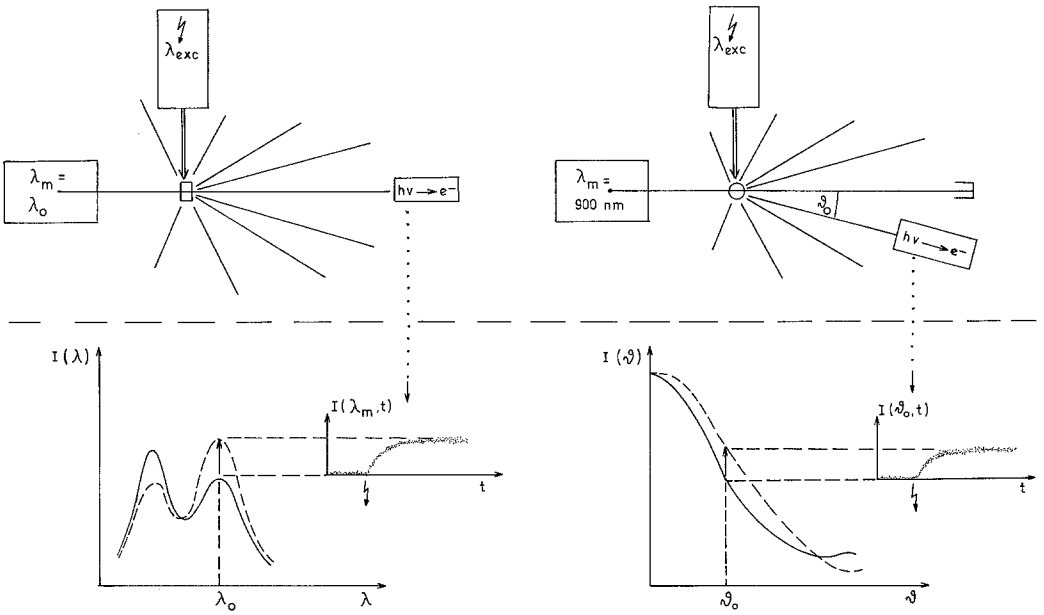

Fig. 1. Comparison of kinetic photometry of absorption (left) and scattering (right). In the upper part of the figure, the measuring arrangements are schematically drawn. In the lower part, spectrum (wavelength-dependence of absorption) and scattering curve are counterposed to each other in analogy. In both cases, an actinic source (e.g., a flash) induces a transition between two chemical resp. structural states which is observed by a change in absorption resp. scattering. In analogy to the difference spectrum (all transitions in a certain range of the wavelength), we define a difference scattering curve. While the measuring wavelength of scattering excludes a contribution of absorption changes, the absorption measurement contains, in general, superimposed scattering contributions. The device shown in the next figure avoids this problem to a high degree

The advantage of the kinetical method may be seen in that both functional and structural pieces of information are obtained. Moreover, for complex biological structures it is not possible to give a general interpretation of light-scattering. In a differential scattering method, however, only these structure characteristics are selected which are labelled by the kinetic parameters of the transition induced by excitation. In favourable cases, the interpretation problems can thus be considerably reduced. As in all differential methods, long term fluctuations are eliminated.

The Measuring Device

The device was developed on the basis of a preceding apparatus described before by Hofmann et al. (1976). Its optical setup is shown in Fig. 2. The apparatus consists of two fast photometers. A flash induces a light-reaction within the organelles of the suspension. Two different kinds of induced processes can be measured simultaneously:

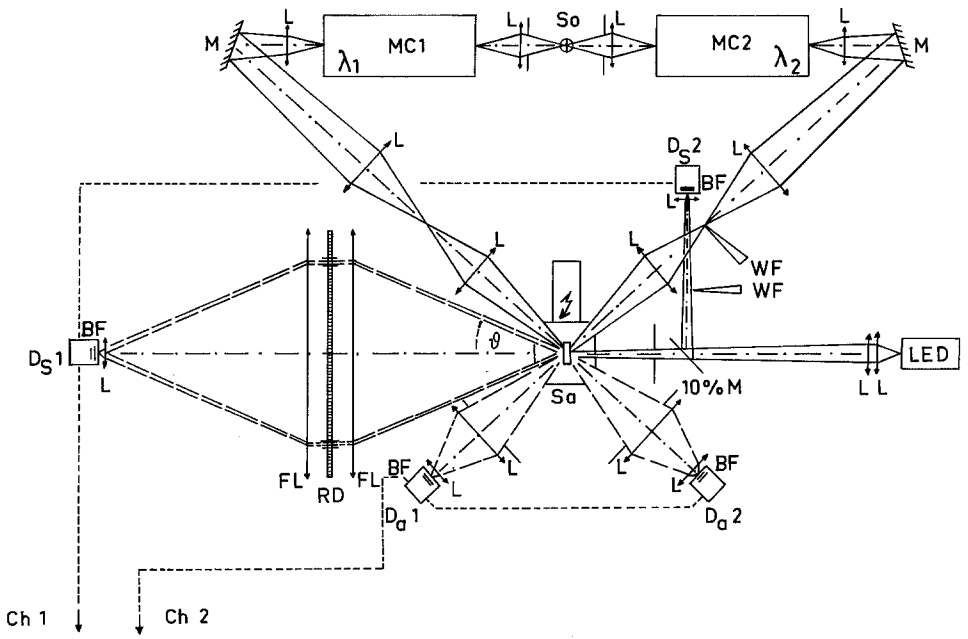

Fig. 2. Optics for the simultaneous time-resolved measurement of pure light-absorption and -scattering changes. The properties and limitations are described in the text. Symbols are: So: light-source for the absorption beams (Halogenium or Xenon high pressure); MC 1: monochromator (Jobin Yvon H 20 UV); MC 2: monochromator (Jobin Yvon H 20 IR); L: lenses; M: mirrors; Sa: sample; ϟ: flash (EG & G, FX 133); WF: wedge filters; D_{a1} and D_{a2}: differential detector for the absorption measurement; LED: semiconductor light source for 950 ± 20 nm (Siemens LD 271); 10% M: mirror reflecting 10% of the incident intensity; FL: Fresnel lenses (Melles-Griot, Arnhem, Holland); RD: ring diaphragms; D_{s1} and D_{s2}: differential detector for the scattering measurement; Ch1/Ch2: Output, separate storage and data manipulation; BF: Blocking filters, for example: flash: Interference broad band $450 < \lambda < 550$ nm (Hugo Anders KG, Diendorf), BG 18, OG 530 (Schott, Mainz); Scattering beam: RG 850 (Schott, Mainz); $\lambda_1 = 387$ nm: UG 5, KG 3 (Schott, Mainz); $\lambda_2 = 417$ nm: 03 SWP 011, 03 SWP 013, 03 SWP 017 (Melles-Griot, Arnhem, Holland), KG 3 (Schott, Mainz)

1. The first given by chemical changes in the system, e.g., production of rhodopsin intermediates, changes of pH, or ionic changes which are indicated by absorption changes (detectors $D_{a1,\,2}$).

2. The second one given by structural changes, e.g., in the optical contrast of the membranes (phase transitions, surface charge changes) or in the form and/or size of the organelles. These are indicated by changes in the scattered near infrared light (detectors $D_{s1,\,2}$).

The scattered light is focussed with the large Fresnel lenses FL. The measurement of both absorption and scattering in two separate double-beam arrangements enhances stability and resolution. Some special features will be discussed in the following.

1. Optical Compensation

The optical compensation considerably reduces the influence of measuring intensity deviations, since they are added to the intensity changes as relative, but not as absolute values. Zeroadjustment is made by neutral wedge filters, automatically positioned just before the measurement takes place. Different operation principles are applied in absorption and scattering:

a) Absorption. In the conventional one-beam device the elimination of the scattering influence on the absorption measurement is usually performed by a separate, subsequent measurement at an isosbestic point of the absorption spectrum or by suppressing one of the effects at the original wavelength (e.g., buffering in the case of alkalisation signals). This procedure mostly yields unsatisfying results because the reference scattering is usually not identical.

Adjusting the wavelength of the absorption beam λ_1 to the peak of the expected absorption change and of the reference beam λ_2 to the isosbestic point or another appropriate wavelength reduces the scattering contribution; the remaining effect is caused by a difference in scattering at wavelengths λ_1 and λ_2 which, in general, is small. This difference is further reduced by focussing the cuvette onto the detector with an aperture angle as large as possible. For this purpose a large Fresnel lense L is used to focus the scattered light. The absorption changes are fed into channel 2 of the signal analyser.

b) Scattering. Scattering changes are measured simultaneously with the scattering optics which is also shown in Fig. 2.

In rod outer segment suspensions, the scattering measurement at 950 nm is certainly free of absorption changes. The reference beam does not pass through the sample and serves only for the reduction of light source intensity deviations as mentioned above. Scattering changes appear in channel 1 of the signal analyser.

2. Detection of the Equivalent Angles of Scattering

For an isotropic sample, the scattering information has a rotational symmetry around the axis of incident light. This property is used in focussing the sample by its scattered light on the detector D_{s1} via the two Fresnel lenses FL.

The scattering angle is selected by a ring diaphragm system. This arrangement leads to an enhancement of the effective measuring intensity by a factor of 10–50 – depending on the angle resolution –, compared with an arrangement where the detector is located at a fixed position on the ring.

3. Application of a Semiconductor Differential Detector

For scattering measurements, an amplitude resolution equivalent to an intensity change $\Delta I/I = 2 \cdot 10^{-5}$ (single flash) and a stability over a 5-min period of the

same magnitude is achieved. The detector is described in detail in a previous paper (Hofmann and Emeis 1979).

4. Application of Semiconductor Light Sources in the Scattering Beam

In general, efficiency-selected light emitting diodes (for example Siemens LD 271) are used. The advantage of the semiconductor lies in the high luminous density; this allows high intensity and at the same time a small aperture, which is necessary in scattering.

Laser diodes could not be used for suspensions: they produced fluctuations probably due to a quasi-elastic scattering effect of cells, which undergo Brownian movement.

5. Storage, Signal Analysis and Evaluation

The signal analyser TN 1500 (Tracor Northern, Middleton, Wisconsin) allows the recording of the absorption and scattering signals in separate memories.

The main advantage of this equipment is the "scratchpad memory": Data manipulation is possible without loss of the original data. 2k data points are used for the representation of each signal. This is essential to perform an expansion of the abscissa which is necessary for detailed kinetic analysis. The signals can be compared on continuously expanded and logarithmic scales. Model fits of the signals are performed with a separate computer.

Performance of Measurement

1. Flash

The flash intensity is controlled and separately recorded so that it can be used for a correction of the signals. The essential parameter for correcting the signals is given by the amount of pigment bleached by the flash; the bleaching rate is directly determined by exhaustion curves as described below.

The excitation of the sample is homogeneous at the concentrations used; with $c_{Rhod} = 5 \cdot 10^{-6}$, only 20% of the flash intensity are absorbed at 500 nm, reflecting mirrors behind the cuvette are additionally used.

2. The Effective Noise Level

Only with careful mechanical, thermal and electrical insulation of the apparatus, the amplitude resolution which is limited by the detector noise can be achieved. In most cases, no electronic filters are used in addition to that given by the apparative time-constant in the detector circuit of 50 μs.

3. *Sample Concentration*

For the interpretation of scattering curves, it must be presupposed that most of the quanta are scattered only once when passing through the sample. Multiple scattering results in an angle-dependent convolution of the scattering curve with itself; therefore, calculation of the true curve is very complicated.

There is analogy to Beer's law for the concentration dependence of total light-scattering. For a transmission of 70%, the deviation from linearity between total scattering and concentration is: $-\ln 0.7-(1-0.7) = 0.06$ or 20% of the total scattered intensity. The experimental curve in Fig. 3 verifies this also for our suspensions. These conditions are acceptable for the scattering measurement but they impose a strong constriction to the simultaneous measurement of absorption changes: a rhodopsin concentration of $4 \cdot 10^{-7}$ mol/l corresponds to the given 80% transmission, far from ideal conditions for the measurement of absorption changes. This concentration is therefore only used for measurements of the angular dependence of a signal. The concentration for simultaneous measurement of scattering and absorption is chosen ten times higher.

With these high concentrations, the effective scattering difference goes through zero at a scattering angle of about 10°–15° (Uhl et al. 1977). The zero point is produced by the influence of multiple scattering and will be explained theoretically in a subsequent study. The measuring example in the next section (Fig. 5) will be performed in the range $6° < \theta < 13°$, where, by experience, a good signal to noise ratio is achieved. No separation of scattering signals with respect to their angular dependence can be performed under these conditions. The following evaluation of the signals will only use their dependence on the excitation light intensity.

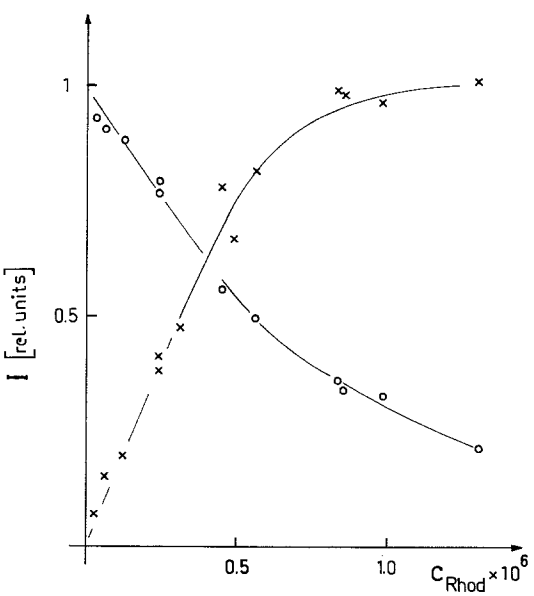

Fig. 3. Light-scattering of rod outer segment suspension as a function of the rhodopsin concentration. Up to a concentration of ca. $c_{Rhod} = 3 \cdot 10^{-7}$ mol/l, the intensity in the primary beam decreases (O) and the scattered intensity at $6° < \theta < 13°$ (×) increases linearily. The onset of multiple scattering is marked by a deviation from the linear slope

4. Signal Evaluation

a) Signal Composition. The whole time course of the intensity change observed at the detector will be called integral scattering (resp. absorption) effect. As a quantitative measure, the apparent absorption change $A\ (t)/= -\Delta I(t)/I$ will be used in this study. In general, an integral effect turns out to be a sum of different superimposed signals. This means an equation

$$A\ (t,\ P_1\ \ldots P_n) = \sum_i S_i\ (t,\ P_1\ \ldots\ P_n)$$

for all values of any parameter P_i. If a S_i cannot be splitted further into a sum, it is understood to be an uniform signal. It is important to separate carefully the different uniform signals from each other. Only uniform signals can be ascribed to uniform processes. An interpretation of an integral effect which can be splitted into a complicated sum of quite different signals, as one uniform phenomenon (Asai et al. 1977) is most probably not correct.

In the case of absorption changes, a measured effect is usually assumed to certainly reflect only one uniform phenomenon if it can be approximated by first order kinetics. For scattering changes, the situation is more complicated, if saturating and s-shaped signals, such as the P-signal, are concerned. In both cases, however, two superimposed signals can be separated by any parameter from which both signals have a different dependence.

b) Signal Separation. (1) Absorption Changes. As shown in Fig. 4, such a separation criterion is provided by the exhaustion of a signal in a series of flashes: the curve 4a appears to consist of two components where the initial part at low flash numbers can be understood as a superposition of an additional effect to the normal logarithmic decrease of the absorption change in the final part.

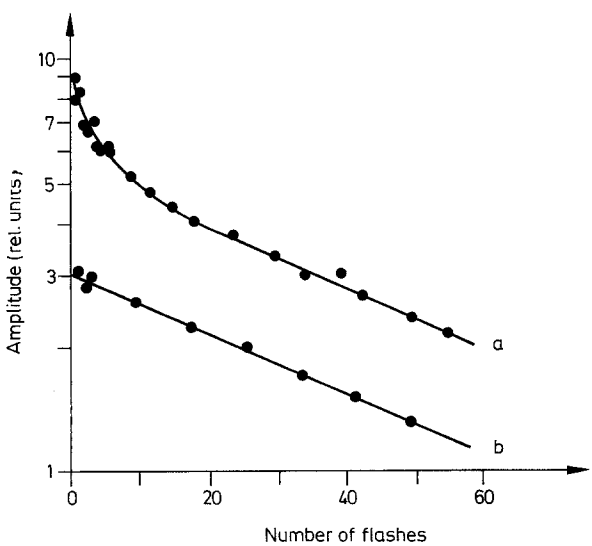

Fig. 4. Successive decrease of photometric changes, induced by 1.1% bleaching flashes (exhaustion curve). a) at 380 nm in an one-beam apparatus (Hofmann et al. 1976); b) as the 386/417 nm difference in the double-beam apparatus. Measuring parameters as in Fig. 5

The superposition is exactly eliminated (Fig. 4b) when measured with the special photometer shown in Fig. 2 using the dual wavelength technique.

(2) Scattering Changes. Scattering effects in the range of seconds (Reichert and Hofmann, unpublished) are easily separated from the ms-effects by their time course. If the rise of a slow effect contributes more than $5 \cdot 10^{-3}$ to the amplitude in the ms-effect range, the slow effect is subtracted as a straight line with the appropriate slope.

The splitting of the integral ms-scattering into signals with different dependence on bleaching is performed in analogy to the procedure described for absorption changes. In this case, too, the exhaustion curve consists of the two components observed already in absorption: In the first, the integral scattering effect is measured in a number of single flash recordings. Here and also in the subsequent flash series, the relative rhodopsin turnover per flash is about one per cent. In this range of bleaching ($\lesssim 10\%$), the integral effect is rapidly decreasing from one flash to the next for the first recordings and goes through zero and a minimum with increasing flash number (Hofmann et al. 1976).

For high flash numbers, a small negative effect is recorded as averaged effect of groups of flashes.

The kinetics between two arbitrary scattering recordings in the linear part of the exhaustion curve are congruent with each other. It is therefore assumed that the same effect is also buried in the integral scattering effect measured in the initial part. The exhaustion curve is extrapolated to flash number one and the so-called P-effect is obtained by a point by point-subtraction of the extrapolated effect in the linear part from the integral scattering effect in the initial part of the signal exhaustion.

c) Identification of Signals. In this last section, we will discuss whether the signals, which were separated by their exhaustion, are really "uniform" and can therefore be attributed to real processes. These considerations lead over to special rhodopsin problems and to subsequent special studies on the scattering signals from rod outer segments.

(1) Concerning the *387/417 nm-difference effect* shown in Figs. 5d–f, the following can be said:

All recordings of the exhaustion in Fig. 4b are kinetically congruent; this is suggested by the zero difference (5f) between the effect of flash number 1 (5d) and the average of all flashes (5e). Therefore, only scattering effects whose exhaustion curve is congruent to that of the metarhodopsin transition could be superimposed, but under the conditions of the measurement, such scattering effects are much smaller than the P-effect, which is not observed at all. It follows that no scattering contributions are present.

Since it is further known that in this spectral and kinetic range essentially only one absorption change of rhodopsin has been detected, we deduce that the average effect in Figs. 5d–f represents a true metarhodopsin signal. Our interpretation of the non-linear exhaustion of the 387 nm-effect as a scattering superposition (Hofmann et al. 1976) is thereby verified.

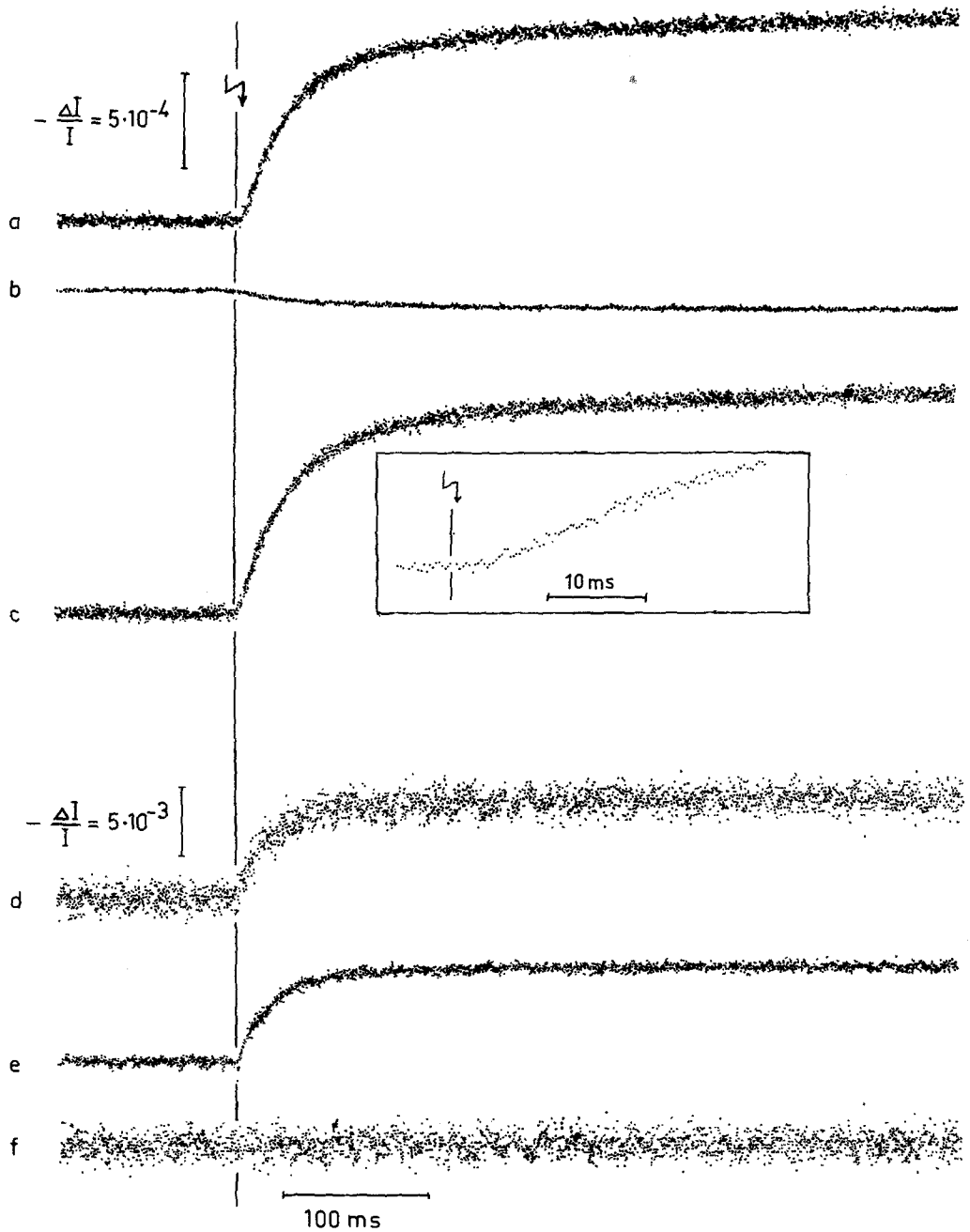

Fig. 5a–f. Simultaneous measurement, comparison and separation of different light-scattering and light-absorption signals from bovine rod outer segments in suspension. Measuring parameters: Bleaching per flash 1.5%, T = 18.1° C, ph = 6, $c_{Rhod} = 5 \cdot 10^{-6}$ M; **a, b, c:** $\lambda_m = 950$ nm, $6° < \vartheta < 13°$; **d, e, f:** $\lambda_m = 387/417$ nm. The traces show (from top to bottom): **a** integral scattering effect in the ms-range (the signals in the range of seconds do not contribute in this time domain); **b** signal N, extrapolated to flash number 1; **c** signal P, which is the difference of traces **a** and **b.** Inset: expanded time base, shows the sigmoidal shape of the signal P; **d** 387/417 nm absorption change, flash number 1; simultaneous measurement with trace **a**; **e** averaged 387/417 nm absorption change, flash numbers 8–47; **f** difference of traces **d** and **e**

(2) The linearly exhausting scattering effect is denoted as *N-signal.* We do not know whether in the strict sense this denotation is correct, since the N-signal could still be a sum of effects with similar exhaustion. However, the simultaneous measurement confirms the parallel exhaustion of this signal and of the metarhodopsin signal. The slope of the N-signal exhaustion is therefore a linear measure of pigment bleaching, which will be essential for the next investigations.

(3) The *P-effect* shows complicated kinetics (cf. the inset in Fig. 5c), which are dependent on bleaching and flash intensity. A further study will show that the measurement of the P-effect in dependence on the scattering angle and the orientation of the rod outer segments allows a further separation; the P-effect consists of two signals with different kinetics. These signals can than be treated in a phenomenological theory as uniform processes and can partly be related to biochemical reactions.

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References

Asai H, Chiba T, Kimura S, Takagi M (1975) A light induced conformational change in rod photoreceptor disc membrane. Exp Eye Res 21: 259–267

Asai H, Chiba T, Watanabe M (1977) Cooperative conformational change in rod photoreceptor disk membrane induced by bleaching. Vision Res 17: 983–984

Baylor DA, Lamb TD, Yau KW (1979) Response of retinal rods to single photons. J Physiol (Lond) 288: 613–634

Eigen M, de Mayer L (1973) Theoretical basis of relaxation spectroscopy. In: Weisberger A (ed) Techniques of chemistry, vol 6, part 2. Wiley, New York London Toronto

Falk G, Fatt P (1968) Conductance changes produced by light in rod outer segments. J Physiol (Lond) 198: 647–699

Falk G, Fatt P (1973a) An analysis of light-induced admittance changes in rod outer segments. J Physiol (Lond) 229: 185–220

Falk G, Fatt P (1973b) Isolation of components of admittance change in rod outer segments. J Physiol (Lond) 229: 221–239

Fatt P, Falk G (1977) Photosensitivity of electrical conductance of the rod disc membrane. In: Barlow HB, Fatt P (eds) Vertebrate photoreception. Academic Press, London New York San Francisco

Harary HH, Brown JE, Pinto LH (1978) Rapid light-induced changes in near infrared transmission of rods in Bufo Marinus. Science 202: 1083–1085

Hofmann KP, Uhl R, Hoffmann W, Kreutz W (1976) Measurements of fast light-induced light-scattering and -absorption changes in outer segments of vertebrate light sensitive rod cells. Biophys Struct Mech 2: 61–77

Hofmann KP, Emeis D (1979) Differential light detector. Rev Sci Instrum 50: 249–252

Kreutz W, Hofmann KP, Uhl R (1974) On the significance of twodimensional super-structures in biomembranes for energy-transfer and signal conversion. In: Jaenicke L (ed) Biochemistry of sensory functions. Springer, Berlin Heidelberg New York, pp 311–328

Liebman PA, Jagger WS, Kaplan MW, Bargoot FG (1974) Membrane structure changes in rod outer segments associated with rhodopsin bleaching. Nature 251: 31–36

Liebman PA, Pugh EN Jr (1979) The control of phosphodiesterase in rod disk membranes: kinetics, possible mechanisms and significance for vision. Vision Res 19: 375–380

Mason WT, Fager RS, Abrahamson EW (1974) Structural response of vertebrate photoreceptor membranes to light. Nature 247: 188–191

McConnell, DG (1975) Relationship of the light-induced proton uptake in bovine retinal outer segment fragments to triton-induced membrane disruption and to volume changes. J Biol Chem 250: 1898–1906

Norisuye T, Yu H (1977) Osmotically induced and photo-induced deformations of disk membranes. Biochim Biophys Acta 471: 436–452

Penn RD, Hagins WA (1972) Kinetics of the photocurrent of retinal rods. Biophys J 12: 1073–1094

Porter G (1963) In: Techniques of organic chemistry. 2nd ed., vol 8, part 2. Interscience Publishers, New York, pp 1055–1106. [First publication: Norrish and Porter (1949) Nature 164: 658]

Rüppel H (1978) Methoden zur Bestimmung schneller Reaktionen. In: Hoppe W et al. (Hrsg) Biophysik, ein Lehrbuch. Springer, Berlin Heidelberg New York

Uhl R, Hofmann KP, Kreutz W (1977) Measurement of fast light-induced disc shrinkage within bovine rod outer segments by means of a light-scattering transient. Biochim Biophys Acta 469: 113–122

Wey Cl, Cone RA (1978) Light-induced light-scattering changes from rod outer segments. Biophys J 21: 135a

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