KFA

KERNFORSCHUNGSANLAGE JÜLICH GmbH

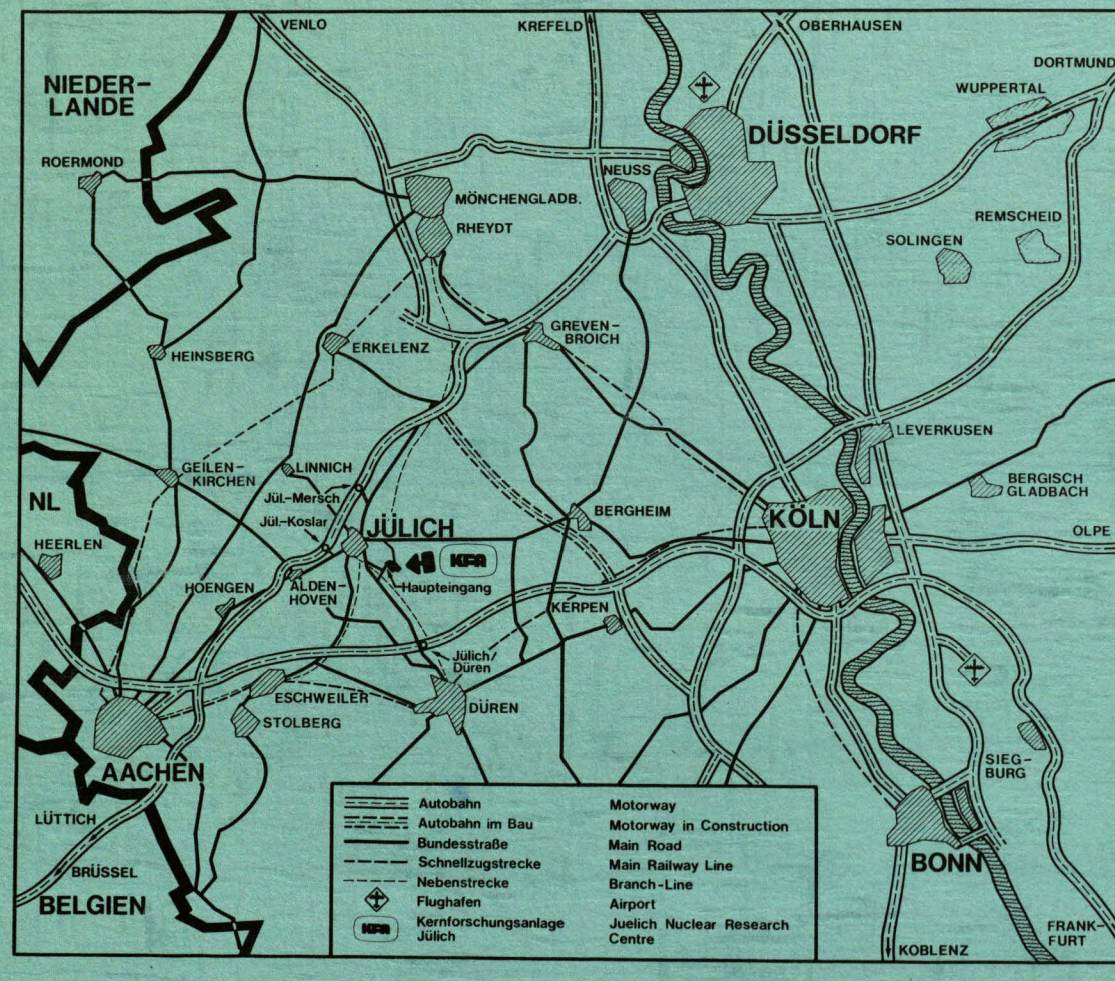
Institut für Neurobiologie

Flash-spectroscopic and electrophysiological investigation of excised retinae from the dark-adapted bos taurus domesticus

by

J.-D. Spalink

Jül - 1665
Juli 1980
ISSN 0366-0885



Als Manuskript gedruckt

Berichte der Kernforschungsanlage Jülich - Nr. 1665

Institut für Neurobiologie Jül - 1665

Zu beziehen durch: ZENTRALBIBLIOTHEK der Kernforschungsanlage Jülich GmbH
Postfach 1913 · D-5170 Jülich (Bundesrepublik Deutschland)
Telefon: 02461/611 · Telex: 833556 kfa d

Flash-spectroscopic and electrophysiological investigation of excised retinae from the dark-adapted bos taurus domesticus

by

J.-D. Spalink

D 82 (Diss. T.H. Aachen)

Acknowledgements

I am sincerely grateful to Professor Stieve for help, guidance and inspiration and for allowing me to work in such a fascinating field.

I would like to thank Dr. W. Sperling as a representative of all who supported and encouraged me during the progress of this work.

The help of K. Noelle-Fischer, Inst. f. Anglistik, for reading the manuscript is gratefully appreciated.

Contents Page

Glossary

DAC	digital to analog converter
DVM	digital voltage meter
ERP	early receptor potential
f	focus
fg	frequency limit
I	transmission
I_o	transmission before the flash
LRP	late receptor potential
λ	wavelength
MI	Metarhodopsin I
MII	Metarhodopsin II
NBS	National Bureau of Standards
PM	photomultiplier
R1	first component of ERP
R2	second component of ERP
R_n	resistance
ROS	rod outer segments
t	time
t_n	time point
T	temperature
∇T	transmission difference
$\tau_{1/2}$	half-time
V	Volts

1. Introduction

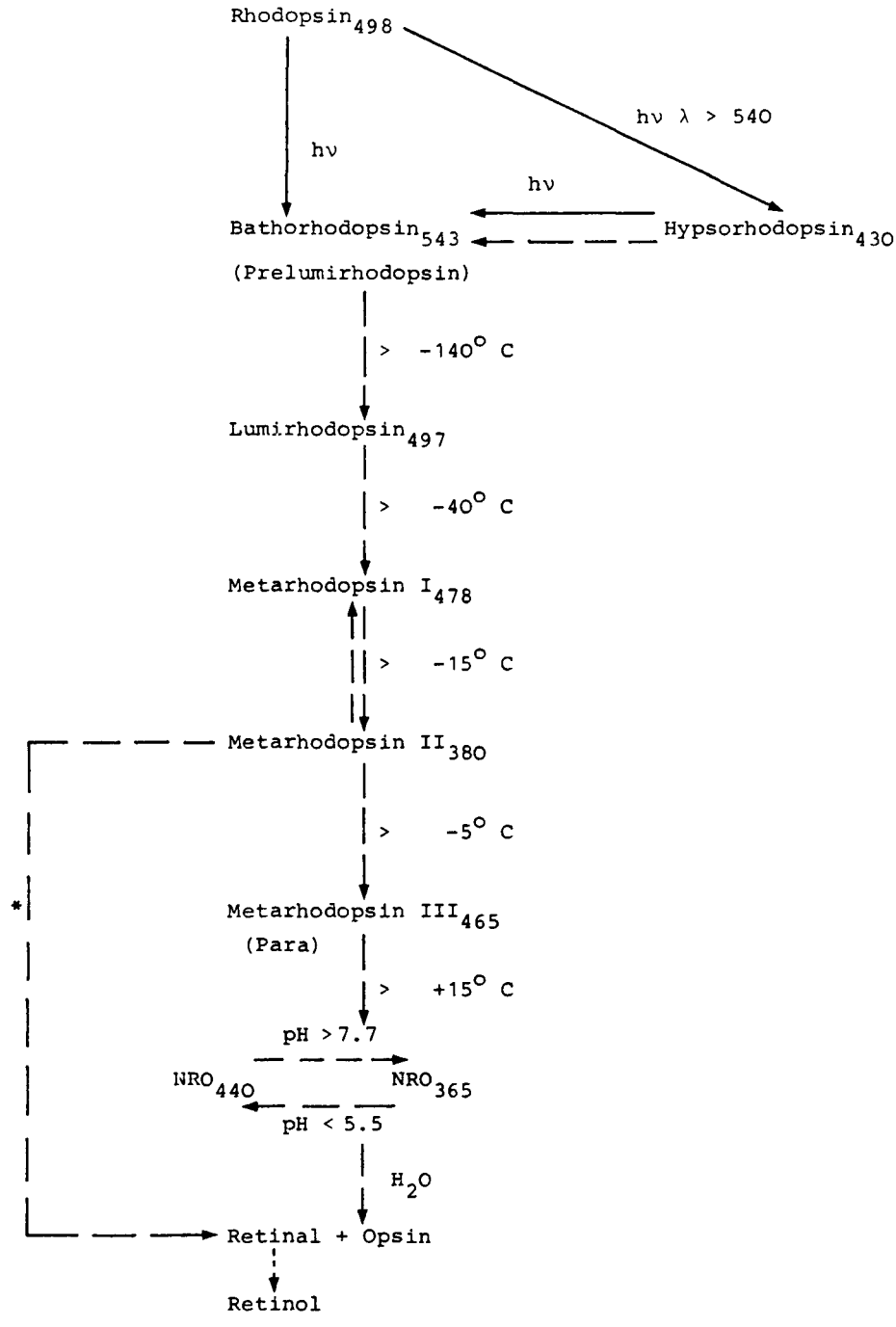
For more than a hundred years great efforts have been made to understand the mechanism of visual transduction. (Hubbard, 1976). Essentially two properties of this process evoked permanent interest for such a long time. First, single photons can already be sufficient to excite a photoreceptor, i.e. to cause a receptor potential (Hecht, Shlaer, Pirenne, 1942). This corresponds to a quantum current gain greater than 10^6 (Hagins, Penn, Yoshikami, 1970). Second, it is not understood, how such a cell gets to "know" that one of the rhodopsin molecules in its outer segment has absorbed a photon.
There seems to be no doubt, that this absorption of a photon is the first step in vision (Hagins, 1957).

When the chromophore of a rhodopsin molecule has absorbed a photon, it runs through an excited singlet state (Abrahamson and Japar, 1971) and several consecutive intermediates, distinguishable by their different absorption spectra. Their sequence for bovine rhodopsin is shown in fig. 1. The formation and decay of intermediates can be recorded with a flash spectrophotometer, as described by Witt and Rüppel (1969). It is supposed that at least one of these intermediates is involved in a process, by which the conductivity of the cell membrane is transiently changed, causing the transmembrane ionic current to be suppressed. This can be measured as a voltage across the membrane, viz. the receptor potential (see Stieve, 1974).
Electrophysiological investigations revealed that at 37° C the first component (a-wave) of the receptor potential already appears about 300 microseconds after the absorption of a photon (Arden and Ikeda, 1968). Thus only intermediates ranging earlier in time than this event might trigger those conductance changes which are supposed to be the origin of the receptor potential.

In 1964 an electrical signal with no detectable latency, the early receptor potential (ERP) was discovered in excised eyes

V e r t e b r a t e R h o d o p s i n

11-cis or 11-cis, 12-S-cis retinal
M.W. 35-40.000 pH 5.4 - 7.7



* Hydrolysis Pathway, Maximum Percentages:
30 % Digitonin (3°)
27 % Frog retina (25°)
20 % Frog retina (10°)
25 % Human retina

Fig. 1. Intermediate sequence in the photolysis of vertebrate (bovine) rhodopsin. Arrows with solid lines denote photoreactions while those with dotted lines denote thermal, dark reactions. (from: Ostroy, 1977)

of albino rats. It was faster than the a-wave and turned out to have an amplitude strongly proportional to the amount of rhodopsin bleached (Brown and Murakami, 1964; Cone, 1964; Pak, 1965). The second one of the two components of the ERP was found to have a half-time and temperature dependence closely matched to the formation of Metarhodopsin II (MII) (Cone and Cobbs III, 1969). Although the reported results indicate a tight relationship between part of the ERP and MII, no definite correlation has been found. The direct involvement of the early receptor potential in the visual transduction process and its origin is still discussed (e.g.: Hodgkin and O'Bryan, 1977; Hagins and Rüppel, 1971; Murakami and Pak, 1970).

An important prerequisite for a better understanding of these events would be a detailed study about rhodopsin intermediates probably involved. Up to now simultaneous measurements of spectroscopical and fast electrical signals were only reported for vertebrates of which specific intermediates have not been investigated. Bovine rhodopsin has been intensely studied by means of biophysical and biochemical experiments and detailed reports about its intermediate kinetics in solutions and suspensions are available, e.g. Ostroy, 1977.

In this work, therefore, flash spectroscopic and electrophysiological experiments with excised bovine retinae and simultaneous measurements of the ERP and the MII-formation are reported. The retinae were taken from dark-adapted animals to avoid problems related to different light adaptation of individual samples and to maximize the available content of unbleached rhodopsin.

2. Materials and Methods

2.1 Preparation of bovine retinae

All experiments reported here were carried out with eyes from dark-adapted cattle. Dark adaptation is achieved by blindfolding the animals during the last two hours before their death in the slaughterhouse. Lighttight masks were used, constructed for male and female animals according to the different shape of their heads. In this way complete dark adaptation was obtained (Spalink, 1978). Subsequent enucleation of the eyeballs took less than ten seconds per eye and was performed after the animals were stunned with a bolt apparatus and before their throat was cut. The light intensity in the slaughterhouse was less than 10 $\mu W/cm^2$. Enucleation under dim red light showed no different results. Immediately afterwards the excised eyes were stored at 4° C in a lighttight Dewar containing oxygen-saturated physiological saline (contents: see appendix A) and then carried back to the laboratory. The successive preparation took about 30 min, allowing experiments to be started about 45 min after enucleation. In order to get optimal signal to noise ratios, samples with the highest rhodopsin concentration were taken. In bovine retinae these are parts situated to the nasal and temporal side of the discus n. optici and include the area centralis. They contain, if any, only small capillaries (Slonaker, 1897), providing samples with minimal light scattering ability. According to the area illuminated by the laser flash a perfusion-cell (Sickel, 1965) was constructed for circular samples with a diameter of about 11 mm.

To prepare these small retinal areas the following steps were carried out under dim red light (Osram No. E27PF7012E).

a) Removal of muscles and adherent tissue from the eyeball.
b) Circular incision along the ora serrata, separating cornea and lens from most of the corpus vitreum (fig. 2).
c) Orientation of the remaining proximal part to apply three incisions versus the proximal pole; one perpendicular to the rim towards the discus n. optici, the other two at an angle of 15° nasal and temporal from the vertical meridian

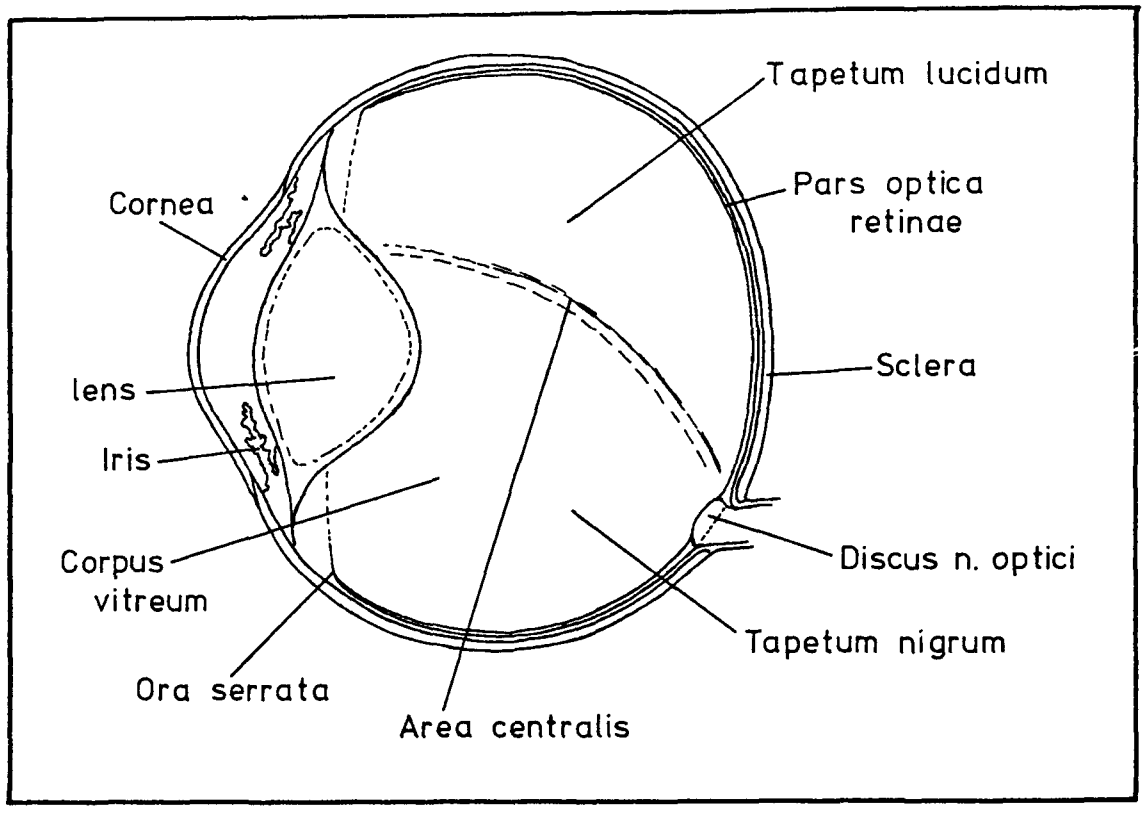


Fig. 2: Section of bovine bulbus

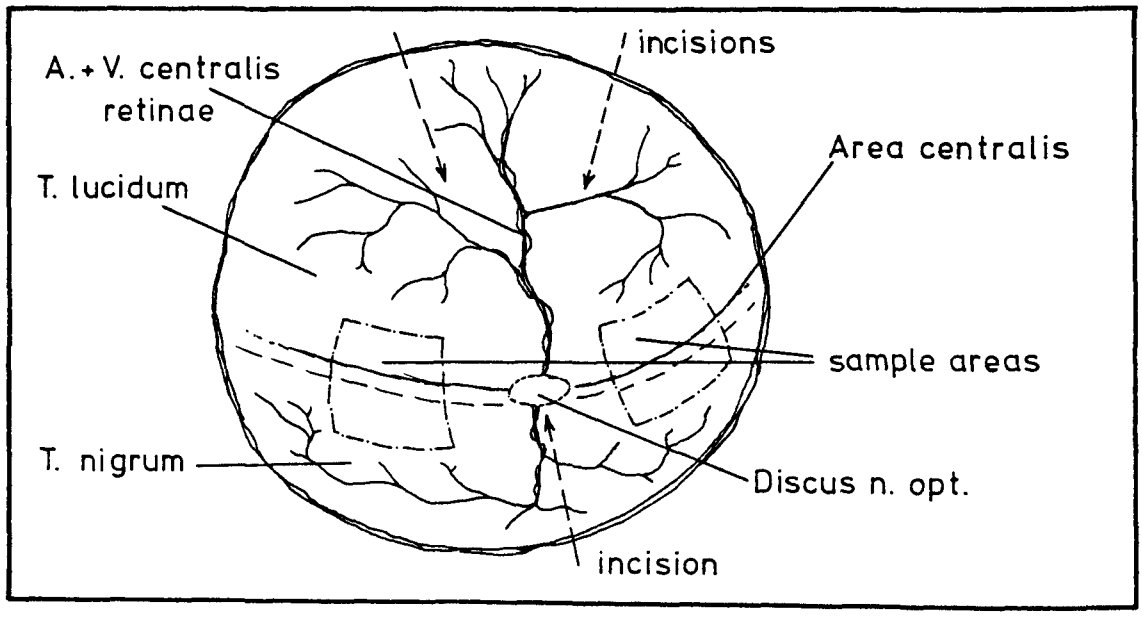


Fig. 3: Location of the sample areas

and opposite to the first incision, so that the main blood vessels are situated in between (fig. 3).

d) After removing the remaining part of the corpus vitreum, each of the two sections containing parts of the area centralis is fastened in a Petri dish filled with oxygenated physiological saline temperatured to about 20° C.

e) After re-orientation, keeping in mind the side where the discus n. optici has been, the area later to be used as a sample is covered by a ring* with a lighttight cap.
The mentioned orientation is possible even under dim red light (which makes the area centralis invisible), because the borderline between the tapetum lucidum and tapetum nigrum can still be identified. In bovine retinae, the area centralis runs parallel to this line at a distance of 1 - 2 mm, within the tapetum lucidum (Krebs, 1979).

f) Under a dissecting microscope the proximal neural layer of the pars optica retinae outside the ring is separated from the distal pigment epithelium by peeling it off carefully. This is done under dark red microscope light with $\lambda > 640$ nm. The loosened tissue is then folded on to the top of the ring's cap very gently, so that it is not hurt where it touches the edge of the cap. Illumination with $\lambda \simeq 800$ nm and preparation with the aid of infrared image converters did not essentially improve the experimental results.

g) The retina-enveloped ring together with the cap is lifted up slowly and transferred to the body of the sample-holding cell.
When the ring is pressed into its position, projecting parts of the retinal tissue are cut along the rim of the ring and are removed. A circular part of it remains inside the cell, carried by a nylon net (Verseidag, Krefeld, type Mon. 560 PA) and fixed into its final position by the ring.

h) Finally the cap, together with excess tissue, is removed from the ring, the cell is closed by quartz windows, filled with physiological saline (PS) and connected to its supply and control devices.

* diameter 11 mm; fits into the body of the Sickel cell.

2.2 Signal recording

For all kinetic measurements a flash spectrophotometer adapted to the special purpose of recording simultaneous spectroscopic and electrophysiological parameters from parts of living retinal tissue was developed, see fig. 4. The apparatus had an amplitude resolution of 2.10^{-4}. The whole set consisted of a central digital timer (Ortec 4610, 2 x 4611) and of four functional units, which will now be described.

2.2.1 Measuring beam

A 250 W tungsten halogen lamp (Osram Halogen-Bellaphot 64655) is connected to a DC power supply (Heinzinger, TN 30-600), modified to have a voltage output stability of $\Delta V/V = 10^{-6}$. This light is focussed on the entrance slit of a grating monochromator (Zeiss M20) whose exit slit is imaged by a second condensor on to the sample inside the perfusion cell. The beam passes an electromagnetic operated shutter (Compur electronic m) and an aperture by which the measuring light spot is matched to the area illuminated by the exciting laser flash.

2.2.2 Sample holding device, fig. 5

Two Peltier elements, supplied by an electronic control unit, allowed the temperature of both the cell and the superfusate to be reproducibly adjusted within .1 centigrade. The temperature of the superfusate is independently measured directly at the inlet of each cell by a calibrated NTC resistor. To avoid moisture on the cell windows at temperatures below 20^{o} C, dry nitrogen gas of adequate temperature was blown against them.

2.2.3 Exciting light source

The dye laser (Molectron DL II, mod. 14) which is pumped by a N_2 laser (Molectron UV 12) was run at a frequency of 20 Hz for optimal amplitude reproducibility of the individual flashes. It was triggered by the central timer to provide exact synchronism with related components. An electromagnetic shutter (s.o.) was used to select single Laser pulses (width 6 ns).

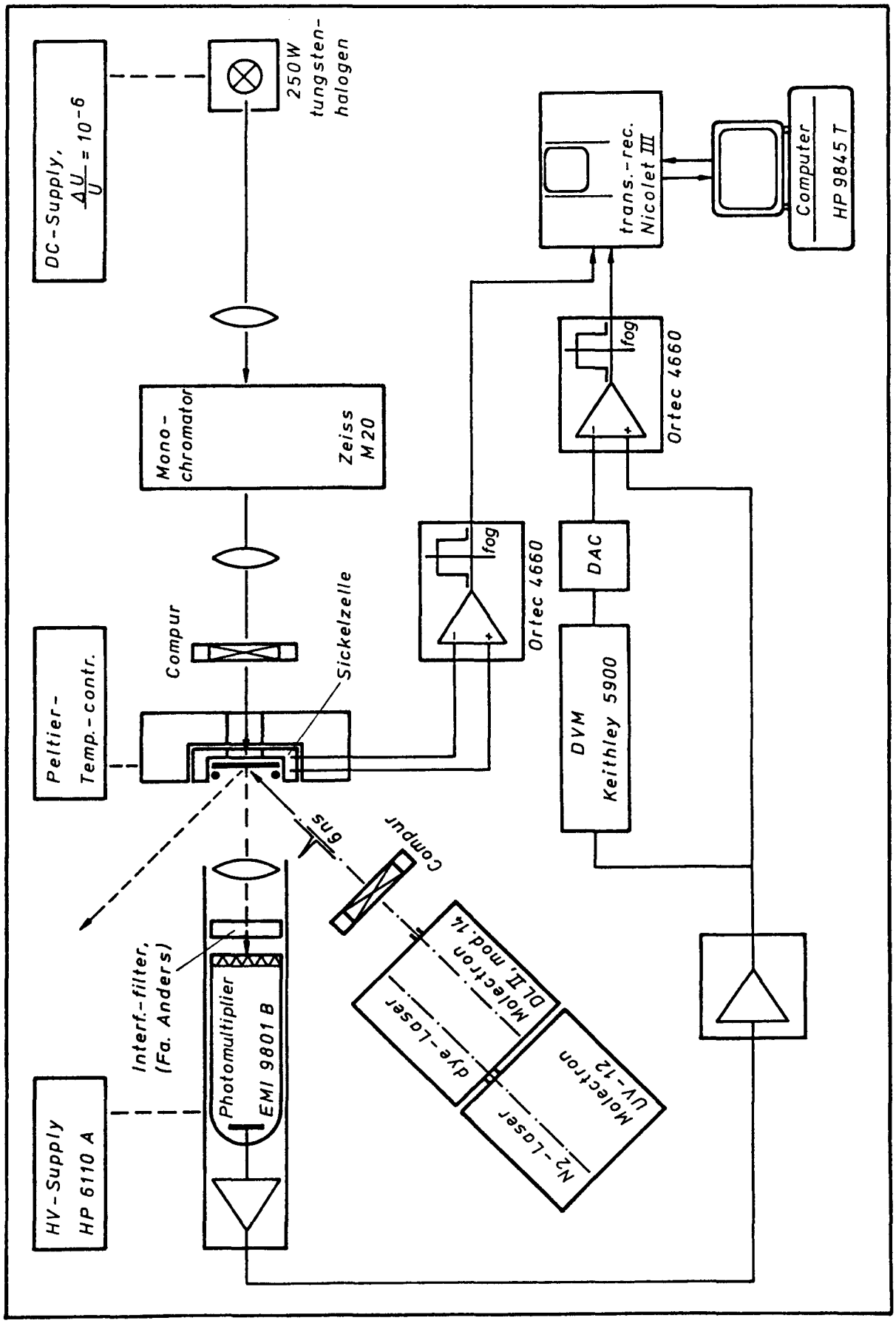


Fig. 4: Flash-spectrophotometer

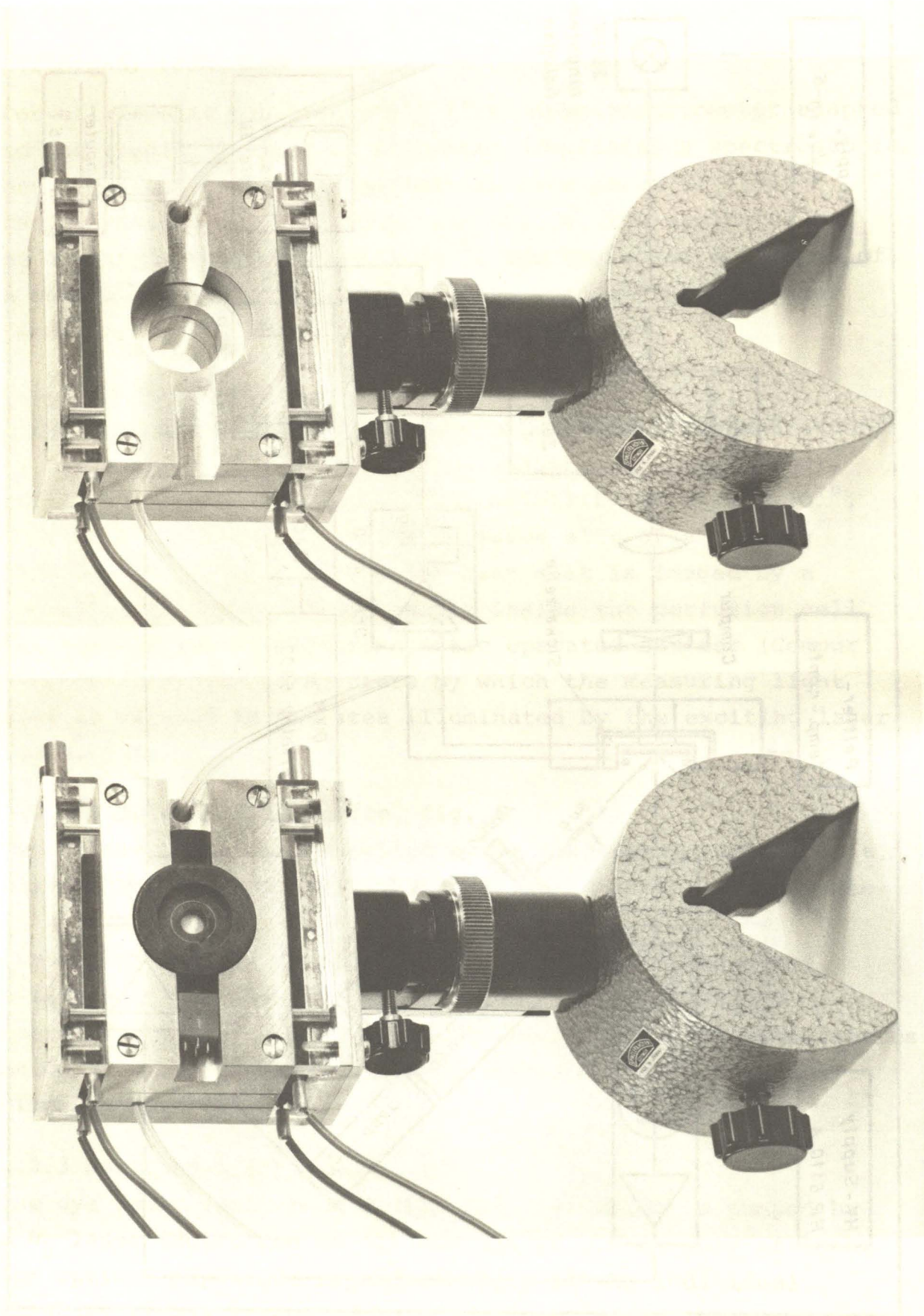


Fig. 5: Sample holder with and without Sickel cell installed

The amount of rhodopsin bleached by one flash (dye: coumarin 500, 10 mM in ethanol) was estimated from spectra recorded on a Cary 118 spectrophotometer equipped with a scattered transmission compartment.

2.2.4 Signal monitoring and storage

The distance between the PM (photomultiplier EMI D214B, eq. to EMI 9801B) and the sample could be varied to capture a maximum of light emerging from the sample under a definite spatial angle. The PM had a prismatic photocathode to absorb as much straylight as possible. A lighttight housing for interference filters was fitted in front of the PM together with an additional condensor (f = 50 mm, Quartz) to meet special spatial requirements. The PM was operated in current mode for the best impulse transmission. The current was kept very low ($\leq$ 5 µA) to assure optimal DC-performance and linearity. The anode was connected via a current-voltage transducer to one input of a differential amplifier (Ortec 4660) and to a digital voltage meter (DVM, Keithley 5900) with a subsequent digital to analog converter (DAC). The DAC was connected to the other input of the diff. amplifier. This provided the possibility to subtract exactly defined DC-levels. The output of the diff. amplifier was fed into one channel of a transient recorder (Nicolet Explorer III, 4096 words (à 8 bit) storage length, min. sample interval = 50 ns). The signals were permanently stored in digitized form on a flexible disc. To avoid aliasing (e.g. Spalink, 1976), the signals were lowpass filtered before being digitized by the transient recorder. The frequency response of the filter unit is shown in fig. 6.
The stored signals were transferred to a general purpose digital computer (HP 9845 T) for signal processing.

The transretinal voltage picked up by two electrodes (Ag/AgCl) of the perfusion cell was taken up by a second diff. amplifier (Ortec 4660), bandpass-filtered (.1 - 20 kHz) and given into the second channel of the already mentioned transient recorder. The parallel recording and storage in two channels allows

direct, time-error free and true-scale correlation between recorded and stored signals.

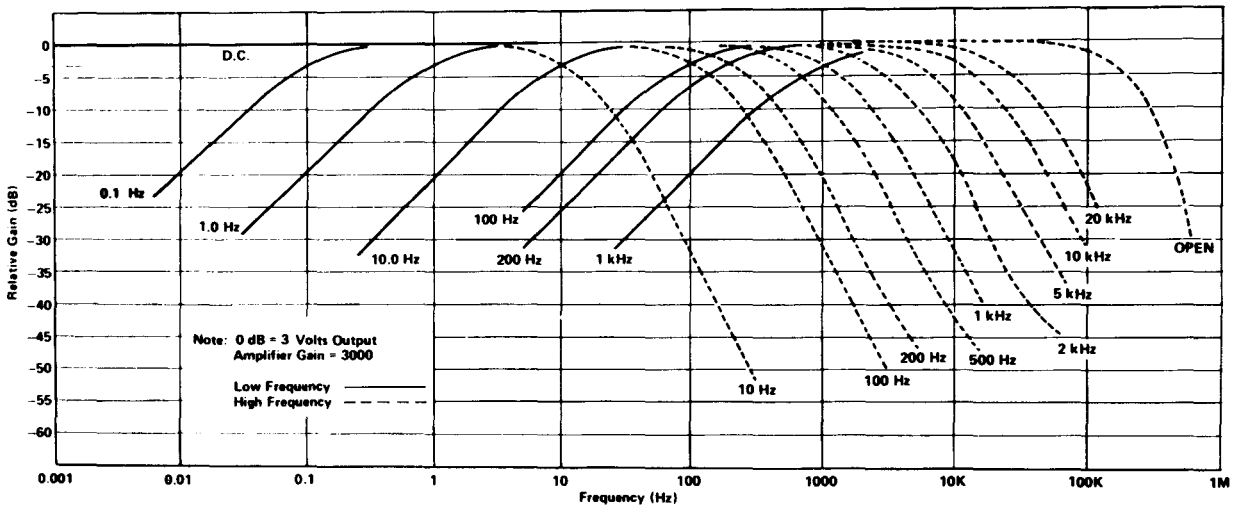


Fig. 6: Frequency response, Ortec 4660 diff. amplifier

2.2.5 Operation

When a cell had been installed in the holder and had reached thermal equilibrium, the experiment was started by triggering the dye laser (20 Hz, one second). 20 ms before the actual flash the shutter in the measuring beam was opened, the steady-light corresponding DC-voltage was measured by the DVM, stored and transferred digitally to the DAC, which returned the analog voltage to one of the inputs of the diff. amplifier. Then a flash was delivered by opening the laser-shutter for one shot. At the output of the diff. amplifier only the difference between the stored DC-level and the actual signal voltage is obtained and was stored by the transient recorder.

2.2.6 Calibration

a) temperature

To get reliable absolute and relative temperature readings, each of the NTC resistors (4,7 kΩ at 25° C) installed in the six utilized Sickel cells, was calibrated individually at 1°, 15°, 25° and 37° C. Calibration was performed by mounting a cell to the holder and perfusing it at constant rate. A

Cu-Constantan thermocouple was inserted from the inflow and positioned exactly at the site of the resistor. Actual temperature was obtained by converting the voltage readings of a DVM (Keithley 5900) into Kelvin using a table published by the NBS. At each temperature step, first of all the superfusate temperature was adjusted, so that the DVM gave exact voltage readings. When no further resistance changes indicated thermal equilibrium between resistor and superfusate, the actual resistance was determined by a Keithley 171 Multimeter. Intermediate values were then calculated according to

$$R_T = A \cdot e^{B/T},$$

A being a constant, and B evaluated separately for each of the three temperature intervals with

$$B = 2{,}303 \cdot \frac{\log R_1 - \log R_2}{1/T1 - 1/T2} \quad \text{(Valvo 1976)}$$

b) wavelength and spectral bandwidth

The monochromator reading was checked by using different monochromatic light sources (Osram Spektrallampen types Cd/10, Hg/13, Na/10); the absolute deviation at 370 nm turned out to be less than .5 nm.

At 380 nm the spectral bandwidth was checked with a second monochromator (Zeiss, M4Q III) as a function of the slitwidth. The result is shown in appendix B.

c) linearity

After a warming-up time of 30 min and connected to a highly stable high voltage supply (HP 6110 A, Hewlett Packard) the PM showed a drift of less than .1 % of the anode current (5 µA) within 20 min.

This allowed easy control of linearity, which was checked for a gain between 10^2 and 10^4 and for transmission changes of $\pm$ 5 % and $\pm$ 10 % by inserting two KG 1 filters (Schott) into the light path; no deviations from the expected values could be detected. Using a light emitting diode as a light source, time resolution was checked and showed to be better then 2 µs;

the limiting component turned out to be the diff. amplifier, whose frequency response is shown in fig. 6.

2.2.7 Comments

Preliminary runs showed that thermal equilibrium between the superfusing PS and the sample was not achieved very easily. The temperature of the PS could be raised from 4^{o} C to 37^{o} C in about 5 min., whereas about 45 min. passed before actual equilibrium between superfusate and the inside of the sample was attained. This was tested by measurement of the MII-formation at 378 nm. Equilibrium was stated, when the kintetics between consecutive records showed no further changes. When light scattering samples are investigated with a flash spectrophotometer, a certain dilemma appears (at least with high amplitude resolution resp. small turnover and short sweeptimes): To get a reasonable signal to noise ratio, as much as possible of the light emerging from the sample has to be cought by the PM. But when this is done, an increasing part of the scattered flash enters the flash blocking interference filter in front of the PM unperpendicularily and is partially transmitted. This causes a spike-like artefact. Lowpass-filtering of the electrical signal broadens this spike, obscuring even a longer part of the record, and is therefore not appropriate. Consequently, for the experiments reported here, an upper frequency limit as high as possible with regard to the Shannon theorem and limited storage length was chosen. This resulted in short artefacts but also in increased shot noise from the PM. The influence of this white noise was usually reduced by a factor of ten by digital lowpass filtering; see next chapter.

2.3 Signal processing

All spectroscopic records start with a short preflash track. The level of this track is measured by the DVM with an accuracy of .5 % (8 bit). Then the actinic flash is triggered. It lasts for about 6 ns, and defines the time point $t_o = 0$. According to the already mentioned mechanism, a spike appears,

masking the start of the transmission change. In order to discard only those parts of the record, which are really affected by the flash artefact, its shape and extension has to be determined. An impulse response of an ideal lowpass filter with upper frequency limit fg is shown in fig. 7 for $t \geq 0$.

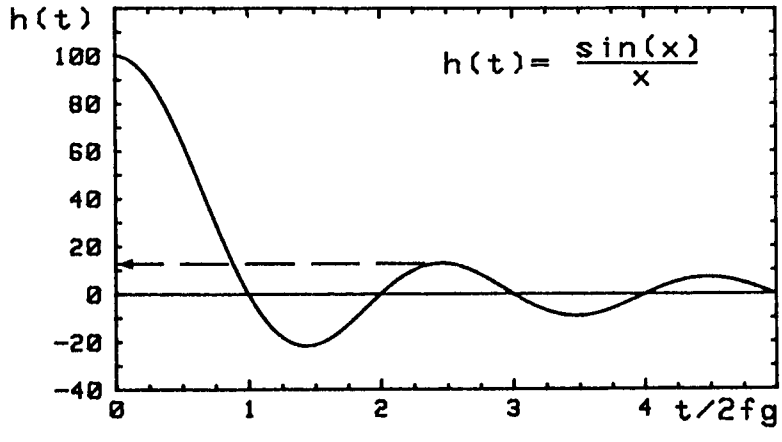


Fig. 7: Impulse response of an ideal lowpass filter, X = 2π tfg. (see: Lüke, 1975)

If the frequency limit is set to 20 kHz, the third halfwave of the impulse response (amplitude: 12,8 % of flash maximum) is terminated after

$$t_3^{20k} = 3 \cdot \frac{1}{2fg} = 75\ \mu s$$

and with a range of 10 kHz after

$$t_3^{10k} = 150\ \mu s, \text{ and so on.}$$

Waiting for the amplitude to decline to about half the previous value takes twice as much time. Flash artefacts observed under experimental conditions declined in a way similar to fig. 7.

To take this into account, records were evaluated starting with data points following the sixth halfwave or even later, if the artefact was additionally broadened by amplifier saturation. To sum it up, an actual time resolution between about 2 µs (no flash artefact detectable) and about 150 µs (prominent flash artefact) is achieved. In the case of amplifier saturation due to the flash artefact, no time resolution can be specified in advance.

For the following digital lowpass filter procedure, records with no major discontinuities are required. Therefore the section affected by the flash artefact is substituted by an appropriatly computed exponential, see appendix C. When software filtering the records, it is taken into account, that the upper frequency limit of the hardware filter was only chosen high enough to keep the flash artefact as short as possible. After its substitution, an upper frequency limit can be taken according to the spectral properties of the actual signal. Power density spectra of exponentials similar to the recorded signals were computed following the method of modified periodograms (Welch, 1967).
From these spectra, upper frequency limits were derived. Controls with test functions showed that under these precautions amplitude-deviations between lowpass filtered and unfiltered records were smaller than .1 %. The complete filter routine is demonstrated in appendix C. One important step of this procedure is the extension of the signal to a time period prior to the flash. This prevents oscillations of the filter response to influence that part of the curve which represents the signal to be analysed. In a final step, the time constant of the record is determined. Computed values for the time constant turned out to be extremely dependent on the exact determination of the exponential's final value. Errors of one or two percent resulted in completely misleading values for the time constant. Thus a computer program has been developed to estimate the required final value under the assumption that the data can be approximated by one exponential. It fails if

more than one exponential is involved. Therefore, a similar procedure based on the assumption of a sum of two exponentials will be developed for those data which have been rejected by the program made for one exponential.

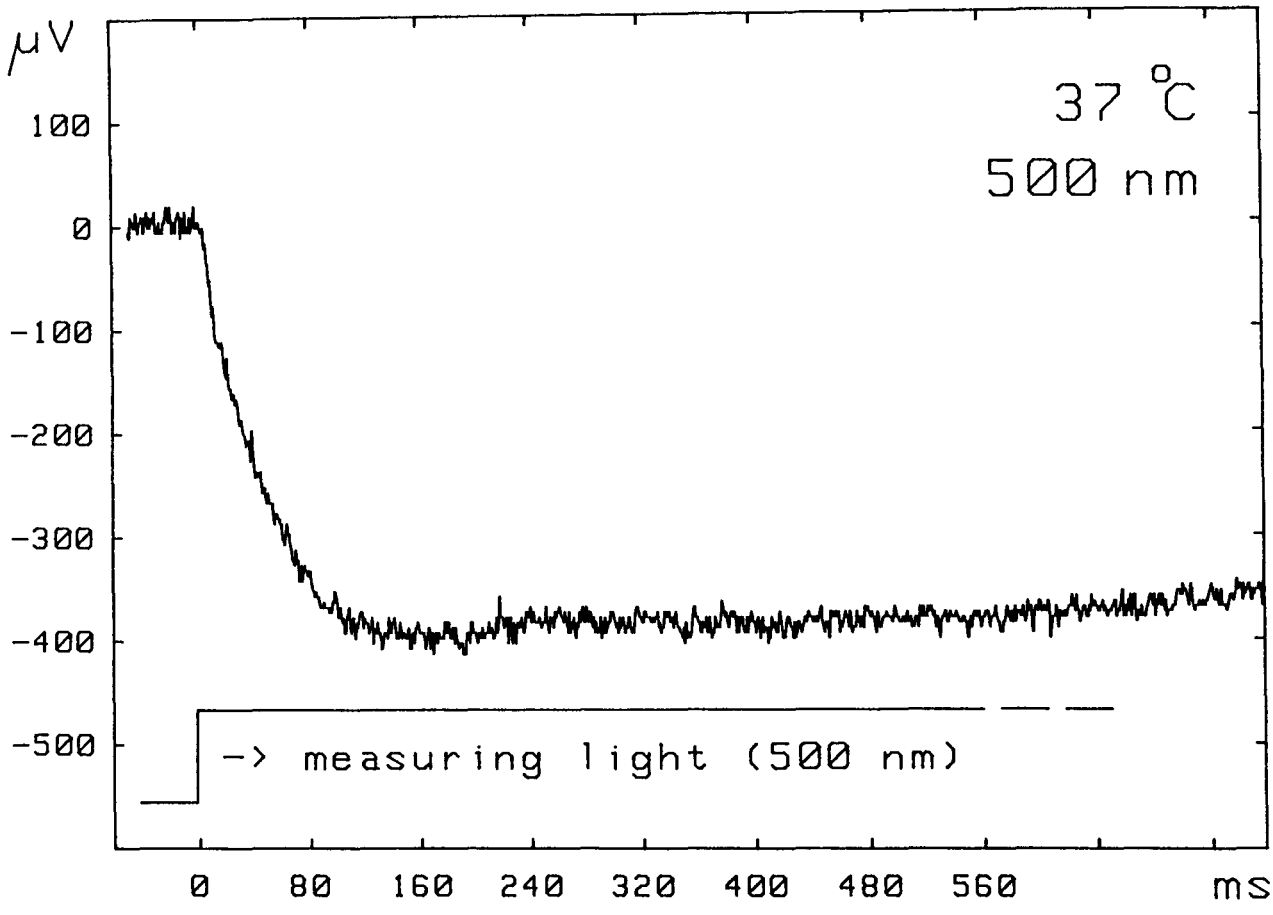


Fig. 8: LRP (a-wave) of a fresh preparation

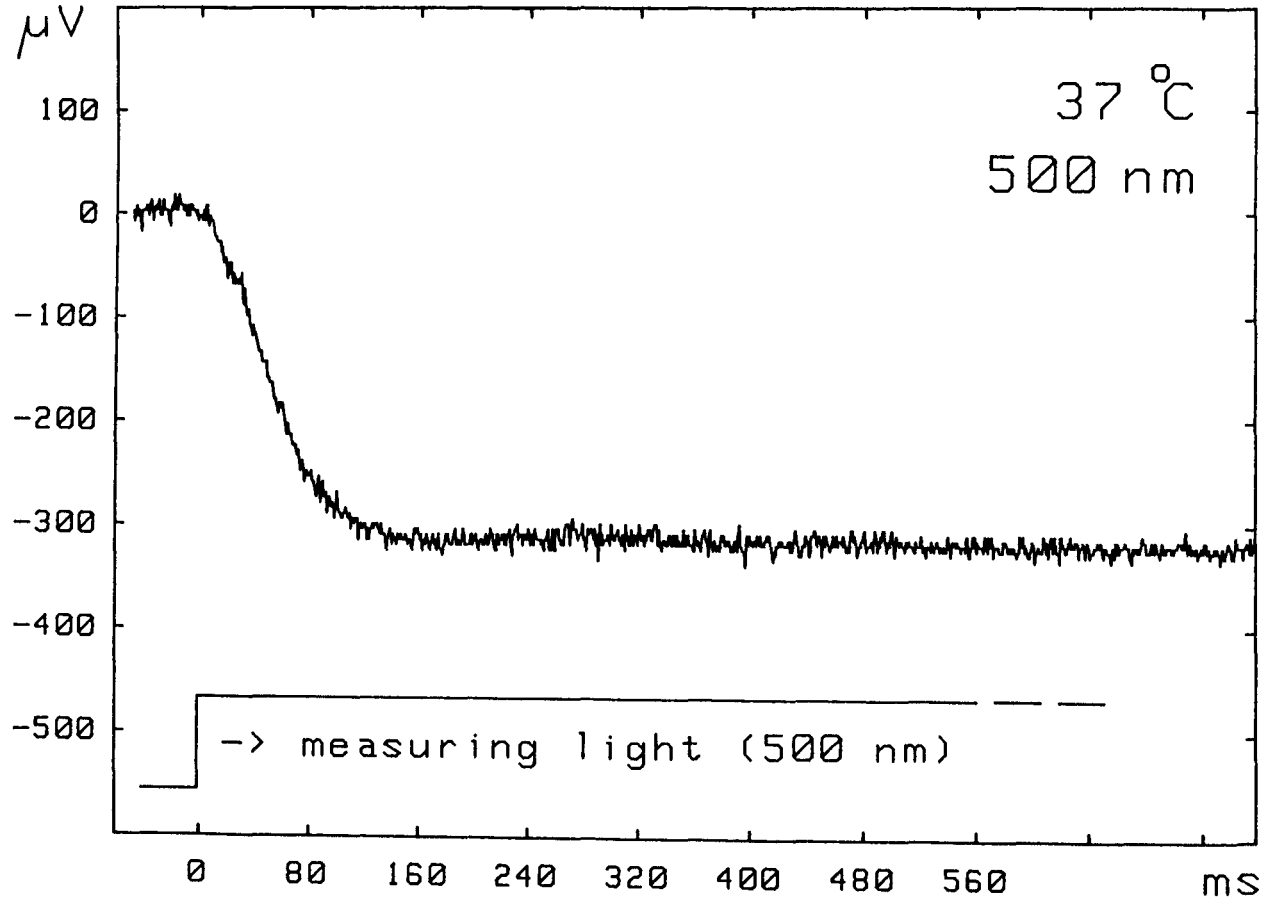


Fig. 9: LRP (a-wave) after 7 h, same retinal area as in fig. 8, after several flash spectroscopic experiments (37° C)

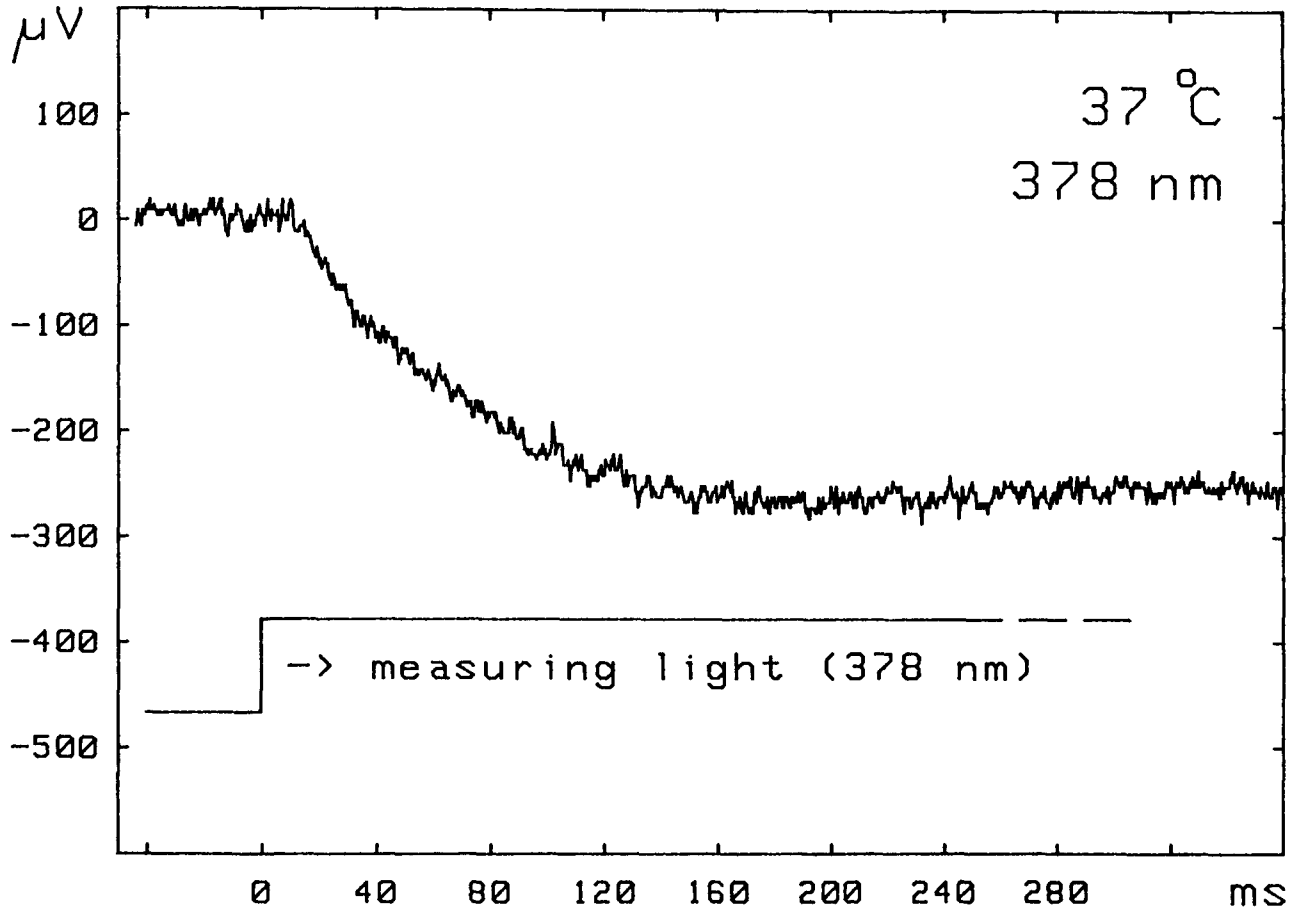


Fig. 10: LRP(a-wave)for low intensity measuring light

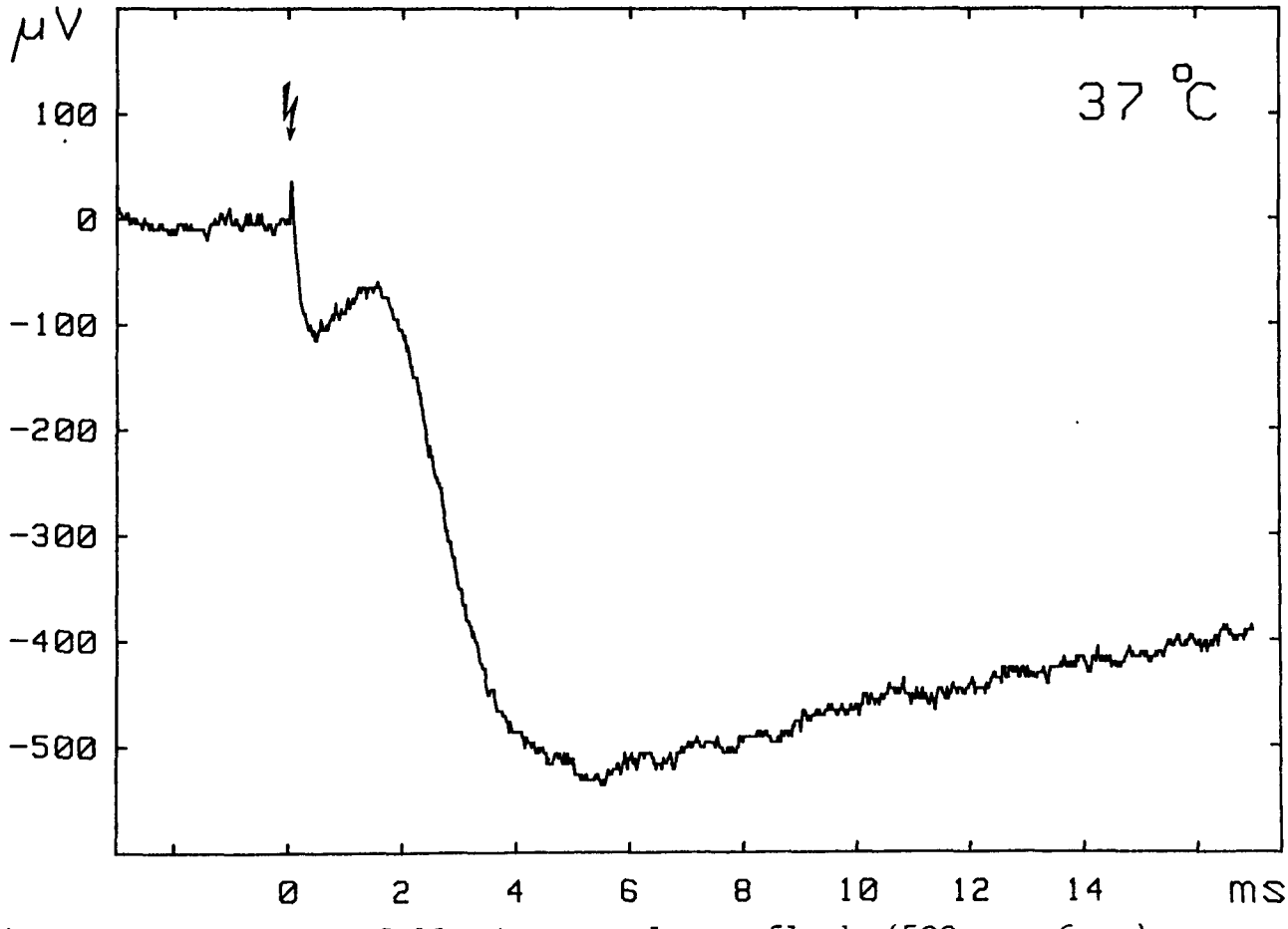


Fig. 11: ERP + LRP following one laser flash (500 nm, 6 ns) without measuring light

3. Results and discussion

3.1 Preparation procedure

Up to now, regarding bovine retinae, no electrophysiological experiments and no preparation methods have been reported. A possible reason is the thin, membrane-like structure. Even a gentle touch definitely destroys them.

Therefore a new procedure had to be developed. The already described method provides retinal pieces, of which the area to be investigated stays completely untouched and is never directly exposed to the red preparation light. Normally obtained samples showed a resistance between 400 and 600 Ω (effective diameter: 9 mm) and an optical density of about .15 (already corrected for light scattering). Eyes from cattle raised outdoors yielded the best results. A major difficulty within the course of development was the separation of the neuroretina from the corpus vitreum and from the pigment epithelium. The usually found adhesion (v. Sengbusch, 1971) depended on the amount of dark adaptation, on the storage temperature and on the degree of disintegration due to the time passed since the enucleation.

With daylight adapted eyes it was not possible to separate the two retinal layers within 30 - 40 min after the animal's death.

Dark adaptation of two or more hours and storage at 37^{o} C resulted in no adhesion at all. To be sure that no disintegration could take place and to ensure a slight adhesion to the pigment epithelium required for orientation purposes, eyes were stored at 4^{o} C in all further experiments.

Because enucleation could only be performed early in the morning between 6 am. and 8 am., the influence of the animal's daily rythms on the process of dark adaptation and resulting adherence could not be evaluated; for details see Young, 1978.

3.2 Transretinal voltage changes

3.2.1 Late receptor potential (LRP)

Preparations according to the procedure described in the methodical section showed stable LRPs for up to seven hours,

when kept in the perfusion cell at 37° C, fig. 8 + 9. For further investigations a saturation of the LRP at the beginning of the experiment was sometimes required. At the start of a spectroscopic measurement the shutter is opened to let the measuring light pass the sample. At least 20 ms of "preflash" time is required for the determination of the light intensity. As shown in fig. 10, this is too short for a LRP to be saturated by such an intensity. As the measuring light has to be kept at a level where it causes no detectable transmission changes due to bleaching of the sample, the preflash time is extended to 200 ms. In this way saturation of the LRP is achieved before the exciting flash is delivered. A single laser flash (500 nm) without adapting measuring light causes an ERP whose tail is overlapped by the a-wave of a beginning LRP, fig. 11. This result is similar to those obtained in the all rod retina of albino rats (Arden and Ikeda, 1968). By recording an ERP with saturated LRP and subtracting it from a record consisting of both ERP and LRP like fig. 11, they found that at 37° C the LRP already starts about 300 µs after the exciting flash. For the bovine retina I found a latency of about 1.3 ms at 37° C, when the a-wave is extrapolated to the baseline, fig. 12. Probably a shorter latency may be found if the shape of the bovine a-wave is similar to the one of albino rats which show a preceding positive component (Arden and Ikeda, 1968).

3.2.2 Early receptor potential (ERP)

A bovine ERP recorded at 37° C is shown in fig. 12. For lower temperatures (e.g. 30° C) no records with a reasonable signal to noise ratio could be obtained.

The reasons will be briefly discussed now. The intensity of the dye laser flashes could not be increased above the level achieved at that time (2,5 µJ), which bleached about 8 % of the rhodopsin. From experiments with albino rats it is known that the amplitude of the R2 response is proportional to the amount of rhodopsin bleached (Cone, 1964). This in turn depends on the polarisation plane of the exciting flash. Light polarisation parallel to the plane of the discs' surfaces

is absorbed 6 or 7 times better than light which is polarized perpendicularily to it (Montal and Korenbrot, 1976). Electron microscopy of bovine retinae reveals that its rods and cones show an inclination towards the optical axis; the inclination increases the more peripherally the receptors are situated (Webb, 1977, Krebs, 1979).

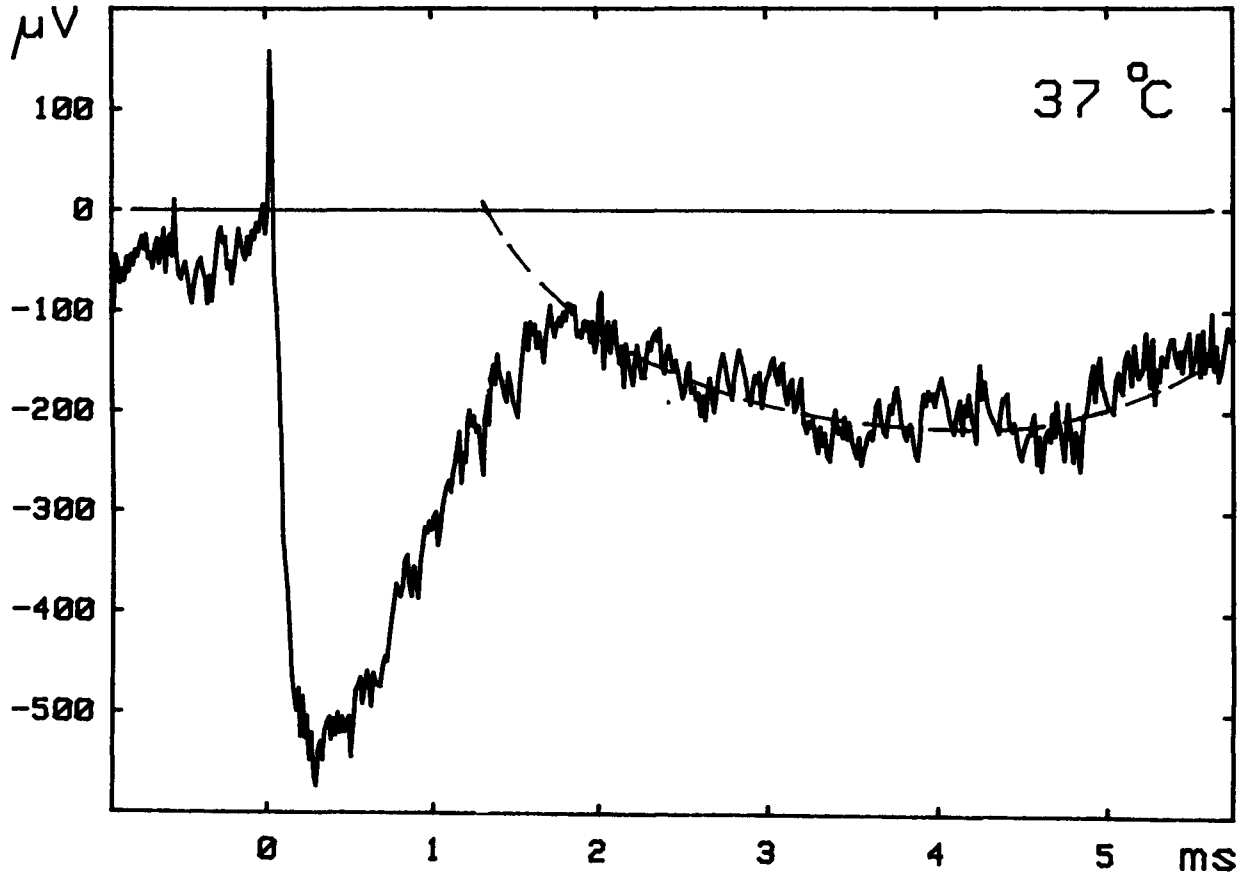


Fig. 12: ERP following a single laser flash (500 nm, 6 ns), weak a-wave due to adaptation to measuring light of 378 nm, dashed line: extrapolation of the a-wave

Therefore, during the preparation procedure, a thorough orientation of the samples in relation to the direction of the incident flash was tried. Because no direct microscopical control was possible, orientation of the outer segments was concluded from visible physiological landmarks like the position of the area centralis and the discus n. optici. Only when this orientation was successful, results as shown in fig. 11 or 12 were obtained at 37° C. Otherwise signal

amplitudes were about 50 % smaller. For lower temperatures higher flash intensities would have been necessary. The possible origin of the ERP will be discussed in section 3.4 (Simultaneous recordings).

3.3 Flash spectroscopy

Before kinetics of rhodopsin intermediates can be studied in excised bovine retinae, essentially two difficulties have to be overcome.
From the spectra of rhodopsin intermediates it can easily be seen that there is no wavelength where only one of them has a considerable absorbance, fig.14. Therefore wavelengths of those isosbestic points have to be determined, where the intermediate to be studied does not interfere with its precursor or successor. A further problem arises when unbleached or dark adapted samples containing intact discs are investigated. Transmission changes due to light scattering occur and have to be taken into account.

3.3.1 Light scattering

Because of their pronounced angular dependence (Hofmann, 1976) it is possible to find a spatial arrangement where positive and negative light scattering signal elements compensate each other. This might be carried out at 705 nm, where no transmission changes caused by rhodopsin intermediates are to be expected. In fig. 13 a record is shown where this is achieved by a proper choice of the distance between the PM and the sample. According to Hofmann (1979) there is strong evidence that for shorter wavelengths too, no such signals will occur if they have been compensated at 705 nm.

3.3.2 Isosbestic points

For 1^{o} C in fig. 14 spectra are shown for those (intermediate) transitional states of partly bleached ROS suspensions which can be detected within the available time resolution. They are calculated from difference spectra for single intermediates, obtained at 1^{o} C by flash spectroscopic and spectrophotometric measurements with ROS suspensions (Rafferty, 1979). To get evident differences between single spectra, a bleaching rate

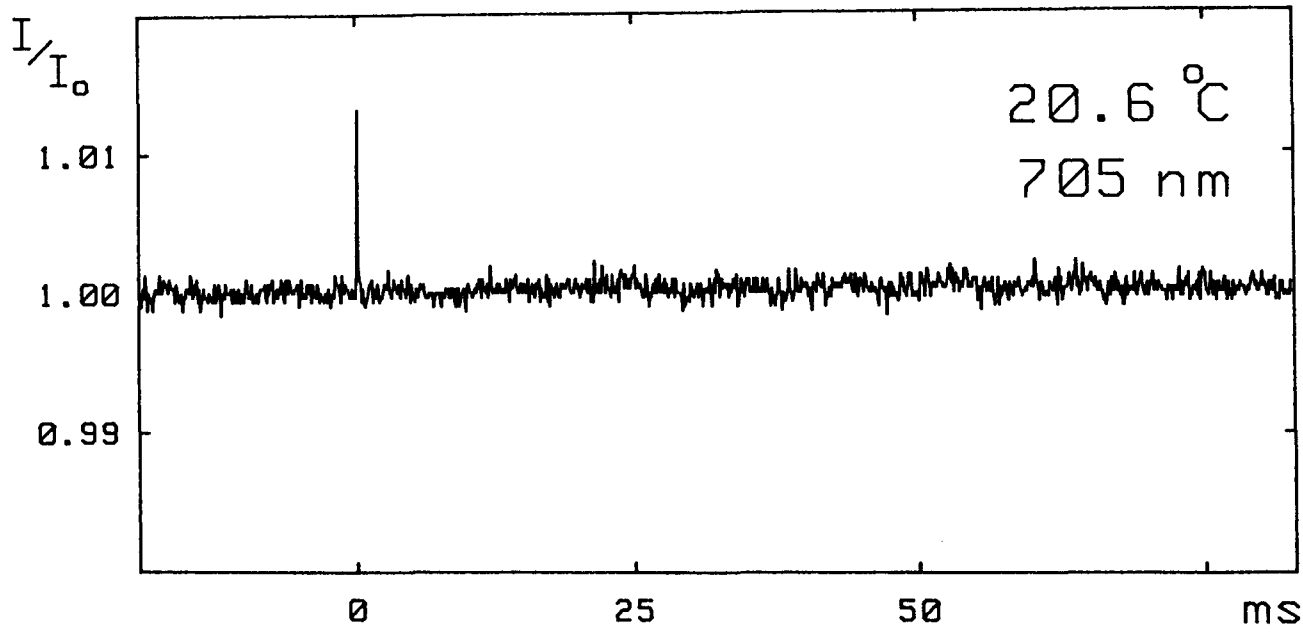


Fig. 13a: No transmission change is detected at 705 nm following the first laser flash

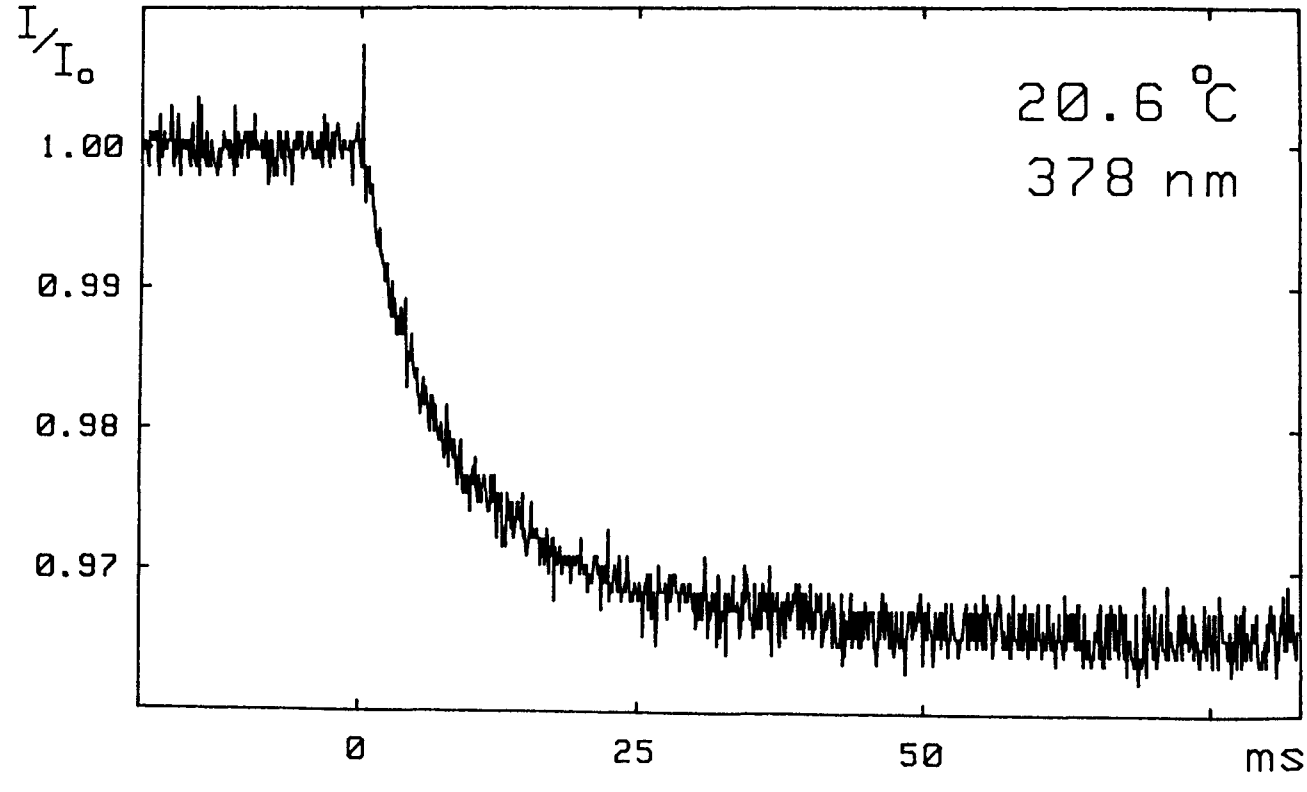


Fig. 13b: Control record; transmission change at 378 nm following the second laser flash

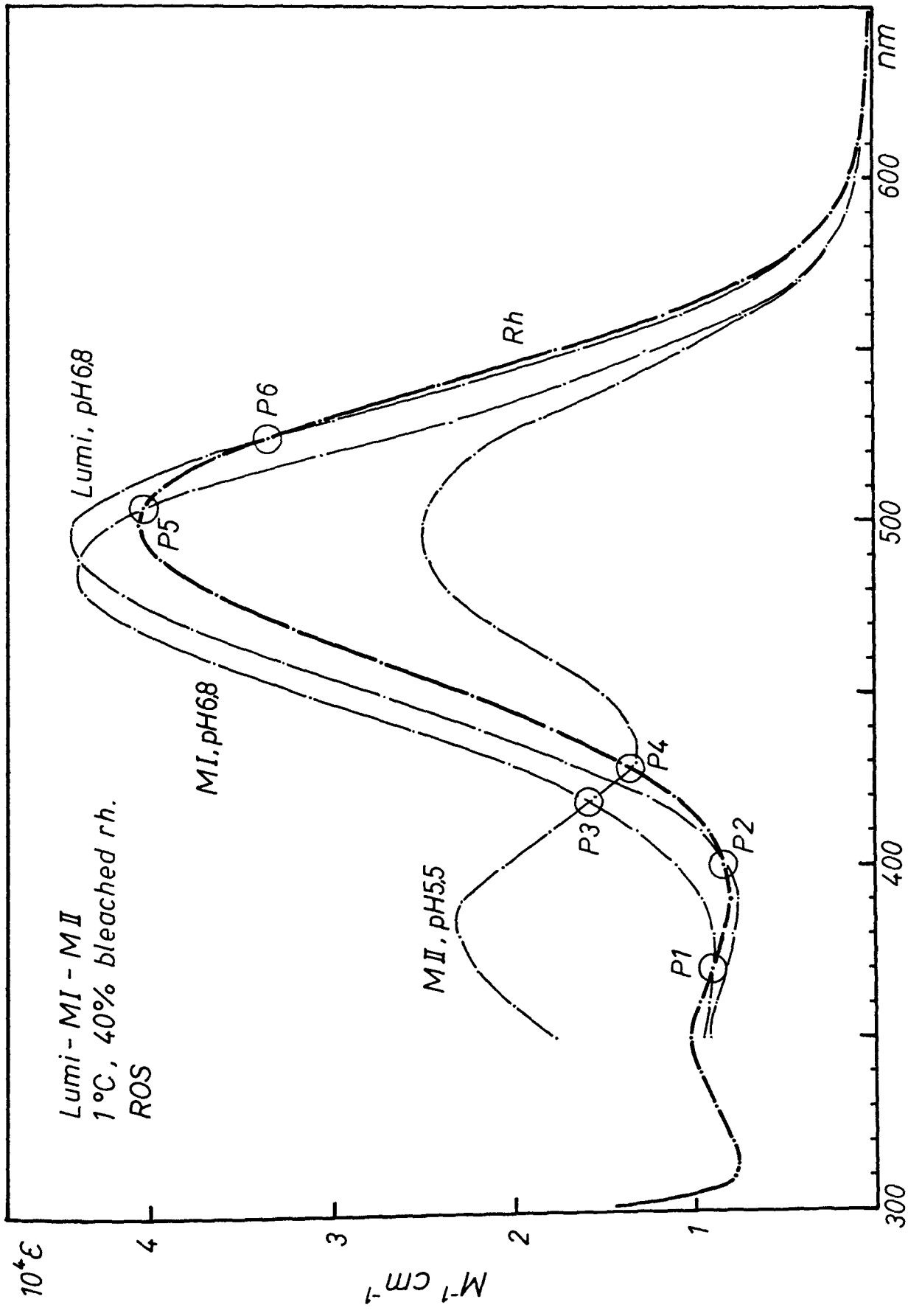


Fig. 14*: Spectra of intermediate states of rhodopsin, Isosbestic points.

*a copy of this figure can be unfolded from the back cover.

of 40 % was assumed for the computations. From these spectra, six relevant isosbestic points can be derived, P 1 - P 6 in fig. 14.

The genuine formation of a certain intermediate can best be observed at a wavelength, where the precursor is isosbest with rhodopsin. In addition, it would be helpful if intermediates ranging even earlier in time than this precursor would not appear at the specific time resolution. Furthermore, a formation can only be studied up to a certain temperature, at which the intermediate starts to decay measurably within the observation time. Analogous limitations have to be taken into account when a decay is to be investigated. Therefore in the next three sections special features related to the measurements of MI (formation and decay) and MII (formation) will be briefly discussed.

a) MI (formation and decay)
From investigations about the equilibrium between MI and MII in digitonin solutions of cattle rhodopsin (Matthews, et al., 1963) it is known that below - 17° C the equilibrium is practically completely on the side of MI and that for higher temperatures the equilibrium is shifted towards MII with nearly 100 % MII at 37° C. To avoid freezing of the sample, experiments with excised retinae are limited to temperatures above 0° C. At 1° C MII appears with a half-time of about one second in ROS suspensions (Rafferty, 1979). Therefore a decay of MI in the same time range has to be expected. From this point of view it seems favorable to measure the formation of MI at a wavelength where its decay is not observable. At P3, where MI and MII are isosbest, the decay of MI is compensated by the formation of MII. However, at P3 part of the signal also shows a contribution of Lumirhodopsin. Above 1° C, the formation of Lumirhodopsin is not resolved and therefore results in a steplike absorption increase. The subsequent decay is assumed to be identical to the formation of MI. If a sample is measured at the isosbestic point between MII and rhodopsin, the observed transmission change after the flash is a direct measure of MI.

To get a more detailed description of the reaction MI $\rightleftarrows$ MII, i.e., to prove that only these two intermediates participate, the decay of MI and the formation of MII will be analysed separately.
Between 300 nm and 700 nm, there is only one wavelength, where rhodopsin is isosbest with MII and only MI-decay is seen. In ROS suspensions and at 1^{o} C this point (P4) is at 428 nm (Rafferty, 1979).

b) MII formation
There are two wavelengths where its precursor, MI, is isosbest with rhodopsin; at P1 (372 nm) and P5 (505 nm) no absorption changes due to MI formation or decay can be observed (ROS, 1^{o}C). P1 represents the wavelength where up to now all reported MII-formation kinetics were determined.
The monochromatic laserflash allows measurements at wavelengths already a few nm apart from the wavelength of the exciting laser flash. An excitation wavelength of 495 nm could be chosen, close enough to the maximal absorption of rhodopsin (498 nm, Abrahamson and Wiesenfeld, 1972) to maintain a sufficient amount (~ 8 %) of rhodopsin bleached per flash. At both wavelengths, P1 and P5, experiments were carried out to get records for a detailed analysis of MII formation kinetics. The results will be compared to those of the independently measured MI decay.
From measurements with prolonged sweeptime (longer than 20 halftimes) it was concluded, that even at 37^{o} C no measurable MII-decay influences the observed formation. This coincides with halftimes (at 37^{o} C) for the MII decay between 63 seconds (rat, Cone and Cobbs, 1969) and 77 seconds (human retina, Baumann and Bender, 1973). The MII formation at 37^{o} C is observed within 5 - 10 ms (halftime about .5 ms).

3.3.3 MI-formation measurements
The formation of MI was observed at the isosbestic point between MI and MII near 420 nm, P3 in fig. 14. The expected signal at P3 should show a steplike transmission decrease, i.e. an absorption increase, due to the formation of Lumi-rhodopsin and a subsequent slower one caused by the formation

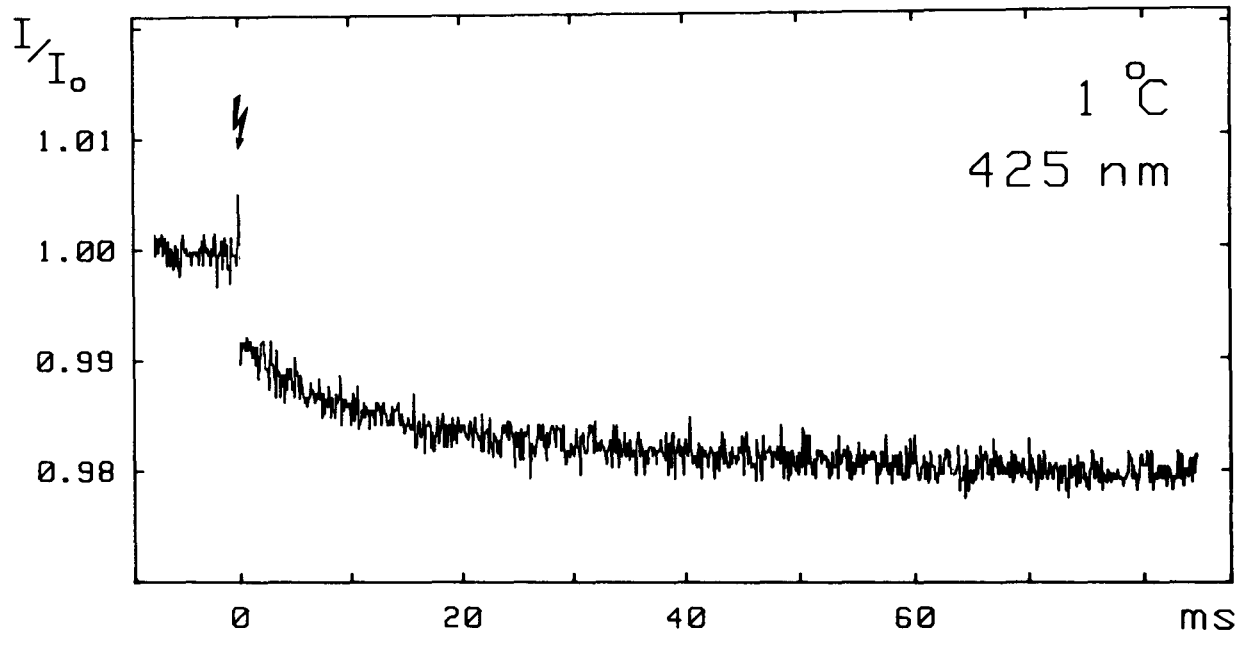


Fig. 15: MI-formation; single flash experiment, first flash record. Transmission step at t = 0 due to formation of Lumirhodopsin, see text.

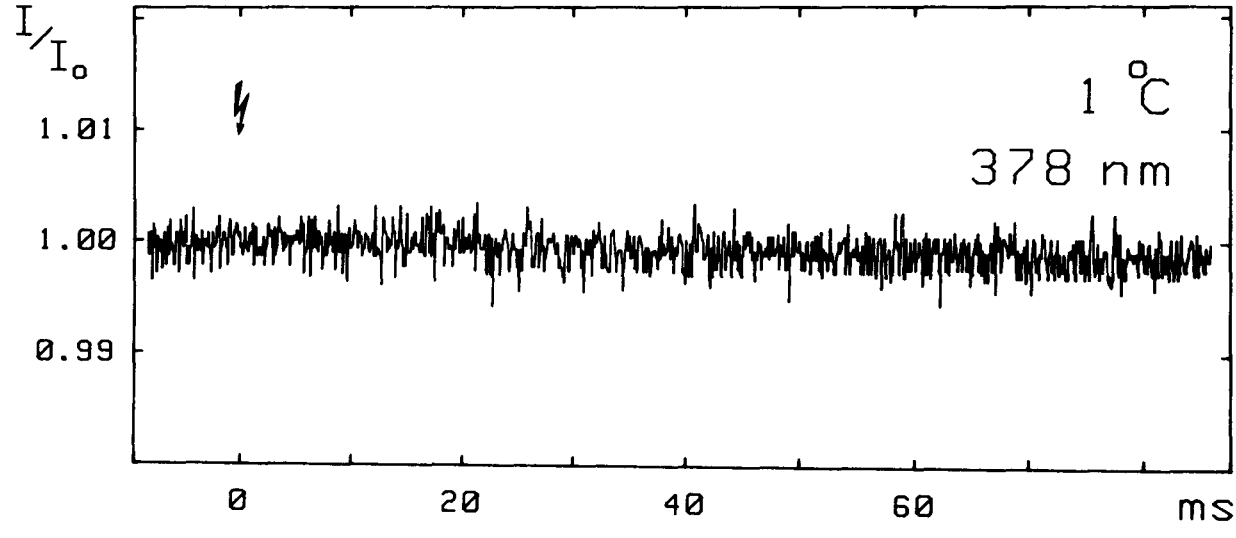


Fig. 16: Control record; same sample as in fig. 15, second flash record.

of MI.
At 1^{o} C and 425 nm such signals were recorded; an example is shown in fig. 15. A half-time of about 12 ms (mean of 5 exp. $\pm$ 2 ms) was graphically derived from the plotted records. (At the time when these experiments were performed, we could not yet store records digitally on a permanent medium).
Experiments at 378 nm showed no MII formation on this time scale and at 1^{o} C, fig. 16. Therefore it can be concluded that only MI-formation and no MI-decay occurs at P3 under these conditions.
MI-formation signals were so small because between 300 nm and 430 nm the molar extinction of all involved intermediates is up to 75 % lower than between 480 nm and 520 nm, see fig. 14. The bleaching of higher amounts of rhodopsin (in order to get greater signals) was not possible with our laser.
The isosbestic point between MI and MII was found to be at 425 nm in the excised retina at 1^{o} C. In ROS suspensions this wavelength is 418 nm (Rafferty, 1979), in digitonin/ glycerol solutions 420 nm were found (Matthews, et al., 1963) for the same temperature. The half-time of 12 ms for the MI-formation at 1^{o} C and 425 nm is in good agreement with results of v. Sengbusch (1970), who found 10.6 ms in a bovine ROS-suspension, but measured at 435 nm. The results of Rapp (1971) cannot be compared, because they were obtained at 480 nm and only results for temperatures higher than 3^{o} C are reported.
Due to light-scattering problems, most of the investigations reported for bovine rhodopsin intermediates were carried out with detergent solutions. Since the kinetics are all speeded up in detergents, (Applebury, et al., 1974; Baker, et al., 1977; Stewart, et al., 1977) no reasonable comparison with these data can be made.

3.3.4 MI-decay measurements

The decay of MI has to be measured at a wavelength, where the following intermediate, MII, is isosbest with rhodopsin, i.e. at P4, fig. 14. The expected signal will suffer from the same limitations due to small molar extinction, as already mentioned in the last chapter.

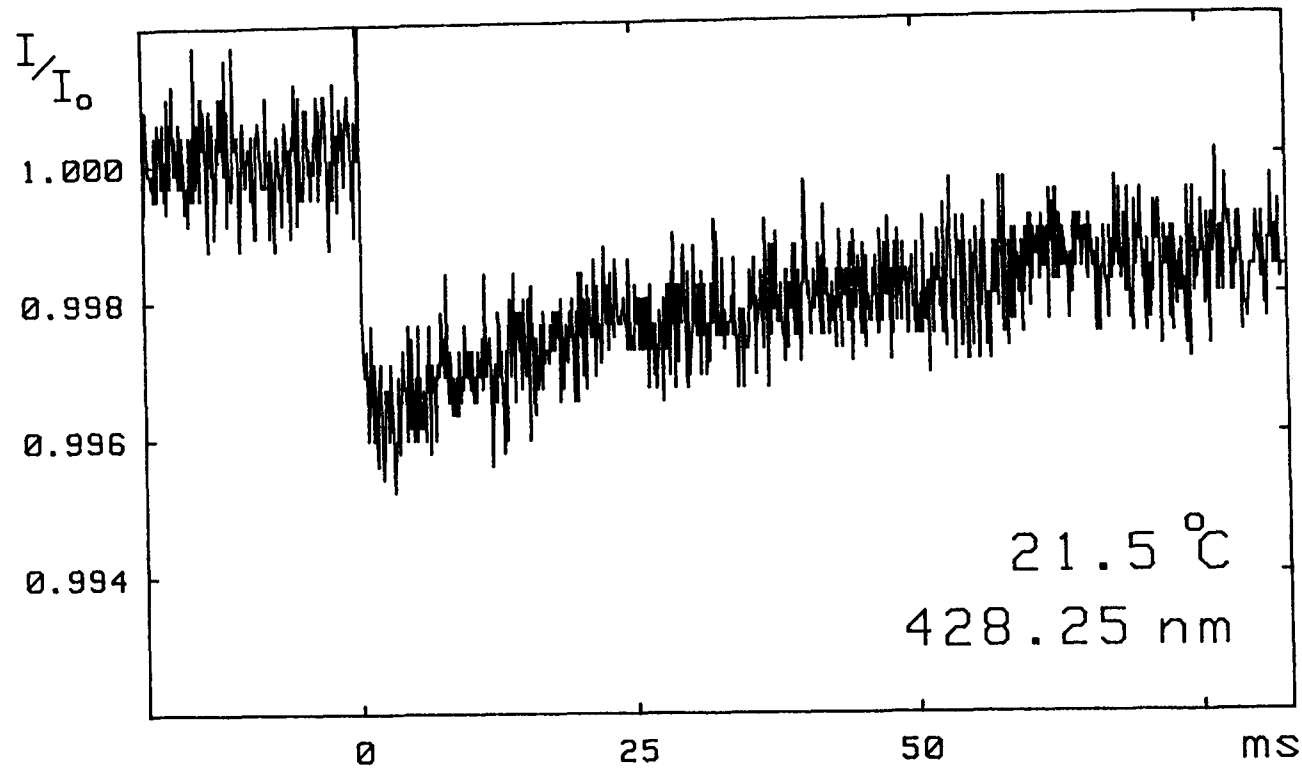


Fig. 17: MI-decay; single flash experiment, first flash record. Transmission step caused by formation of Lumirhodopsin; further small decrease due to MI-formation.

There is, however, one advantage: The isosbestic point can be easily identified because at this point the signal has to return to the preflash transmission level. The signal should be composed of one step of absorbance increase (formation of Lumirhodopsin) and a further increase (formation of MI) which is followed by the decay to be determined. Measurements were made between 426 nm and 432 nm and in the temperature range 5° C to 37° C; an example is shown in fig. 17. A rather strong temperature dependence of the isosbestic point was found. It turned out to be difficult to obtain records at the exact wavelength*. If the exact wavelength of the isosbestic point

*The flash-blocking interference filter had to be inclined at a certain angle to yield a transmission maximum for the required specific wavelength. This wavelength had to be matched by the measuring light.

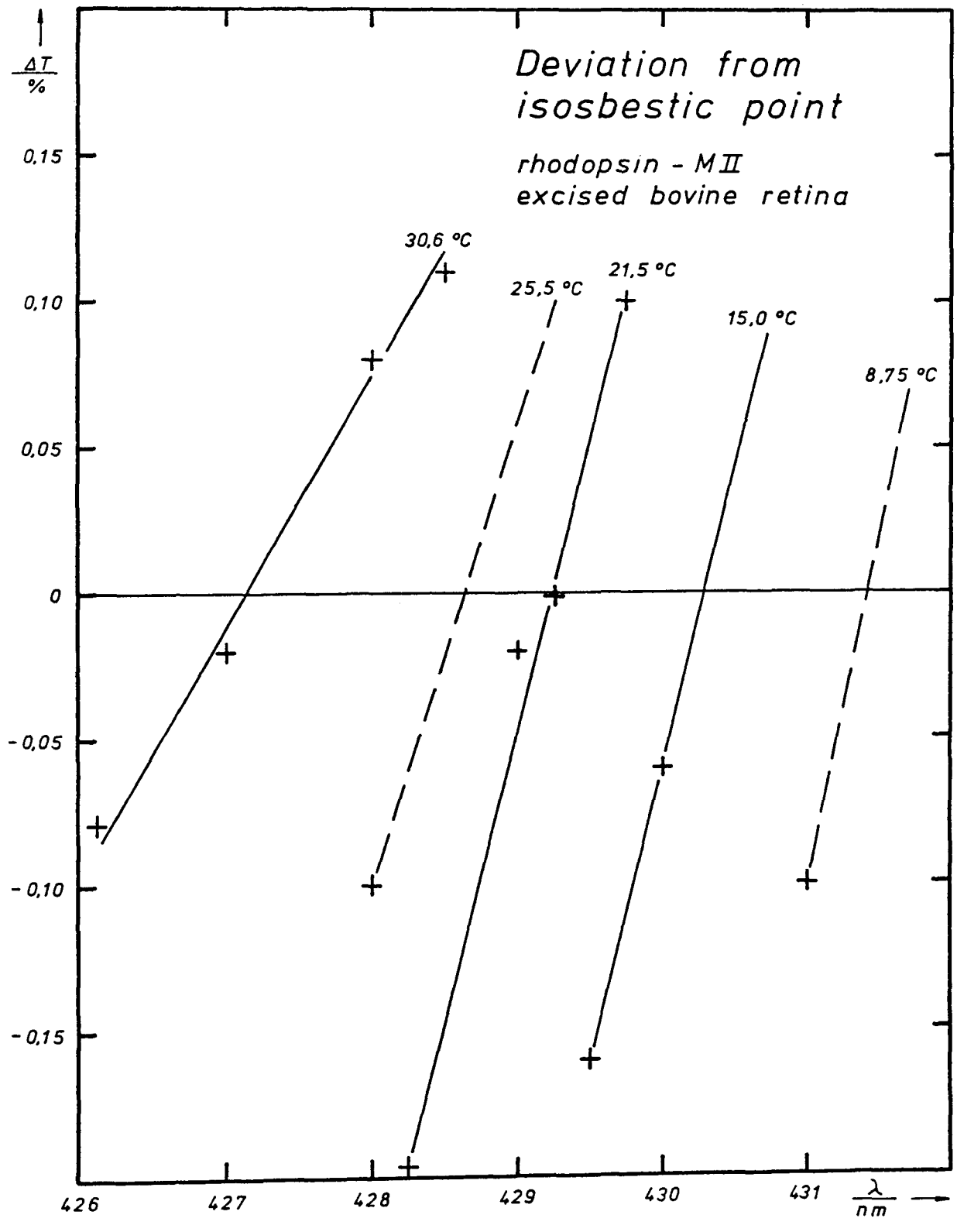


Fig. 18: Difference between the preflash and the final transmission versus wavelength for different temperatures. Dashed lines denote extrapolation.

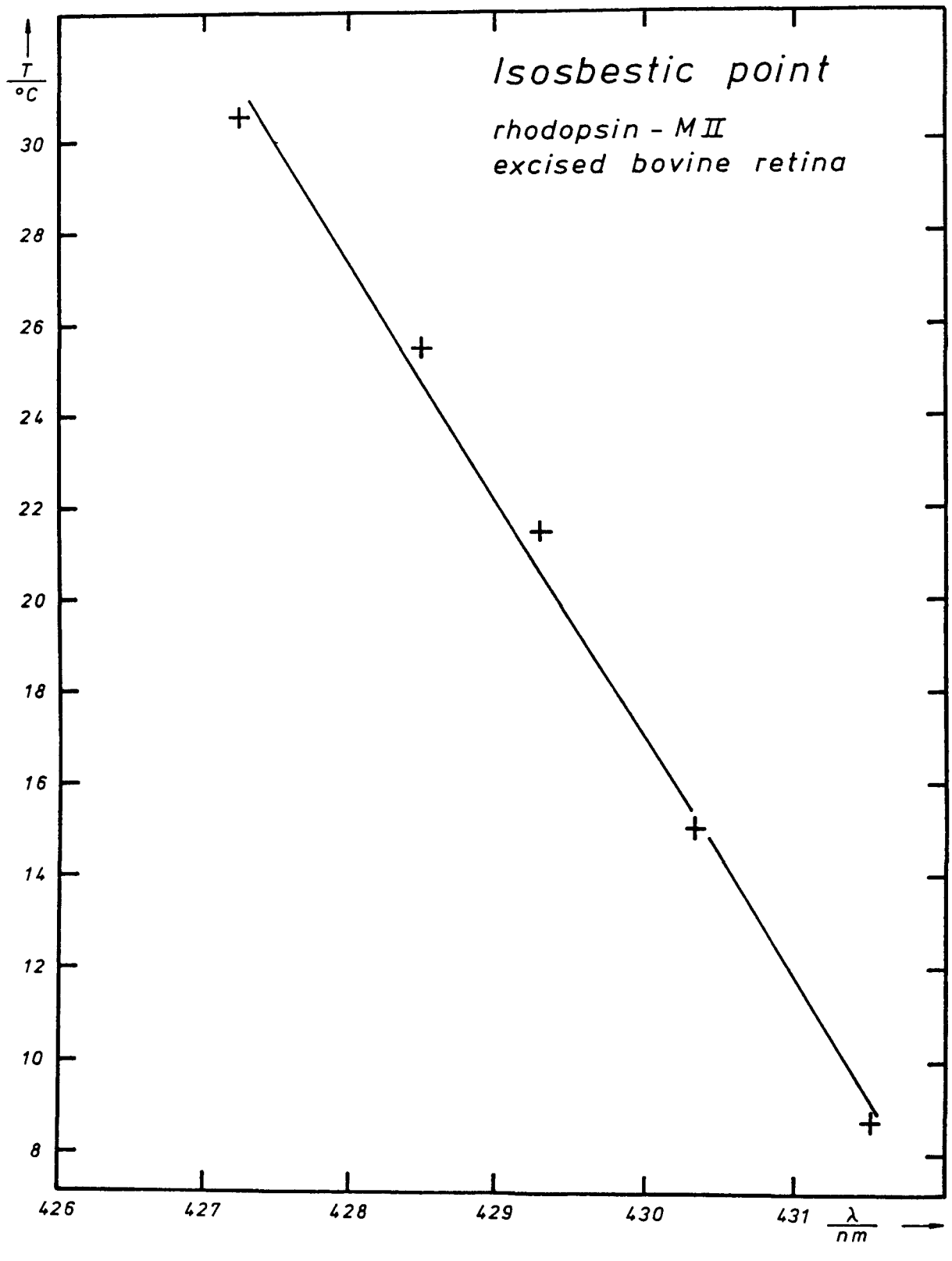


Fig. 19: Temperature dependence of the isosbestic point between rhodopsin and MII.

at a certain temperature was missed by even half a nm, the records showed that the transmission did not return to the preflash level and a deviation remained, see fig. 17. The deviation can be expressed as a transmission difference, measured in % ΔT, positive or negative according to the direction of the deviation. In fig. 18 this transmission difference is plotted against corresponding wavelengths for five different temperatures.

From this figure the wavelengths for the isosbestic point were taken by interpolating between results obtained at the same temperature and slightly different wavelengths or by extrapolation to the baseline. This results in fig. 19, where the estimated wavelength of the isosbestic point is plotted against the temperature. A hypsochromatic shift roughly proportional to the temperature is found. This can be interpreted as an increase in the amount of MII for higher temperatures. The isosbestic point between MI and MII, P3, is situated at a shorter wavelength than P4, whose temperature dependence I have just discussed. A shift in the MI/MII equilibrium towards MII results in an absorption decrease of MII at P4, meaning a hypsochromatic shift for this point. This may be regarded as part of an answer to a question raised by Abrahamson and Wiesenfeld (1972), asking whether the MI/MII equilibrium is also found under physiological conditions.

Records which are composed of more than one exponential (like those obtained for the MI-decay) are rejected by the present computer program. A graphic analysis of the MI-decay records was therefore carried out. The obtained half-times for seven records at five different temperatures are shown in an Arrhenius-plot in fig. 20. A preliminary description by half-times is preferred because in this way kinetics can be described without previous determination of their order; compare Rafferty (1979) and Nöll (1974). The results will be compared to those obtained for the MII-formation and discussed in the following chapter.

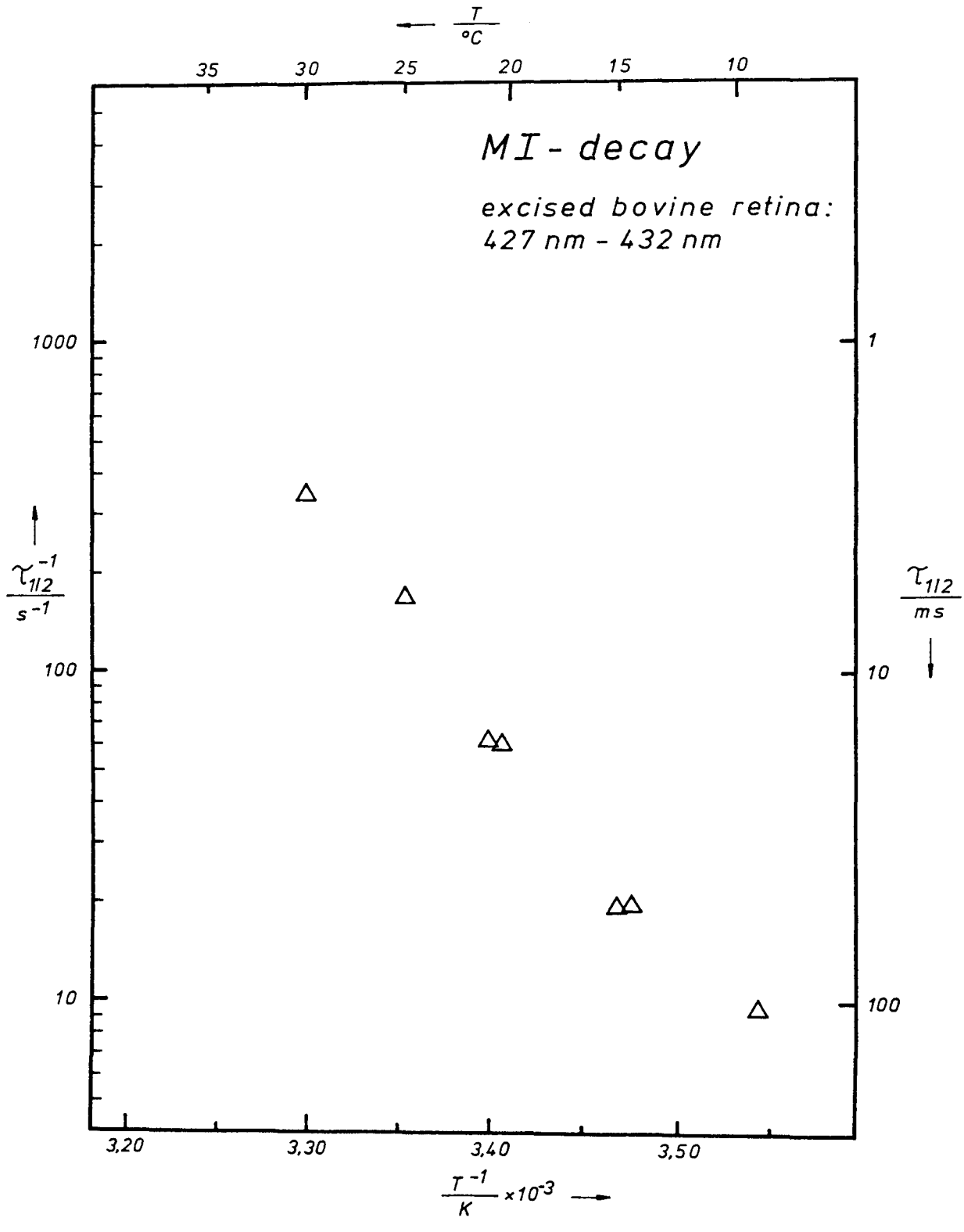


Fig. 20: Arrhenius-plot of graphically derived half-times of the MI-decay.

3.3.5 MII-formation measurements

A lot of divergent results on this topic have been reported; for a review see Ostroy (1977). There is general agreement on the time scale of the formation; the exact description of the kinetics still remains doubtful. Different results for the formation kinetics of MII certainly originate in part from different experimental conditions like the applied method of preparation, different solutions or the amount of rhodopsin bleached - to mention only a few. In my own experiments it was tried to approach physiological conditions as far as could be realized. Excised retinae were taken and only the record of the first flash-induced reaction is evaluated if not mentioned otherwise. No averaging is applied to avoid a mixture of records from partly bleached samples; for the same reason dark adaptation of the animals was performed. The amount of bleached rhodopsin was kept below 10 % and a series of experiments with reduced turnover was carried out. Certainly this is still far above any physiological level; further improvement in signal processing methods and of light detecting devices may help to reduce the amount of bleached rhodopsin in the future. Compared to former investigations the duration of the flash could be reduced to 6 ns because a dye laser was available. Under these circumstances only Bathorhodopsin appears in the same time range as the flash (Busch, 1972). Thus photoregeneration of later intermediates can be excluded.

The wavelengths for optimal measurements of the MII-formation have already been discussed (section 3.3.3 Isosbestic points). The obtained results will now be described. All samples were in good electrophysiological condition, proved by measuring the a-wave of the LRP as a response to weak red light ($\lambda \sim 640$ nm) before and after the flash spectroscopic experiments.

a) Measurements at 378 nm

Analysis of records obtained at 378 nm revealed that the formation of MII cannot be described by a single first order reaction. By no means a curve fit with one exponential was achieved. Therefore only half-times are reported here and a

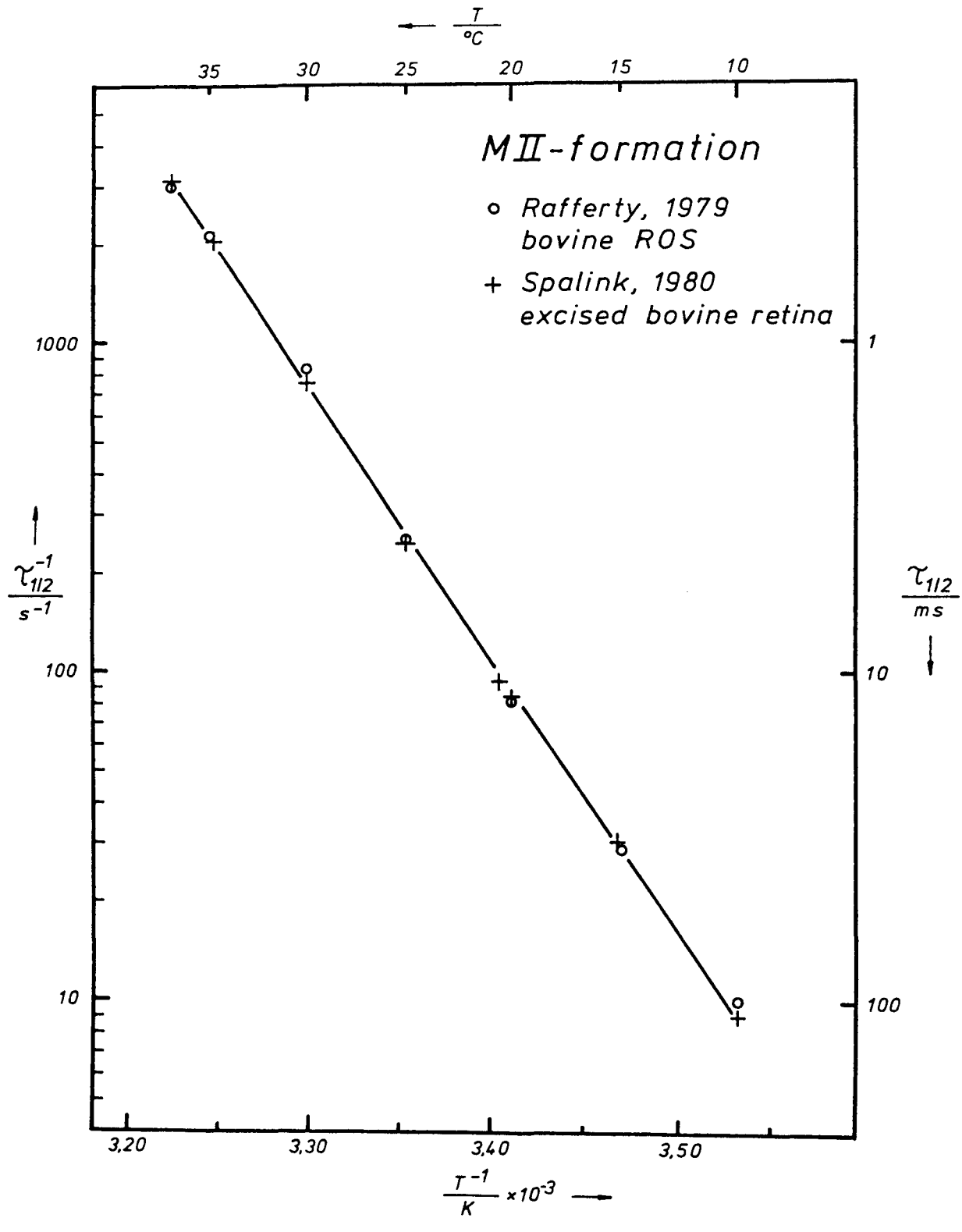


Fig. 21: Arrhenius-plot: graphically derived half-times of the MII-formation in excised bovine retinae compared to those in bovine ROS

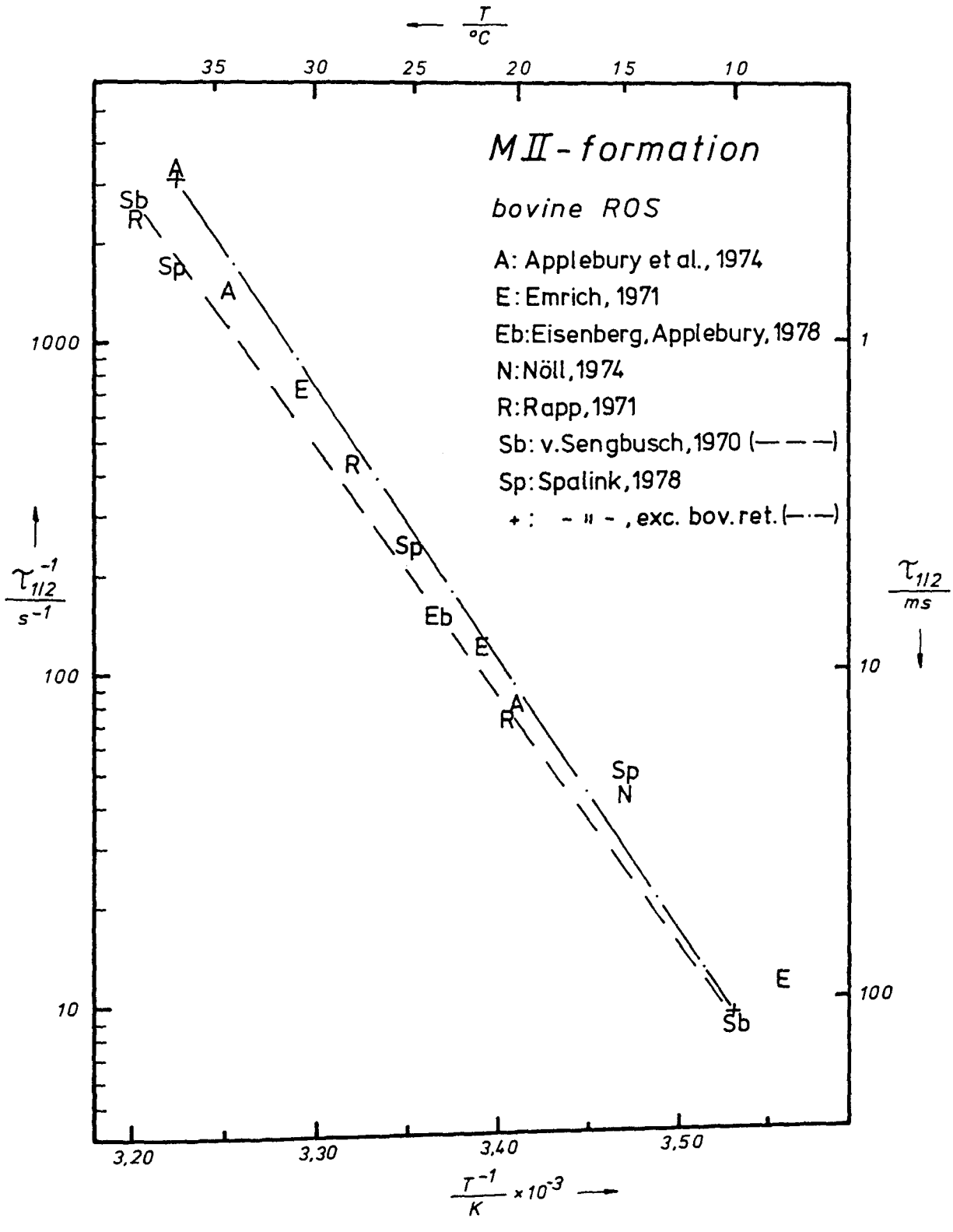


Fig. 22: Arrhenius-plot: Comparison of MII-formation half-times from different authors.

more distinctive analysis will be performed by a computer program which is in preparation. An Arrhenius plot, fig. 21, shows that a complete correspondence is achieved between half-times graphically determined here for excised retinae and those obtained by Rafferty (1979) for bovine ROS suspensions (70 mM phosphate buffer, pH 6.88). From this correspondence of the formation kinetics it can be concluded that concerning the environment of the rhodopsin molecule there seems to be no important difference between excised retinae and ROS preparations (which followed a method developed by McConnell, 1965).
In a second Arrhenius plot in fig. 22 the half-times obtained with excised retinae are compared to results with ROS suspensions published up to now, and own unpublished results obtained following the method described by Rafferty (1979), but using a 70 mM Imidazole buffer of pH 6.8.

b) Measurements at 506 nm
At first, records obtained at this wavelength were evaluated like those of 378 nm. The confusing result was a larger half-time for the 506 nm records. Rhodopsin seemed to decay to MII with a slower reaction than the appearance of MII at 378 nm. A computed analysis showed that at least 95 % of a 506 nm record, corresponding to an observation time of three life times, can be described by a single first order reaction. In a premature evaluation of the 378 nm signals it was tried to separate them into two exponentials, a possibility proposed by several authors, see Stewart, et al., 1977. Although the computer program was not appropriate for this attempt, rough estimates for the half-times of a slower component were obtained. In fig. 23 these half-times are shown together with computed results for the 506 nm signal and graphically derived half-times for the MI-decay in an Arrhenius plot. Two conclusions are rather evident:
1.) There seems to be little doubt, that the slow component appearing at 378 nm and at 506 nm are the same. In addition, the MI-decay fits into the time range where the MII-formation occurs. To assure these findings, more detailed studies of the

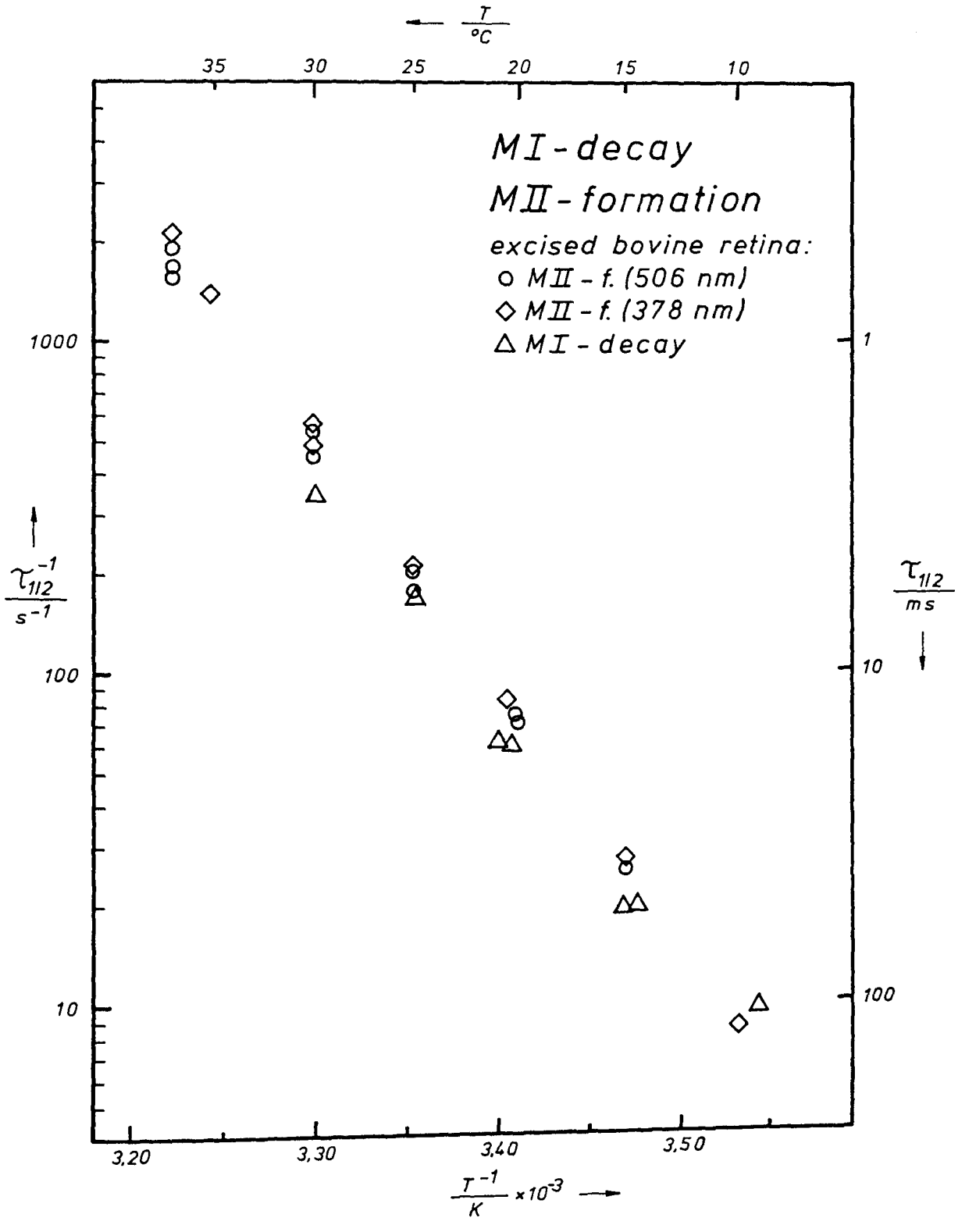


Fig. 23: Arrhenius-plot: MI-decay compared to the MII-formation measured at different wavelengths.

MI-decay with higher amplitude resolution are obviously required.
2.) At 378 nm a further, faster component seems to be involved, with increasing participation in the signal amplitude for higher temperatures.
To confirm the fact that the observed differences between the 378 nm signal and the 506 nm signal did not result from the application of different cells and from (most improbable) different temperatures, the following experiments were performed.

c) Measurements at 378 nm and at 506 nm
For two temperatures, 30° C and 20° C, measurements with two cells for each temperature were performed under identical conditions. The first cell was taken to measure the MII-formation at 378 nm with the first flash and at 506 nm with the second one. For the second cell this sequence was inverted, with the temperature-controls unchanged: MII-formation at 506 nm with the first flash and at 378 nm with the second flash. All flashes had the same wavelength of 495 nm, of course. No significant differences could be found between the first and the second records with the computational methods available at that time.

d) Measurements with reduced flash intensities
To find out if the formation kinetics of MII are influenced by different flash intensities, two different absorption filters (Schott NG) with a transmission of 54.5 % and of 25 % at 500 nm were used to reduce the intensity of the laser flash. Experiments were performed at 372.5 nm, at 378 nm and at 506 nm. The analysis is postponed until the multiexponential analysis program has been developed. An example of the records obtained is shown in fig. 24.

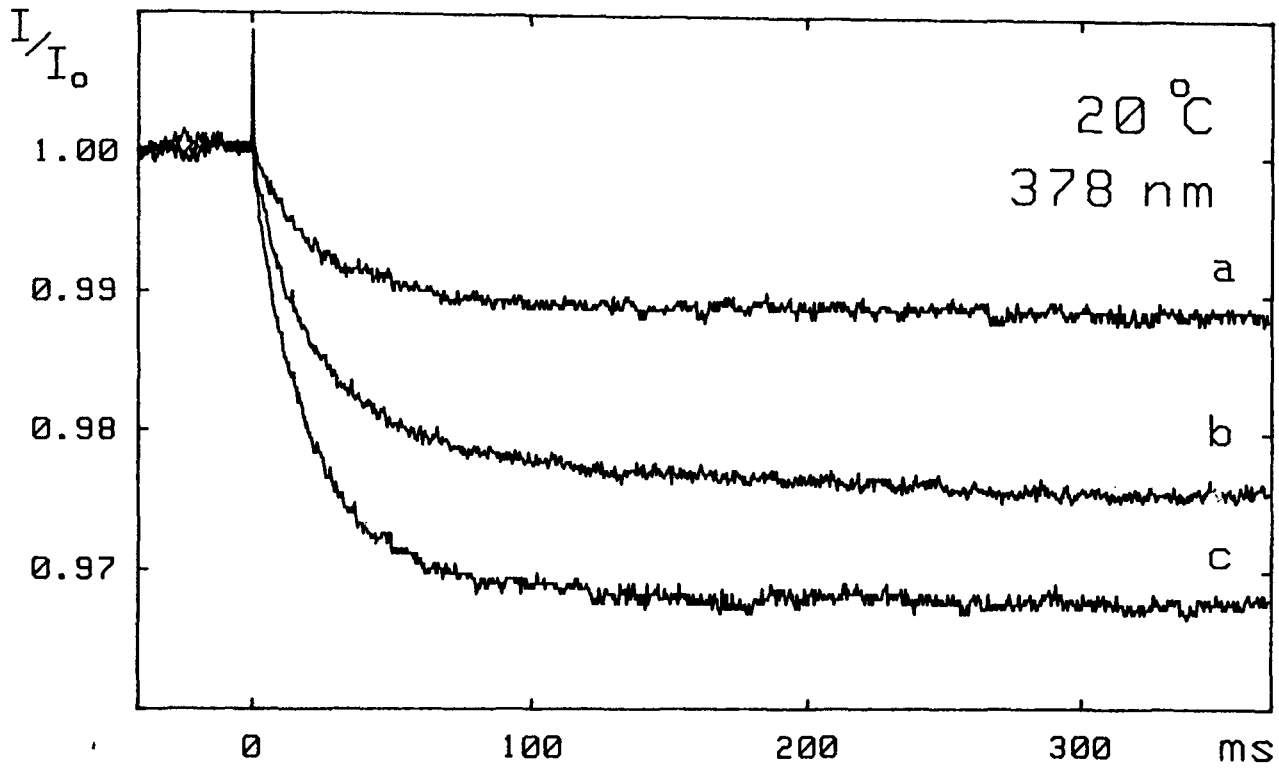


Fig. 24a: MII-formation: a) first flash 25 % flash intensity b) second flash, 54,5 % flash intensity c) third flash, full flash intensity.

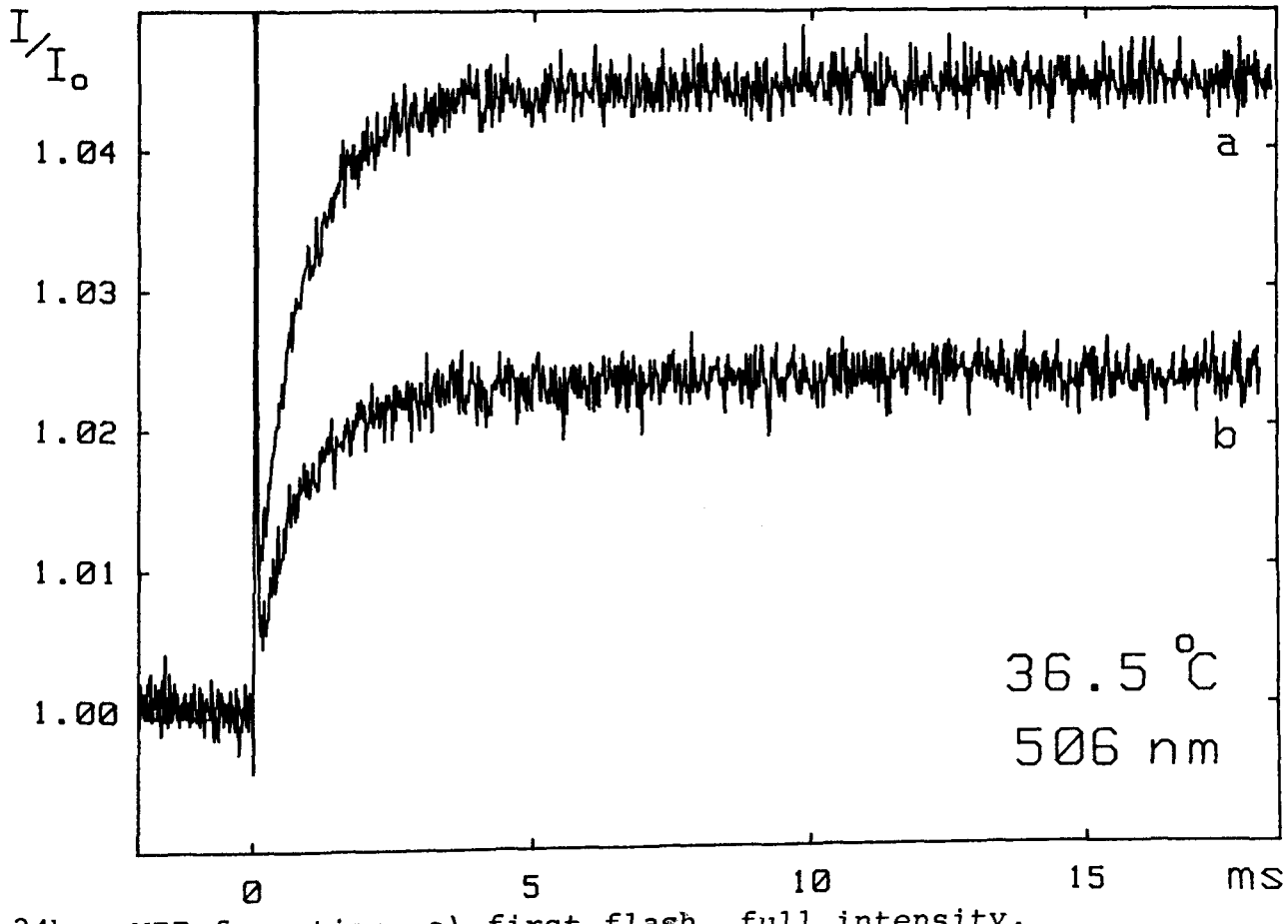


Fig. 24b: MII-formation: a) first flash, full intensity, b) second flash, 54,5 % intensity.

3.4 Simultaneous recordings

Transmission changes at 378 nm and transretinal voltage changes were simultaneously recorded, fig. 25. In 1969 it had already been found that part of the early receptor potential, the R2-response (Cone, 1965), coincides with the formation of MII. Half-time and temperature dependence were shown to closely match the formation of MII; however no direct correlation was found (Cone and Cobbs III, 1969). The origin of this electrical signal was proposed to be a rotation of molecular dipoles (Arden and Ikeda, 1965), a proposition later discussed in detail by Cone (1969). If this hypothesis is true, a direct coupling between spectroscopically observable changes of the molecular structure and electrical signals should be measurable. Under this aspect the present flash-spectroscopic investigation was undertaken to provide detailed kinetic information on probably involved intermediates like MI and MII. It was shown in section 3.3.5 that the formation of MII measured at 378 nm might be described by a sum of two first order reactions. The simultaneously recorded ERP, fig. 25, shows two different responses: a cornea-positive peak, the rise of which could not be resolved, and a second, negative one decaying within the same time range as the formation of MII. Detailed studies have revealed, that these two parts of the ERP have rather different properties. When the temperature is lowered, the R2-response decreases strongly, whereas tne R1-response can be measured even at 0^{o} C (Pak and Cone, 1964). This indicates that their origin might be rather different. A model proposed by Govardovskii (1978) is also based on different assumptions concerning the amount of charge displacements for the generation of R1 and of R2. The model is able to reproduce the changes of the ERP waveform for temperatures between 10^{o} C and 45^{o} C. A separation of the two responses in experiments at physiological temperatures has not been reported up to now. Assuming that the R1-response behaves at 37^{o} C like it does below 5^{o} C, i.e. that it can be described by the rise and decay of the same amplitude, the R2 response might be regarded as a separate signal at this temperature.

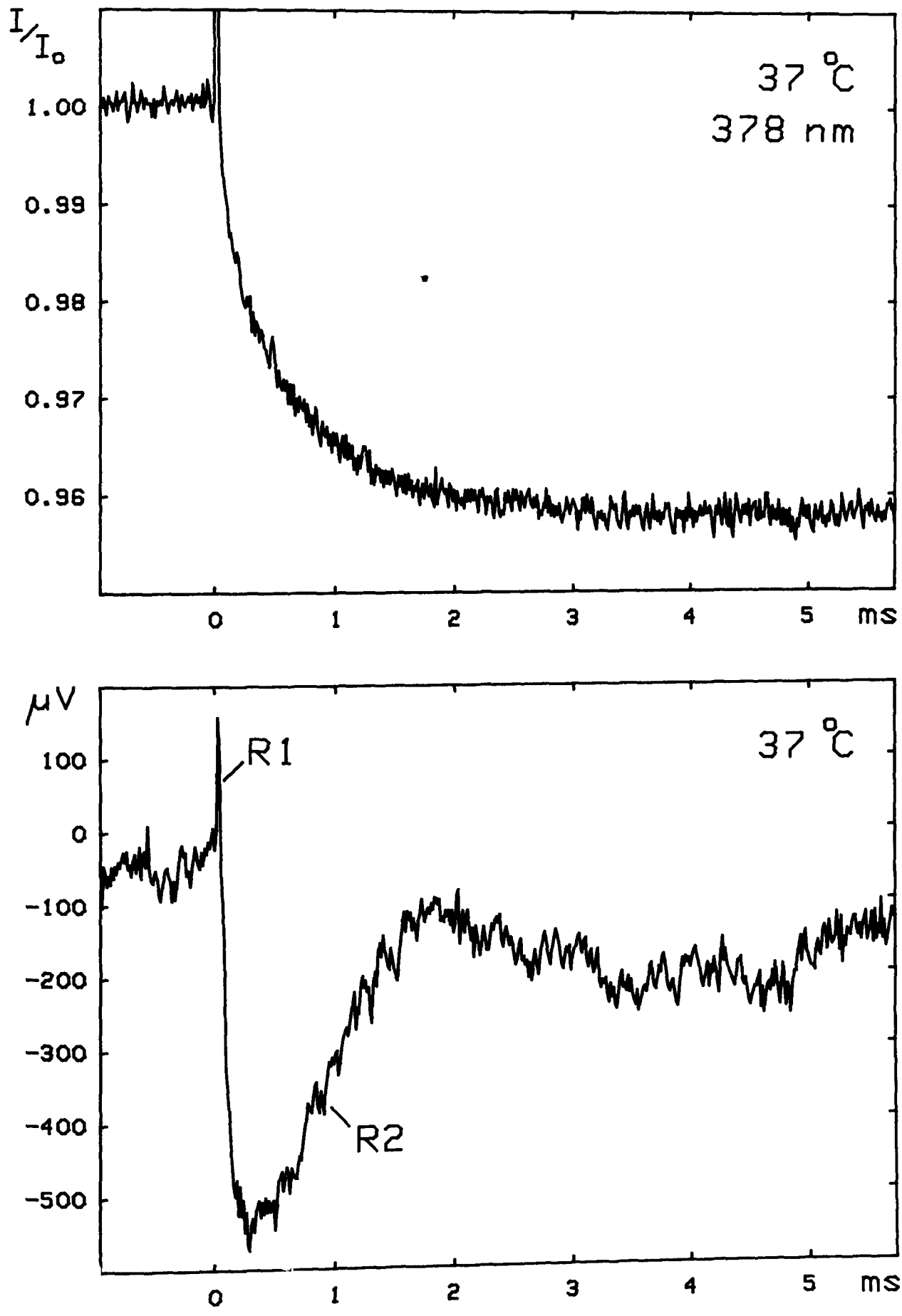


Fig. 25: MII-formation and ERP simultaneously recorded: single flash experiment, first flash record.

A computer analysis of my experimental results shows that under these circumstances the R2-response can be closely fitted by the difference of two exponentials with time constants identical to those obtained for the formation of MII. A description of this correlation is given in appendix D, representing a short communication submitted to the journal "Biophysics of Structure and Mechanism" (accepted in February 1980). If an approximation of the R1-response, based on the assumption mentioned before, is added to the published one, a complete fit of the ERP at 37° C is established, fig. 26. Future experiments with higher amplitude resolution or more intense flashes will have to show whether this correspondence can also be found for temperatures below 37° C.

From the simultaneous measurements it cannot be concluded that there is a causal linkage between the formation of MII and the R2-response. For bovine retinae, it is known that in the region of the area centralis rods and cones are found with a ratio of 15 : 1 (rods : cones); the cones are homogeneously distributed (Krebs, 1979). Investigations of early receptor potentials in rodents (Pak and Ebrey, 1966) showed that both types of photoreceptors produced ERPs similar in waveform, time course, and flash-intensity dependence ("stimulus-response curves"). The authors concluded that the ERP is caused by a similar mechanism in all-rod and all-cone retinae.
To decide whether the ERP measured in excised bovine retinae is caused by rods or by cones would require the measurement of action spectra for these signals.

Furthermore, a detailed investigation of the MII formation at different wavelengths in the near UV might help to find the spectroscopic analogon. The problem is complicated by the fact, that for different conditions such as higher flash intensities and/or longer flash durations other relations are found for the fast and slow components of the MII-formation. With 23 % bleached rhodopsin and a flash duration of 500 ns (30 % width) at 37° C a relation of 80/20 (fast/slow) was found (Stewart, et al., 1977) in contrast to 30/70 (fast/slow) which I found for the excised retina.

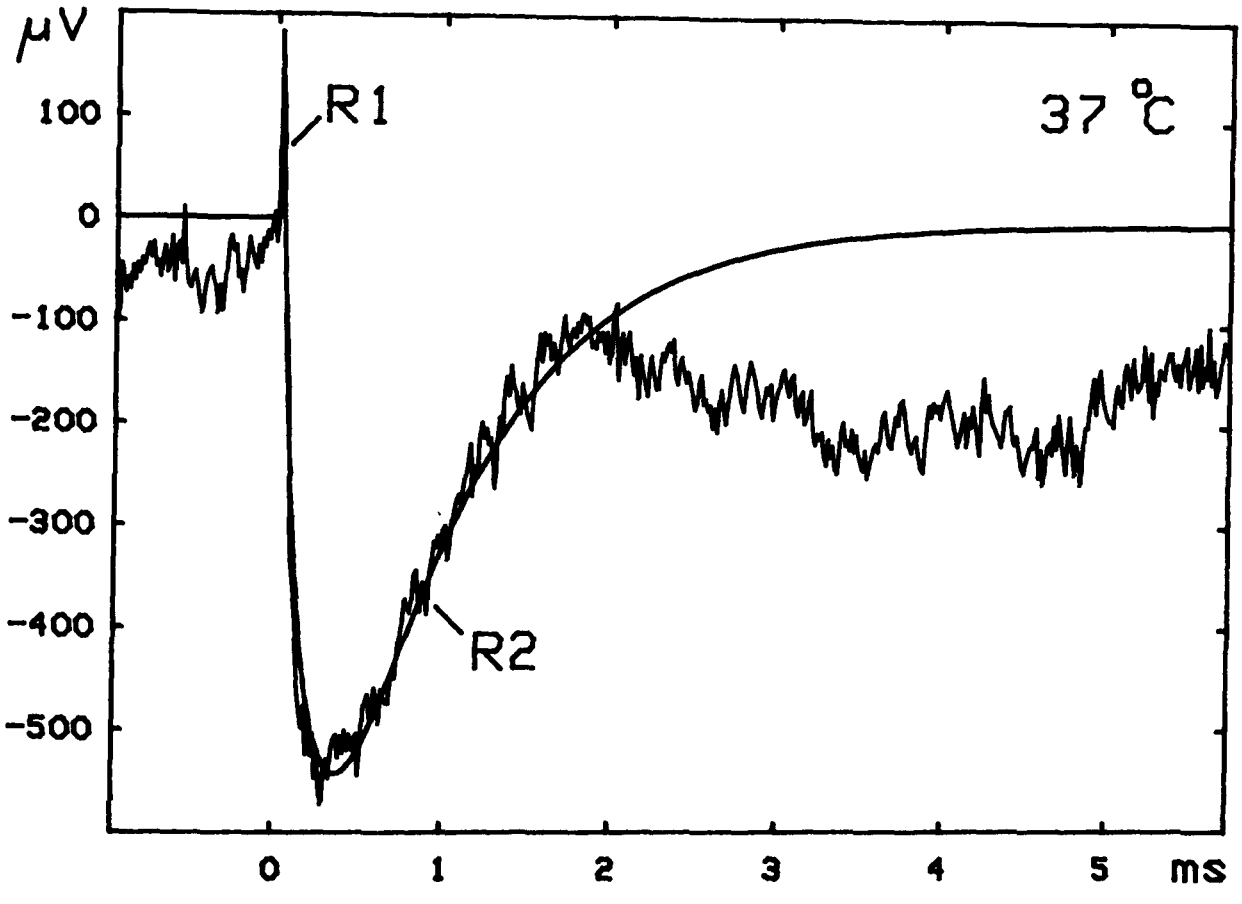


Fig. 26: Computed fit for both components of the ERP.

4. Summary

1.) A flash spectrophotometer adapted to the simultaneous recording of spectroscopic and electrophysiological parameters from excised bovine retinae was developed, with a maximum time resolution of 2 µs and an amplitude resolution of 2×10^{-4}.

2.) All experiments were performed with dark adapted eyes from blindfolded animals to avoid problems related to different light adaptation and to maximize their content of unbleached rhodopsin.

3.) A new preparation method is described, which results in selected parts of bovine retinae for spectroscopical and electrophysiological investigations. The method is gentle enough to keep the samples in a condition which allowed measurements of the late receptor potential for more than seven hours.

4.) The latency of the a-wave was determined to be not greater than 1.3 ms at 37° C.

5.) Records of the formation and decay of MI had small amplitudes and did not allow a detailed kinetic analysis. The half-times of the MI-decay between 10° C and 30° C fit into the time range of the MII-formation. From the temperature-dependent shift of the isosbestic point between MII and rhodopsin it is concluded that an equilibrium between MI and MII does exist for excised retinae.

6.) The formation of MII was traced at two different wavelengths about 120 nm apart. At both wavelengths the signals show the same first order reaction; at 378 nm an additional, faster, component is detected. The formation of MII at 378 nm can be described by a sum of two first order reactions. The origin of this difference between the 378 nm signal and the one at 506 nm is not known.

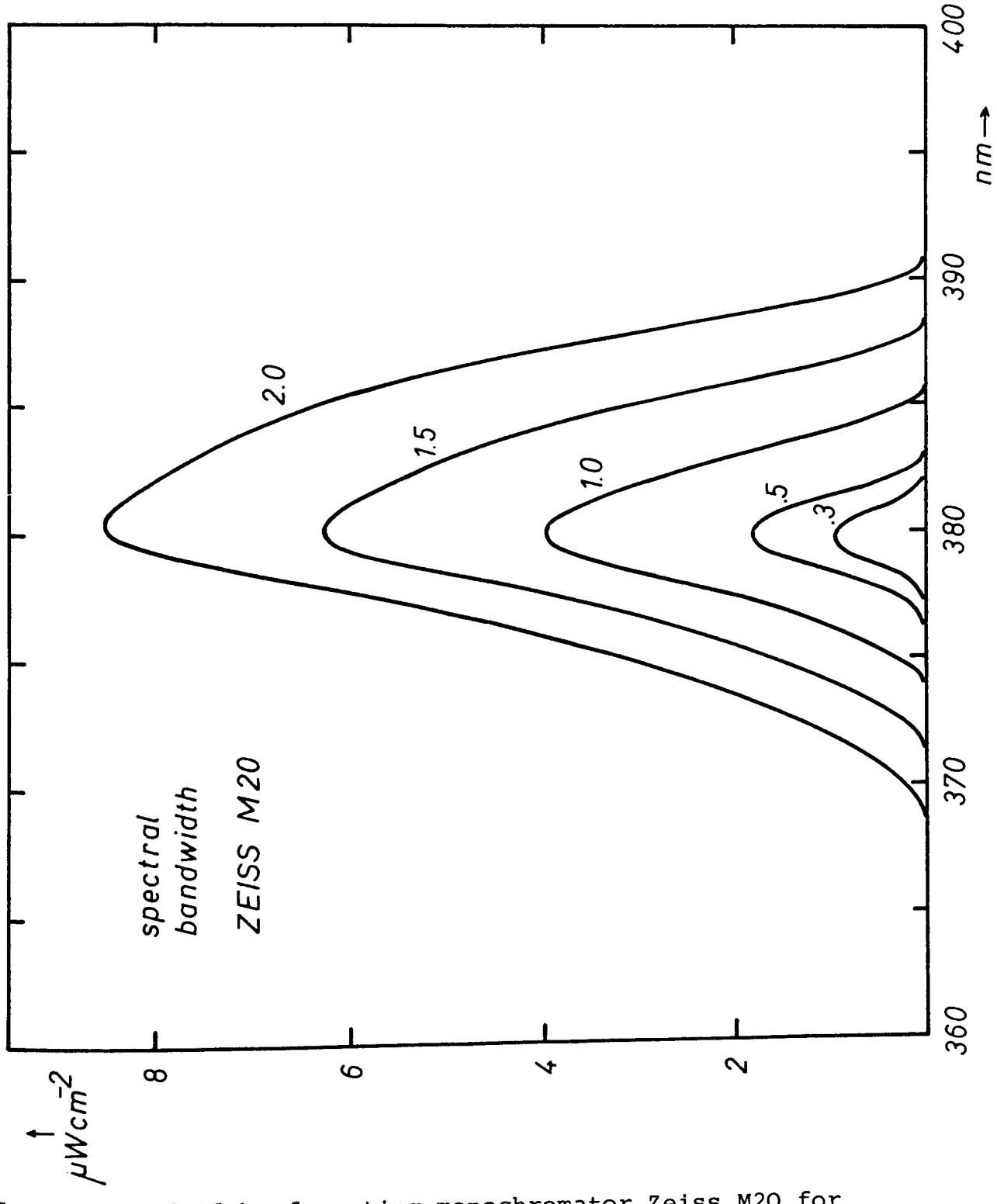
7.) For 37^{o} C a direct correlation between the R2 component of the early receptor potential and the formation of MII was found. The R2-response could be matched by a difference of two weighted exponentials, whose time constants were identical to those obtained for the MII-formation measured at 378 nm.

8.) The recorded signals were digitally processed by routines especially programmed by the author for this purpose. The data could be subjected to digital lowpass filtering, computation of their power density spectra, and determination of time constants if they represent single first order reactions.

Contents of the physiological saline:
(Kühn, 1979; Leser, 1979; Hamacher, 1979)

Na Cl	137.0	mM
K Cl	5.4	mM
$Na\ H_2\ PO_4$	1.25	mM
$Na_2\ H\ PO_4$	3.75	mM
$Mg\ SO_4$	1.0	mM
$Ca\ Cl_2$	1.0	mM
$Na\ H\ Co_3$	2.0	mM
Glucose	10.0	mM

Oxygen saturation was achieved with purified Oxygen supplied by Linde, purity: 99.998 %.



Spectralbandwidth of grating monochromator Zeiss M20 for different slitwidths (.3 to 2.0).

Digital lowpass filter procedure :

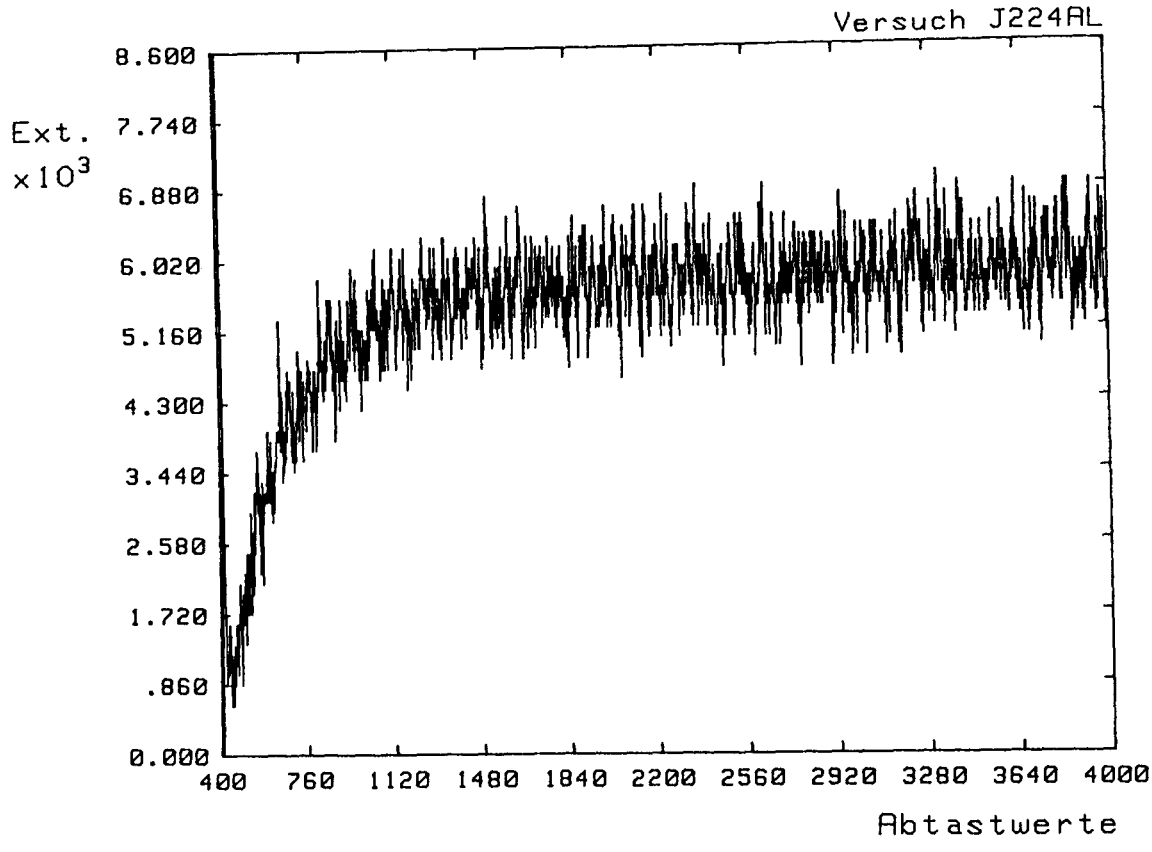


Fig. 27

At the beginning of the procedure recorded transmission changes are transformed to extinction values, fig. 27. Only values for t > 0 (following the flash) are treated. The artefact caused by scattered flashlight is shown in more detail in fig. 28 and is substituted by an appropriate computed part of an exponential based on data taken from fig. 28 interactively. The substitution is shown in fig. 29. The complete track is represented by the right half of fig. 30. The left half is a mirror picture of the right one. This is done to provide continuance at t = 0. Then the left half is multiplied, fig. 32, by the cosine function shown in fig. 31. To the end of the right half a short cosine track is added. When the whole function shown in fig. 32 is subjected to the digital lowpass filter program, two advantages are obvious:

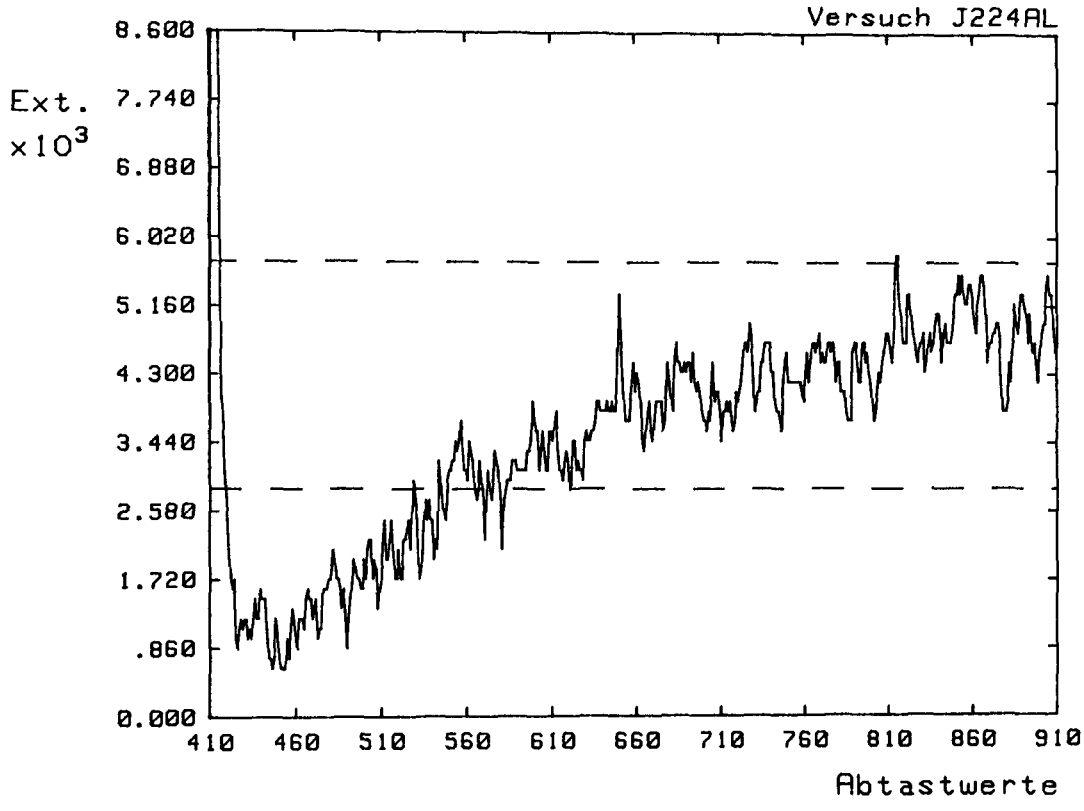

Versuch J224AL
Ext.
x10^3
8.600
7.740
6.880
6.020
5.160
4.300
3.440
2.580
1.720
.860
0.000
410
460
510
560
610
660
710
760
810
860
910
Abtastwerte

Fig. 28

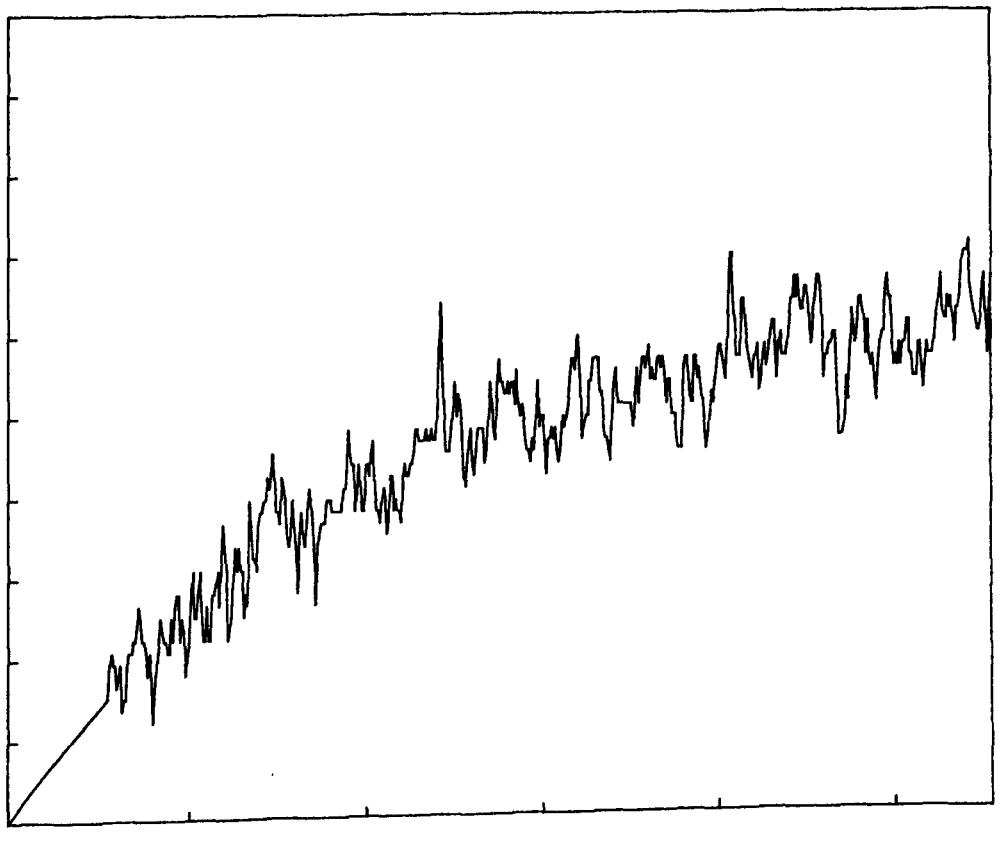


Fig. 29

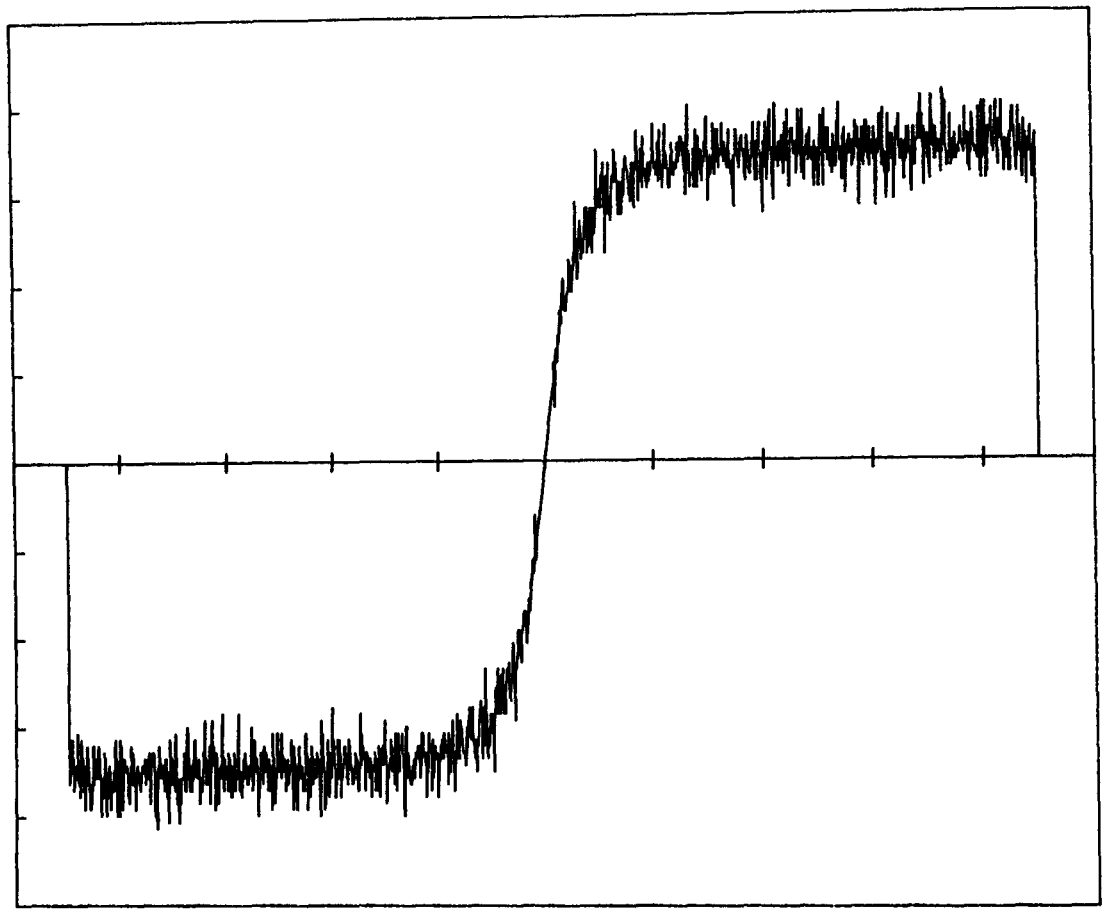


Fig. 30

1.) The smooth start of the function causes no major onset oscillations of the filter response.
2.) The continuous transition from the left to the right half allows undisturbed filtering of the right half from the beginning.

The result of the procedure is shown in red in fig. 33. The green curve is identical to the right half of fig. 30; for further evaluation (e.g. determination of time constants) only data following the substituted part are taken.

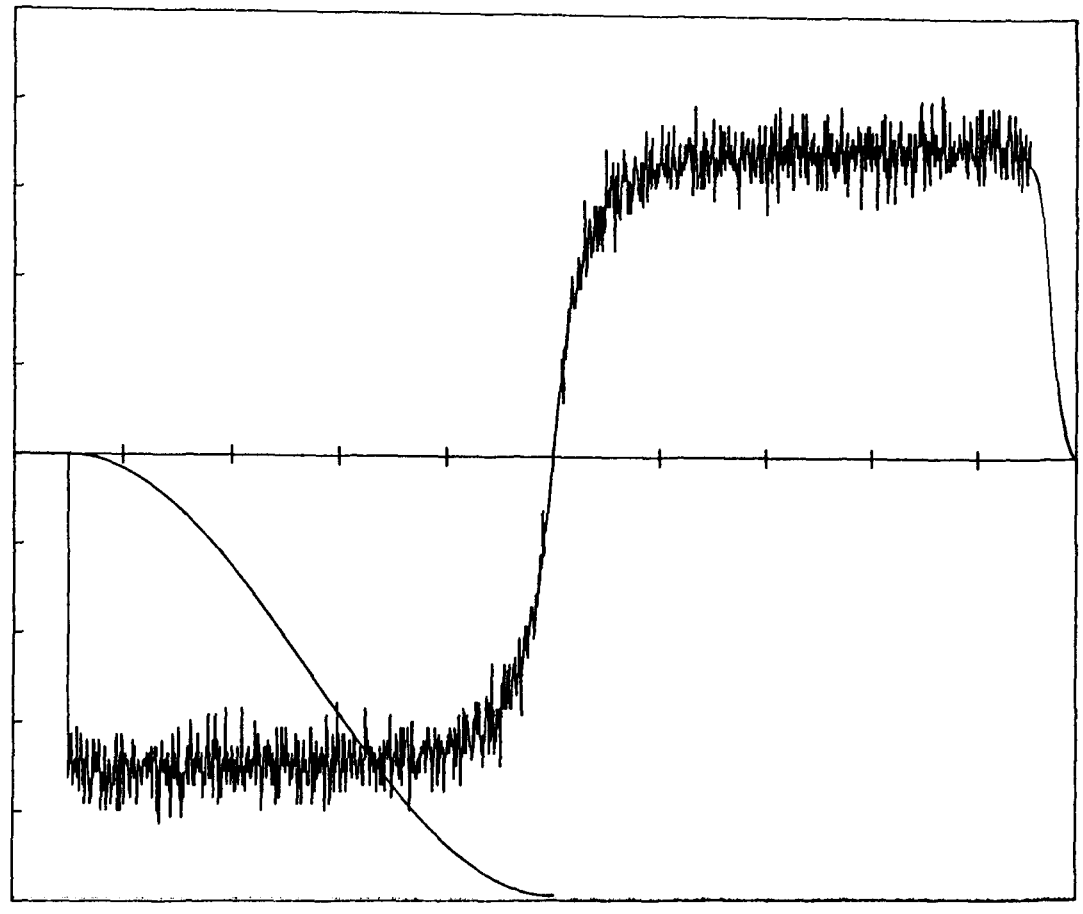


Fig. 31

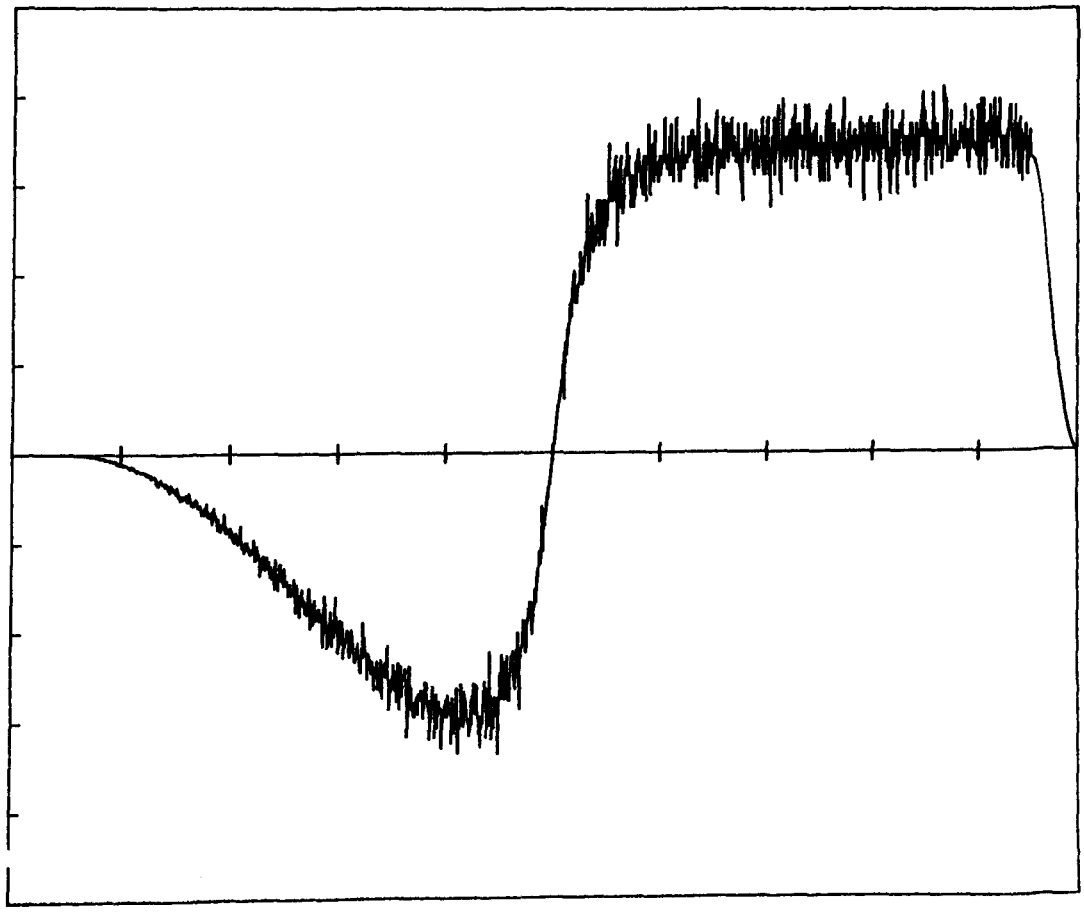


Fig. 32

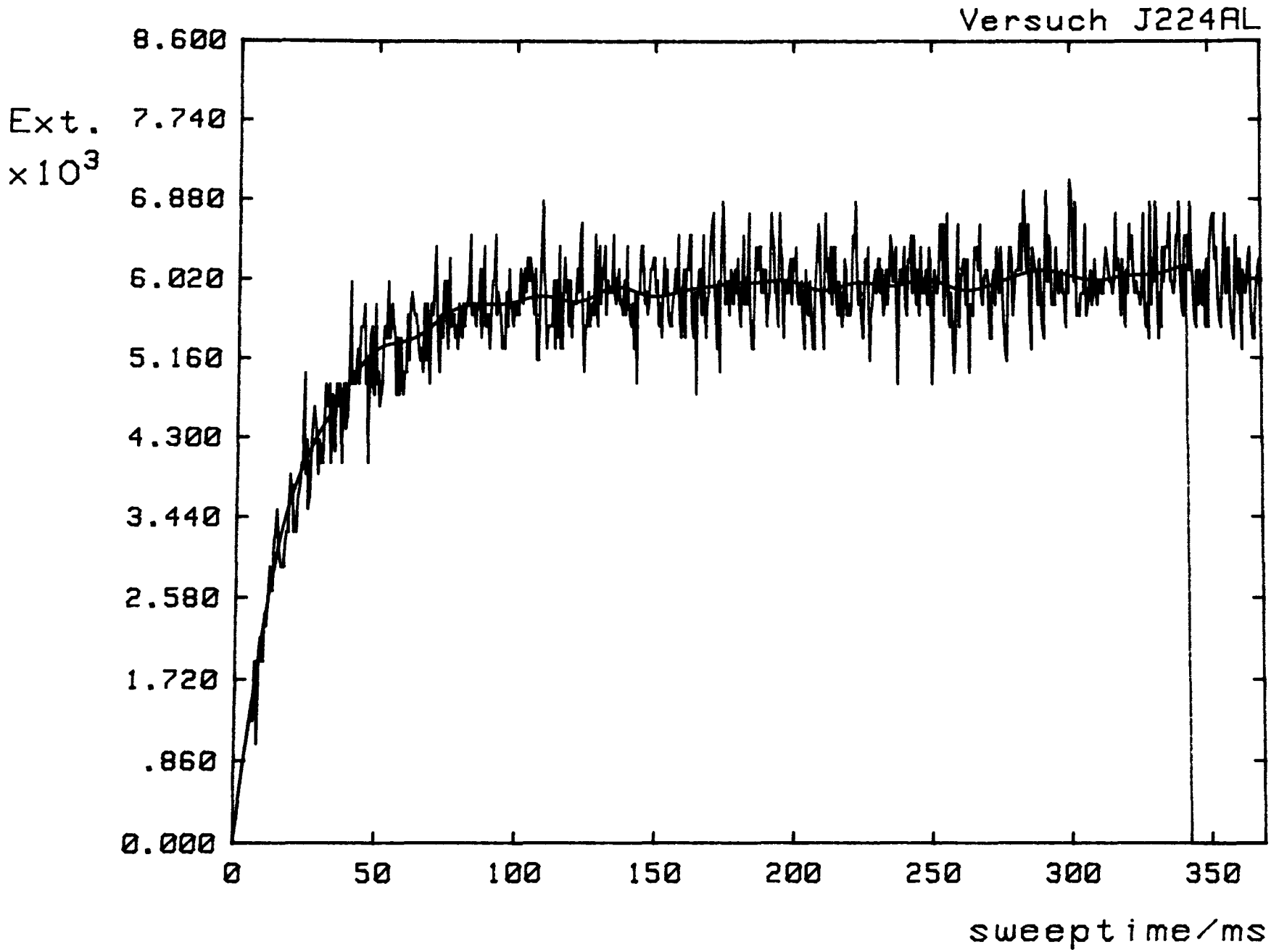

Versuch J224AL
Ext.
x10³
8.600
7.740
6.880
6.020
5.160
4.300
3.440
2.580
1.720
.860
0.000
0
50
100
150
200
250
300
350
sweeptime/ms

Fig. 33

Biophys. Struct. Mechanism 00, 000–000 (1980)

Biophysics of Structure and Mechanism

Short Communication

Direct Correlation Between the R2 Component of the Early Receptor Potential and the Formation of Metarhodopsin II in the Excised Bovine Retina

J.-D. Spalink, and H. Stieve

Institut für Neurobiologie der Kernforschungsanlage Jülich, D-5170 Jülich, Federal Republic of Germany

Abstract. The formation of Metarhodopsin II, measured spectroscopically in the excised bovine retina at 37° C, can be described by a sum of two exponentials. The R2 response of a simultaneously recorded early receptor potential can be matched by a difference of two weighted exponentials, whose time constants are identical to those obtained for the spectroscopic signal.

Key words: Flash-spectroscopy – Early receptor potential – Simultaneous recording – Bovine retina.

Since the discovery of the early receptor potential (ERP) in 1964, (Brown and Murakami 1964; Cone 1964) it has often been tried to find a relation between this fast photovoltage signal and spectroscopically observable rhodopsin intermediates. In the excised eye of albino rats, the R2 response of the ERP at 37° C was found to occur in the same time range as an absorbance change observed at 404 nm (Cone 1969) and to have a half-time and temperature dependence closely matched to the formation of Metarhodopsin II (M II), measured at 380 nm in the excised retina of the same animal (Cone and Cobbs III 1969). A detailed analysis of the formation of M II revealed that a possible description of its time course is a sum of two exponentials (see Stewart et al. 1977). The molecular nature of the probably two components is still uncertain.

We report here, that in excised bovine retinae, kept under physiological conditions, i.e., 37° C, and superfusion with oxygenated physiological saline, the formation of M II as well as the R2 response is described by the same set of two time constants, a possibility already proposed by Rüppel (1979).

Using a method described elsewhere (Spalink 1979), selected areas of bovine retinae were prepared from dark adapted animals. The samples showed a stable electrophysiological response for up to seven hours when they were kept in a superfusion cell according to Sickel (1965). The cell allowed simultaneous recordings of both absorbance and transretinal voltage changes. At 37° C, ERPs as well as late receptor potentials (a-wave) were measured following a single laser flash (495 nm, 6 ns), which bleached about 8% of the rhodopsin. The formation of M II was traced at 378 nm and 505 nm, which are isosbestic points between the precursor of M II and rhodopsin

Springer-Verlag – Heidelberg

Betr.: Bio. 155

Mskr.-Seiten: 5 Spalten: 4

Druckerei: Carl Ritter, Wiesbaden Versand: 14.3.1980

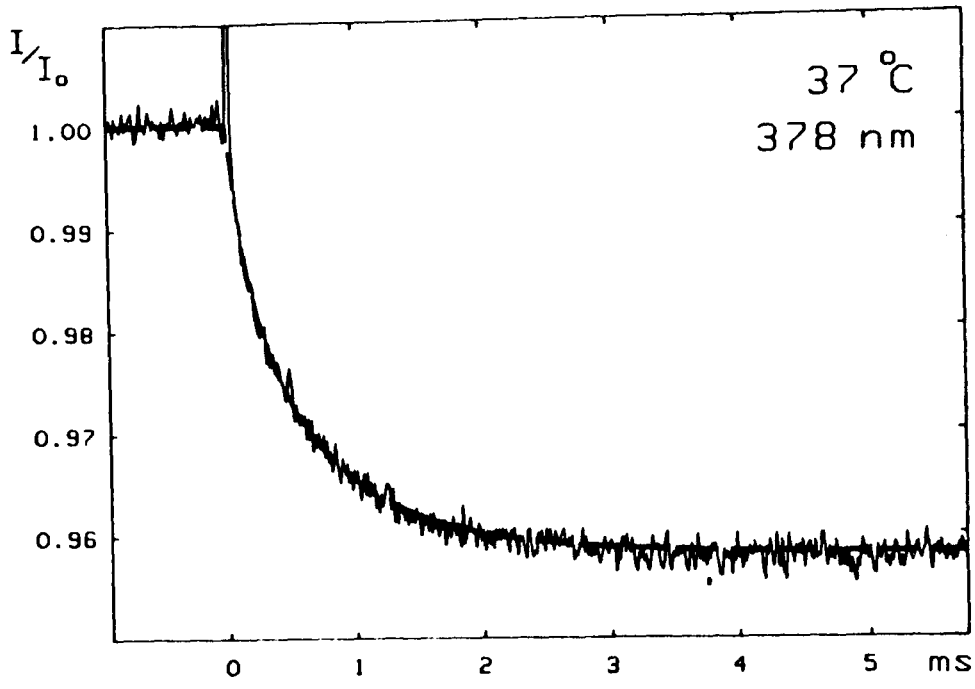


Fig. 1. Formation of M II in an excised bovine retina: transmission change vs. time, measured at 378 nm and 37° C. Single flash experiment, original data (solid line), about 8% rhodopsin bleached, laser flash (495 nm, 6 ns) delivered at t = 0, initial peak due to scattered light of the flash. Dashed line shows computed approximation with a sum of two exponentials (time constants $k_1 = 1250\ s^{-1}$, $k_2 = 5000\ s^{-1}$), weight turned out to be 30/70 (fast/slow). Bandwidth DC – 20 kHz

(Rafferty 1979). At these wavelengths the formation of M II can be measured without interference of other intermediates. Care was taken to avoid transmission changes due to light scattering (Spalink 1979).

For low temperature the formation of M II can be described essentially by one exponential, whose time constant, k1, is the same at 378 nm and 505 nm. With increasing temperature, the 378 nm signal shows an increasing contribution of an additional component, whose time constant, k2, is about four times larger than k1. At 505 nm, no such fast component can be found. For the amplitude of the R2 response a temperature dependence similar to that of the faster component was found by Pak and Ebrey (1966) and Arden and Ikeda (1968). Our analysis of the rise or decay of the R2 response at 37° C shows no strict first order kinetics. The rise seems to have about the same time constant as the fast M II component and the decay might follow the slower one. The typical shape of a R2 response is obtained, when the faster M II component is subtracted from the weighted slower one, with both functions starting at zero time. This operation together with an original ERP is shown in Fig. 2. The ERP was simultaneously recorded with the M II signal of Fig. 1.

Because of the small amplitude of the ERP the measurements at lower temperatures could not yet be evaluated.

Since two time constants for the M II formation are found not only for membrane-bound rhodopsin, but also in various detergent solutions (Stewart et al. 1977), it seems

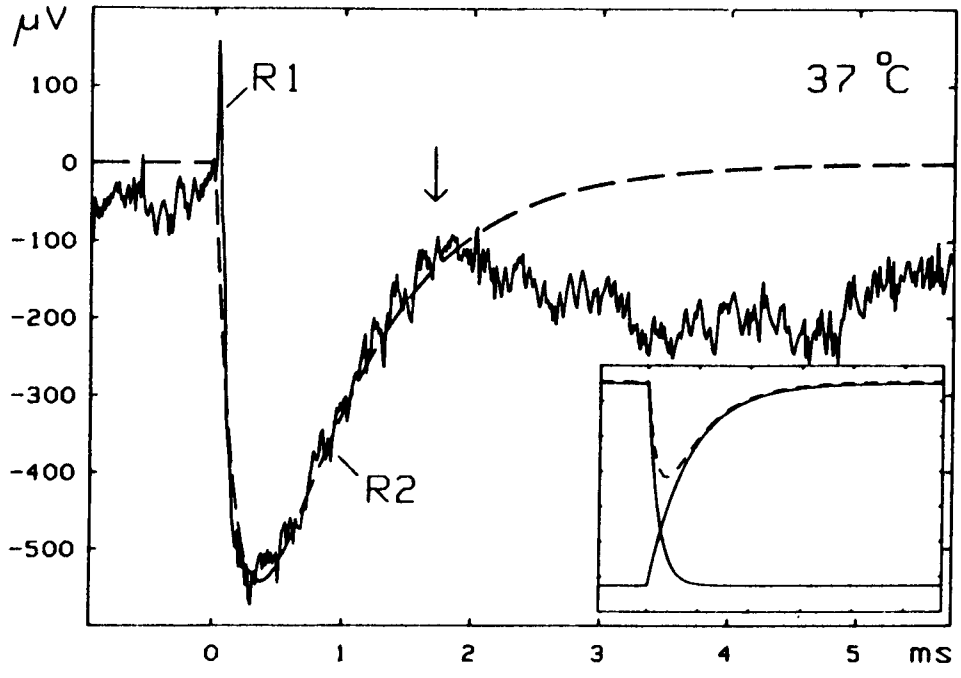


Fig. 2. Transretinal voltage change, recorded from the same retinal area simultaneously with the transmission change of Fig. 1; original data (solid line). Dashed line shows computed approximation with a difference of two exponentials (time constants k_1 and k_2, weighted 1 : 1), for further details see Fig. 1. The arrow indicates the transition between the R2 response and the a-wave, which is weak due to adaptation to the measuring light (378 nm). The insert shows a graph of the two separate exponentials and their computed difference. Bandwidth 0,1 – 20 kHz

ressonable to assume that this effect directly reflects changes in the rhodopsin molecule. It has been suggested (Cone 1969; Hodgkin and O'Bryan 1977), that the ERP is caused by changes in the dipole moment of rhodopsin. Thus it seems to be conceivable to us that the two components may represent two states of the visual pigment, which differ in magnitude and/or orientation of their dipole moment. However, the correspondence may be a mere coincidence. Hodgkin and O'Bryan (1977) showed that the ERP can be described by a capacitive transmembrane current, whose time course depends strongly on the time constant of the plasma membrane.

Acknowledgements. We want to thank W. Sperling for helpful and critical discussions, Chr. Baumann and H. Rüppel for valuable suggestions concerning the manuscript. This work was supported by the Deutsche Forschungsgemeinschaft (SFB 160).

References

Arden GB, Ikeda H (1968) The minimum latency of the a-wave. J Physiol 197:529–549

Brown KT, Murakami H (1964) A new receptor potential of the monkey retina with no detectable latency. Nature 201:626–628

Cone RA (1964) Early receptor potential of the vertebrate retina. Nature 204:736–739

Cone RA, Cobbs III WH (1969) Rhodopsin cycle in the living eye of the rat. Nature 221:820–822

Cone RA (1969) In: Reichardt W (ed) Processing of optical data by organisms and by machines. Academic Press, New York

Hodgkin AL, O'Bryan PM (1977) Internal recording of the early receptor potential in turtle cones. J Physiol 267:737–766

Pak WL, Ebrey TG (1966) Early receptor potentials of rods and cones in rodents. J Gen Physiol 49:1199–1208

Rafferty CN (1979) Light-induced perturbation of aromatic residues in bovine rhodopsin and bacteriorhodpsin. Photochem Photobiol 29:109–120

Rüppel H (1979) personal communication

Sickel W (1965) Respiratory and electrical responses to light stimulation in the retina of the frog. Science 148:648–651

Spalink JD (1979) Flash spectroscopic studies on electrophysiologically intact bovine retinae. In: Adam G, Stark G (eds) Annual meeting of the Deutsche Gesellschaft für Biophysik. Abstracts of Poster Presentations B 59. Springer-Verlag, Berlin Heidelberg New York, p 81; and (1980) Ph. D. Thesis, TH Aachen

Stewart JG, Baker BN, Williams TP (1977) Evidence for conformeric states of rhodopsin. Biophys Struct Mech 3:19–29

Received February 5, 1980/Accepted February 21, 1980

Appendix E

Tables

1) MI-decay (426 nm - 432 nm)
graphically derived half-times

°C	8.75	14.5	15.5	20.5	21.5	25	30
$\tau_{1/2}$	104.2	51.28	52.08	16.66	16.13	6.06	2.93

2) MII-formation (378 nm)
graphically derived half-times

°C	10	15	20	25	30	35	37
$\tau_{1/2}$	105.26	33.3	11.76	4	1.33	0.5	0.325

3) MII-formation (378 nm)
computed slow component half-times

°C	10	15	20.5	25	30	35	37
$\tau_{1/2}$	117.65	36.23	12.05	4.76	2.02 1.78	0,71	0,47

4) MII-formation (506 nm)
computed half-times

°C	15	20	20.2	25	30	37
$\tau_{1/2}$	39.2	14.18	13.1	5.68 5.07	2.25 1.88	0.64 0.61 0.53

6. Literature

Abrahamson, E.W.; Japar, S.M.:
Principles of the interaction of light and matter
in: Loewenstein, W.R. (ed.)
Handbook of Sensory Physiology, Vol. I, Springer-Verlag,
Berlin, Heidelberg, New York, 1971

Abrahamson, E.W.; Wiesenfeld, J.R.:
The structure, spectra and reactivity of visual pigments
in: Dartnall, J.A. (ed.)
Handbook of Sensory Physiology, Vol. VII, 1, Springer-Verlag,
Berlin, Heidelberg, New York, 1972

Abrahamson, E.W.:
The kinetics of early intermediate process in the photo-
lysis of visual pigments
in: Biochemistry and Physiology of visual Pigments
Langer, H. (ed.), 47 - 56, Springer-Verlag, New York, 1973

Applebury, M.L.; Zuckermann, D.M.; Lamola, A.A.; Jovin, T.M.:
Rhodopsin
Purification and recombination with phospholipids assayed
by the metarhodopsin I - metarhodopsin II transition
Biochemistry 13, 3448 - 3458, 1974

Arden, G.B.; Ikeda, H.
A new property of the early receptor potential of rat retina
Nature 208, 1100 - 1101, 1965

Arden, G.B.; Ikeda, H.:
The minimum latency of the a-wave
J. Physiol. 197, 529 - 549, 1968

Baker, B.N.; Williams, T.P.; Donovan, W.J.:
Extractant effects on some properties of rhodopsin
Vision Res. 17, 1157 - 1162, 1977

Baumann, Ch.; Bender, S.:
Kinetics of rhodopsin bleaching in the isolated human
retina
J. Physiol. 235, 761 - 773, 1973

Brown, K.T.; Murakami, M.:
A new receptor potential of the monkey retina with no
detectable latency
Nature 201, 626 - 628, 1964

Busch, G.E.; Applebury, M.L.; Lamola, A.A.; Rentzepis, P.M.:
Formation and decay of prelumirhodopsin at room temperatures
Proc. Nat. Acad. Sci. USA, Vol. 69, No. 10, 2802 - 2806, 1972

Cone, R.A.:
Early receptor potential of the vertebrate retina
Nature 204, 736 - 739, 1964

Cone, R.A.:
The early receptor potential of the vertebrate eye
Cold Spring Harbour Symp. Quant. Biol. 30, 483 - 491, 1965

Cone, R.A.:
The early receptor potential
in: Reichardt, W. (ed.)
Processing of optical data by organisms and machines
Academy Press, New York,1969

Cone, R.A.; Cobbs III, W.H.:
Rhodopsin cycle in the living eye of the rat
Nature 221, 820 - 822, 1969

Eisenberg, A.; Applebury, M.L.:
Meta I Meta II transition of bovine rhodopsin: kinetics and proton flux
6th Annual Meeting, Am. Soc. f. Photobiol., Progr. a. Abstr.
Univ. of Vermont, 1978

Emrich, H.M.:
Optical measurements of rapid pH-change in the visual process during the Metarhodopsin I - II reaction
Z. f. Naturf. 26b, 352 - 356, 1971

Govardovskii, V.I.:
The mode of generation of the early receptor potential and an electric modul of the retinal rod
Biofizika 22(3), 514 - 519, 1978

Grellmann, K.-H.; Livingston, R.; Pratt, D.C.:
Flash photolysis of rod particle suspensions
Photochem. Photobiol. 3, 121 - 127, 1964

Hagins, W.A.:
Rhodopsin in a mamalian retina
Ph. D. Thesis, Univ. Cambridge, 1957

Hagins, W.A.; Penn, R.D.; Yoshikami, S.:
Dark current and photocurrent in retinal rods
Biophys. J. 10, 380 - 412, 1970

Hagins, W.A.; Rüppel, H.:
Fast photoelectric effects and the properties of vertebrate photoreceptors as electric cables
Fed. Proc. 30, Nr. 1, 64 - 68, 1971

Hamacher, K.:
Pers. comm., 1979

Hecht, S.; Shlaer, S.; Pirenne, M.H.:
Energy, quanta, and vision
J. Gen. Physiol. 25, 819 - 840, 1942

Hodgkin, A.L.; O'Bryan, P.M.:
Internal recording of the early receptor potential in turtle cones
J. Physiol. 267, 737 - 766, 1977

Hofmann, K.P.:
Pers. comm., 1979

Hofmann, K.P.; Uhl, R.; Hoffmann, W.; Kreutz, W.:
Measurements of fast light-induced light-scattering and absorption changes in outer segments of vertebrate light sensitive rod cells
BSM 2, 61 - 77, 1976

Hubbard, R.:
100 years of rhodopsin
Trends in Biochemical Sciences 1(7), 154 - 158, 1976

Krebs, W.:
Pers. comm., 1979

Kühn, H.:
Pers. comm., 1979

Leser, K.-H.:
Pers. comm., 1979

Lüke, H.D.:
Signalübertragung
Springer-Verlag, Berlin, Heidelberg, New York, 1975

Matthews, R.G.; Hubbard, R.; Brown, P.K.; Wald, G.:
Tautomeric forms of metarhodopsin
J. gen. Physiol. 47, 215 - 240, 1963

McConnell, D.G.:
Isolation of rods, large prep.
J. Cell. Biol. 27, 459, 1965

McConnell, D.G.; Rafferty, Ch.N.; Cassim, J.Y.:
CD, ORD and absorption studies on the conformation of bovine rhodopsin in situ and solubilized with detergent
Biophys. Struct. Mechanism 2, 277 - 320, 1977

Montal, M.; Korenbrot, J.I.:
Rhodopsin in cell membranes and the process of photo-transduction
in: Martonosi, A. (ed.)
The enzymes of biological membranes, Vol. 4,
Wiley, London, 1976

Murakami, M.; Pak, W.L.:
Intracellular recorded early receptor potential of the vertebrate photoreceptors
Vision Res. 10, 965 - 975, 1970

Seiferle, E.:
Nervensystem, Sinnesorgane, Endokrine Drüsen
in: Nickel, R.; Schummer, A,; Seiferle, E. (eds.)
Lehrbuch der Anatomie der Haustiere, Bd. IV
Parey, Berlin u. Hamburg, 1975

Nöll, G.N.:
Reaktionskinetische Untersuchungen an Rinder-Rhodopsin und Stäbchenaußensegmenten sowie 11-cis-Retinal
Ph. D. Thesis TH Aachen, 1974

Ortec:
Operating manual for Ortec 4660 bandpass differential amplifier
Ortec Inc., Oak Ridge, 1978

Ostroy, S.E.:
Rhodopsin and the visual process
BBA 463, 91 - 125, 1977

Pak, W.L.; Cone, R.A.
Isolation and identification of the initial peak of the early receptor potential
Nature 204, 836 - 838, 1964

Pak, W.L.:
Some properties of the early electrical response in the vertebrate retina
Cold Spring Harbour Symp. Quant. Biol. 30, 493 - 499, 1965

Pak, W.L.; Ebrey, T.G.:
Early receptor potentials of rods and cones in rodents
J. of Gen. Physiol. 49, 1199 - 1208, 1966

Rafferty, Ch.N.:
Light-induced perturbation of aromatic residues in bovine rhodopsin and bacteriorhodopsin
Photochem. Photobiol. 29, 109 - 120, 1979

Rapp, J.:
The kinetics of intermediate process in the photolysis of bovine rhodopsin
Ph. D. Thesis, Case Western Res. Univ., 1970

Sickel, W.:
Respiratory and electrical responses to light stimulation in the retina of the frog
Science 148, 648 - 651, 1965

Slonaker, J.R.:
A comparative study of the area of acute vision in vertebrates
J. Morphol. 13, 445 - 503, 1897

Spalink, J.D.:
Untersuchung der Stationarität stochastischer Signale biologischer Natur
Diplom-Arbeit, TH Aachen, 1976

Spalink, J.D.:
unpublished results, 1978

Stewart, J.G.; Baker, B.N.; Williams, T.P.:
Evidence for conformeric states of rhodopsin
BSM 3, 19 - 29, 1977

Stieve, H.; Wilms, M.; Nöll, G.:
Measurements on the pH dependence of metarhodopsin reactions in sonicated rod outer segments
Z. f. Naturf. 28c, 600 - 602, 1973

Stieve, H.:
On the ionic mechanism responsible for the generation of the electrical response of light sensitive cells
in: Jaenicke, L. (ed.)
Biochemistry of Sensory functions
Springer-Verlag, Berlin, Heidelberg, New York, 1974

Valvo Handbuch:
Temperaturabhängige Widerstände, Heißleiter, 1977
Valvo, Hamburg, 1976

von Sengbusch, G.
Reaktionskinetische Untersuchungen an Stäbchen-Suspensionen und Rhodopsin-Lösungen mit der Blitzlichtmethode
Ph. D. Thesis, TH Aachen, 1970

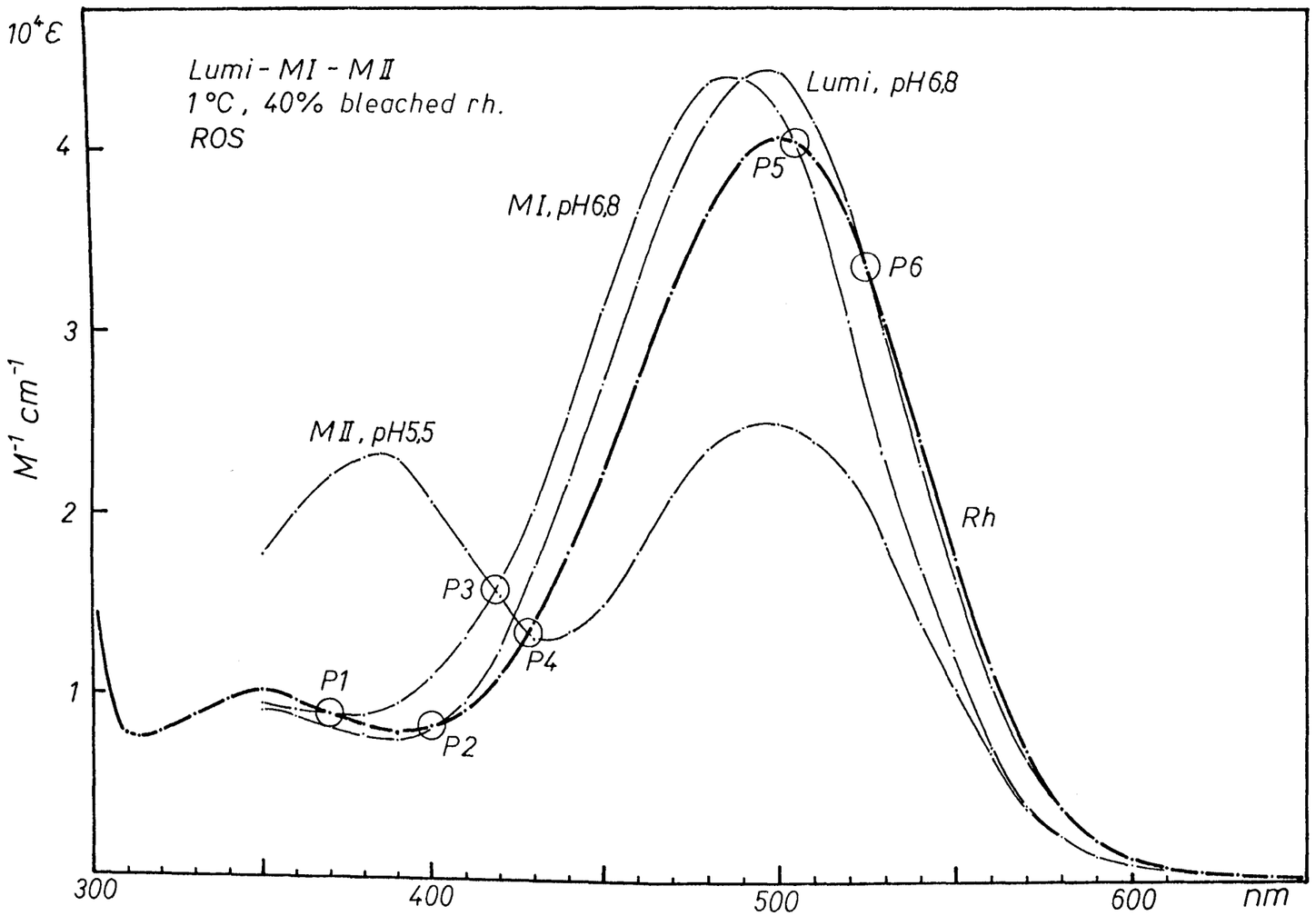
von Sengbusch, G.; Stieve, H.:
Flash photolysis of rhodopsin
Z. f. Naturf. 26b, 488 - 489, 1971

Webb, N.G.:
Orientation of retinal rod photoreceptor membranes in the intact eye using x-ray diffraction
Vision Res. 17, 625 - 631, 1977

Welch, P.D.:
The use of fast fourier transform for the estimation of power spectra
IEEE Trans. AU-25, 2, 70, 1967

Witt, H.T.; Rüppel, H.:
Measurement of fast reactions by single and respetitive excitation with pulses of electromagnetic radiation
in: Kustin, K. (ed.)
Fast reactions Methods in Enzymology Vol. XVI
Academic Press New York, 1969

Young, R.W.:
Visual cells, daily rythms and vision research
Vision Res. 18, 573, 1978


$10^4\varepsilon$
Lumi - MI - MII
1 °C, 40% bleached rh.
ROS
Lumi, pH6,8
P5
MI, pH6,8
P6
MII, pH5,5
Rh
P3
P4
P1
P2
4
3
2
1
$M^{-1}\,cm^{-1}$
300
400
500
600
nm