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Photoactive Retinal Pigments in Haloalkaliphilic Bacteria

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By D.B. Bivin[1*] and W. Stoeckenius[1,2]

Cardiovascular Research Institute[1]

and

Department of Biochemistry and Biophysics[2]

University of California

San Francisco, CA 94143

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Running Title: RHODOPSINS IN HALOALKALIPHILES

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Abbreviations:

P_f — fast cycling pigment, P_s — slow cycling pigment, bR — bacteriorhodopsin, hR — halorhodopsin, sR — slow rhodopsin,

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SUMMARY

Light-induced fast transient absorbance changes were detected by time-resolved spectroscopy in 38 out of 51 haloalkaliphilic isolates from alkaline salt lakes in Kenya and the Wadi Natrun in Egypt. They indicate the presence of two retinal pigments, P_f and P_s, which undergo cyclic photoreactions with half-times of 2 ms and 500 ms respectively. P_f absorbs maximally near 580 nm and P_s near 500 nm. The pigments differ in their sensitivity to hydroxylamine and detergent bleaching and the photoreactions of P_f are strongly dependent on chloride concentration. Of the 38 pigment-containing strains, 29 possess both P_f and P_s, 9 possess only P_s.

Inhibition of retinal synthesis with nicotine blocks pigment formation and addition of retinal restores it. Hydroxylamine-bleached pigments can be reconstituted with retinal or retinal analogues. Their similarity to the retinal pigments of Halobacterium halobium strongly suggests that they are also rhodopsin-like retinyledene proteins. P_f in all properties tested is almost identical to halorhodopsin, the light-driven chloride pump of H. halobium, and may serve the same function in the haloalkaliphiles. P_s has photocycle kinetics similar to sensory rhodopsin and a far blue-shifted long-lived photocycle intermediate but its ground state absorption maximum is near 500nm instead of 587nm. We have not found a bacteriorhodopsin-like pigment in the haloalkaliphiles.

INTRODUCTION

Halobacteria possess the only biological light energy-transducing system that does not use chlorophyll as the energy converter. Instead they use the rhodopsin-like pigments bacteriorhodopsin (bR) and halorhodopsin (hR), which function as light-driven ion pumps. Through these pigments the bacteria can substitute light for respiration as their main energy source. A third very similar pigment, sensory or slow rhodopsin (sR) appears to function as a signal transducer which mediates the phototactic responses of these cells [for review see (Stoeckenius & Bogomolni, 1982, Lanyi, 1986)]. All three pigments undergo cyclic photoreactions which can be detected with time-resolving spectrophotometers by their absorbance changes in the visible and near-UV region of the spectrum; the observed kinetics cover the time range from approximately 1 ps to 1 s. Since these are fast cyclic changes it is possible to average over a large number of measurements, typically a few hundred, and to detect the presence of these pigments with high sensitivity. While nearly all detailed investigations into their structure and function have been made on H. halobium and a few very closely related strains, we have found in a survey that similar or identical pigments are widespread among the bacterial populations of natural salt lakes and salterns (Stoeckenius, 1981; Javor et al., 1982; Stoeckenius et al., 1985). However, the phenotypically distinct alkaliphilic halophiles (Tindall et al., 1980; Soliman & Trüper, 1981), recently classified into two new archaebacterial genera Natronobacterium and Natronococcus (Tindall et al., 1984) have not been systematically surveyed for such pigments. These bacteria pose interesting questions with respect to bioenergetics in view of their vigorous growth at pH values above 9.5. We report here that alkaliphilic halobacteria have two photoactive pigments, one whose spectroscopic properties are very similar to those

of hR and a second pigment similar to sR in its kinetics but absorbing at shorter wavelengths.

METHODS

Strains and growth. The 44 strains isolated from alkaline salt lakes in Kenya have been described before (Tindall et al., 1980). *H. pharaonis* from Wadi Natrun (Soliman & Trüper, 1981) was obtained from H. Trüper. We have isolated six similar strains from material also collected at Wadi Natrun by one of us (W.S.) in August 1982.

The growth medium (Tindall et al., 1980), was autoclaved for 20 min and stored in sealed flasks. For agar plates NaCl and Na_2CO_3 were dissolved and autoclaved separately, cooled to approximately 70 °C before mixing, and poured into culture dishes. Agar plates were sealed with Parafilm to prevent drying.

Cells from single colonies grown on agar were transferred to culture tubes containing 2 ml liquid medium and shaken for 3 d at 37 °C. The cells were then transferred to 10 ml medium in 25 ml flasks and when they had reached early exponential phase the 10 ml cultures were used to inoculate 250 ml medium in 1 litre flasks. The flasks were shaken vigorously at 37 °C and after 4 d the cultures had reached the end of the exponential phase and contained approximately $2x10^9$ cells ml^{-1}. The cells were collected by centrifugation, and washed in basal salt (medium without Casamino acids and yeast extract).

The optimum pH for growth in the above medium is 9.5 (Ross et al., 1981). We confirmed this by monitoring growth of strain SP1W in media adjusted to pH 6.5, 7.5, 8.0, 9.5, 10.0, and 11.0. Growth rates dropped sharply between pH 10 and 11 and between pH 8.0 and 7.5 but significant growth still occurred at pH 6.0 and 11.0 (data not shown).

Nicotine blocks biosynthesis of retinal in H. halobium (Danon et al., 1977; Sumper et al., 1976; Hwang et al., 1981) and also in the haloalkaliphiles. A standard 10 ml cell culture at the end of the exponential phase was added to a 1 litre flask containing 250 ml culture medium and 2mM nicotine. After 4 days a 10 ml sample of the cells grown in the 2 mM nicotine medium was transferred to a second 1 litre flask containing growth medium and 2mM nicotine and the last step was repeated twice. Cells from the last culture were harvested at the end of the exponential phase.

Preparation of cell membranes. Washed cells from 250 ml culture were lysed by resuspension in 250 ml 100 mM NaCl, 50 mM HEPES (pH 7), to which DNAase had been added, and dialysed against 2 litres of the same solution for 24 h. Membrane fragments were collected by centrifugation for 50 min at 200,000 g and washed once in 100 mM NaCl, 50 mM HEPES (pH 7). The membranes were then resuspended in the desired salt solution for spectroscopic analysis or centrifuged again at 250,000 g for 1 h if a membrane pellet was needed. When necessary, membranes were stored in 3 M NaCl/50 mM HEPES (pH 7) at 4 ^{o}C in the dark.

Assays for photoactive pigments Single colonies of various strains of alkaliphilic bacteria growing on agar plates were examined for photocycling pigments in a flash spectrophotometer designed by R.A. Bogomolni (Weber & Bogomolni, 1982) with the position of the condenser lens and microscope reversed. The light beam from the quartz iodine lamp was first passed through a monochromator and then focussed through a microscope objective lens onto the colony. The transmitted light was collected with the large-aperture condenser lens and directed onto the cathode of a photomultiplier. A tunable dye laser provided the actinic light flashes of 250 ns duration from the side and the pho-

tomultiplier was protected against scattered actinic light by filters. Time-resolved absorbance changes at 560 and 480 nm were recorded and stored in a Nicolet 1074 signal-averaging computer and either plotted on paper or transferred to magnetic tape.

For higher sensitivity and better time resolution isolated membrane preparations were used either in suspensions or as pellets. Pellets scatter much less light than a suspension of membranes of the same optical density, but are more difficult to mount and to align in the spectrometer. Therefore, membrane suspensions were used whenever possible. Membrane pellets were placed in demountable cuvettes (Hellma) of 0.1 mm or 0.2 mm pathlength and examined for photocycling activity in a spectrophotometer as described (Lozier, 1982). Under optimal conditions, this technique can detect $\geq$5 bR molecules per cell. Membrane suspensions were placed in 10 mm x 10 mm or 10 mm x 4 mm cuvettes (Hellma) and examined in the same apparatus. All experiments were done at 20 ^{o}C.

Bleaching and reconstitution of pigments. Hydroxylamine hydrolyses Schiff bases and can bleach the chromophore in retinylidene pigments (Oesterhelt et al., 1974) and the chromophore reconstituted by addition of retinal (Oesterhelt & Schuhmann, 1974). Bleaching in rhodopsin-like pigments is very sensitive to reaction conditions. Membrane suspensions from haloalkaliphiles adjusted to 2.5 mg total protein ml^{-1} were bleached with 0.5M hydroxylamine in 3 M NaCl, 50 mM HEPES (pH 7) either for 2 h in the dark at 20 ^{o}C or for 24 h in white light (0.2 Joules cm^{-2} sec^{-1}) at 40 ^{o}C, as described by Taylor et al., (1983). Membranes of *H. halobium*, used for comparison, were bleached in the dark for 24 h at 40 ^{o}C. The bleached membranes were then dialysed against 3 M NaCl, 50 mM HEPES (pH 7) for 36 h to remove residual hydroxylamine. One sample of the dialysed membrane preparation was assayed for photocycling pigments as described above. To

another sample all-trans retinal (Sigma) was added to a final concentration of 1 µM. Membrane preparations from cells grown in the presence of nicotine were used without bleaching. After 3 h in the dark at room temperature the suspension was examined in the flash spectrophotometer. Absorbance spectra of the bleached and reconstituted membrane suspensions were taken with an Aminco DW2A spectrophotometer in the single-beam mode, first stored in a Nicolet 1180 computer and then tranferred to magnetic disk.

Reversible photobleaching of membrane suspensions. Membranes in suspension (3 M NaCl/50 mM CHES, pH 9, 2.5 mg protein ml^{-1}) in both the reference and sample chambers of the spectrophotometer were first illuminated with blue light (Corning 5-60 filter and a 250 W projector) for 2min. Then the suspension in the sample chamber only was illuminated with orange light (Corning 3-69 filter) and again with blue light. Spectra were recorded with the Aminco DW2A spectrophotometer before and after each illumination.

Solubilization of membrane proteins. Membranes were placed in 15mM octylglucoside, 3 M NaCl and 50 mM HEPES (pH 8.6), protein/detergent ratio 1:2 (w/w). After 24 h at 4 ^{o} in the dark the suspension was centrifuged for 50 min at 200,000g and the supernatant used for spectroscopic analysis.

RESULTS

Fig. 1 shows averaged transient absorbance changes from a single colony of haloalkaliphilic strain F34 excited with 256 flashes of 510nm light. A fast absorbance decrease with a recovery halftime of ~2 ms is seen at 560 nm and a much slower decrease at 480 nm, which recovers with a halftime of ~500 ms. The cells obviously produce pigment(s) undergoing fast light-induced cyclic reactions. Colonies on agar plates can be rapidly screened for light-induced absorbance changes and photoactive pigment-rich strains selected. Fractionation of the cells showed that the pigments are always contained in the cell membrane, and we have carried out further studies mostly with cell membrane preparations.

Fig. 2 shows the transient absorbance changes of a membrane pellet from cells of strain SP1W, a spontaneous mutant of the natural isolate SP1, which lacks the major carotenoid pigments. An initial, fast decrease in the absorbance at 580 nm and 480 nm and an increase at 400 nm are not time-resolved. The absorbance recovers completely in approximately 4 s. At least two first order kinetic components with halftimes of 2 ms and 500 ms can be distinguished. The most likely interpretations are: (1) that there is a single pigment with at least two spectroscopically distinct intermediate states and a branch in its photocycle, so that pigment molecules return to the initial state via either a path of 2 ms halftime or a path of 500 ms halftime; or (2) that there are two pigments, one cycling with a halftime of 2 ms and the other with a halftime of 500 ms. In case (2), the cycle observed may depend upon the wavelength of the actinic light. If there were two pigments absorbing at different wavelengths and cycling with different time constants, the absorbance changes with one time constant should be enhanced over absorbance changes with the other, when the wavelength of the actinic light is changed.

The fast response characterized by an absorbance decrease in the 560-600 nm region of the spectrum is present when membranes are excited at either 590 nm or at 510 nm. The slow response, which is characterized by an absorbance decrease in the 480-520 nm region, is undetectable when membranes are excited at 590 nm (compare Fig. 3a with 3b). We conclude tentatively that two photoactive pigments are present in these membranes.

Selective bleaching also allows one to distinguish the two pigments. After treatment in the dark with 0.5 M NH_2OH, only the fast response remains (Fig. 3d). The fast response, however, is more sensitive to detergent treatment. Fig. 3c shows the absorbance changes generated in membranes solubilized in octylglucoside at low ionic strength. Only the slow component of the absorbance change remains.

Using flash photolysis of colonies growing on agar plates we assayed 51 strains of alkaliphilic halophiles for photoactive pigments. Responses similar to those seen in SP1W membranes were seen in colonies of most strains and are summarized in Table 1. Note that some strains showed only the slow response. These results further support our postulate that the slow and fast responses are due to the two distinct pigments. We have selected some strains for further study, in particular strain SP1W, which shows both the fast and slow photoresponses and strain SP1(24), which exhibits only the slow response (Fig. 3c). We shall provisionally term the fast pigment P_f and the slow pigment P_s.

Fig. 4a shows difference spectra from SP1W membranes for several times after excitation with 590 nm light, i.e. under conditions where only P_f is detected. The absorbance decrease is greatest in the region around 580 nm and an absorbance increase occurs around the 500 nm and later around 640 nm. Evidently P_f absorbs maximally near 580 nm and has two distinguishable photocycle

intermediates, one which absorbs in the 500 nm region and another following it which absorbs in the 640 nm region. The absorbance decrease at 580 nm recovers with a halftime of approximately 2 ms (Fig. 2a or Fig. 4a). Therefore, 15-20 ms after excitation essentially all of the fast pigment molecules have returned to their initial state and the transient absorbance changes at later times in samples excited with shorter wavelengths should be due only to P_s. This conclusion is verified by the data shown in Figs. 4b and 4c, which compare the absorbance changes at 1, 3, 6 and 15 ms with the absorbance changes at 100, 300, 600 and 1000 ms after excitation with 510 nm light. We see a single absorbance minimum near 500 nm and absorbance maxima in the 400 and 560 nm regions 100 ms and 300 ms after the flash while the spectra at shorter times show a more complicated pattern with contributions from both pigments. Thus we conclude that P_s absorbs maximally near 500 nm and has a relatively long-lived intermediate absorbing in the 400 nm region followed by an intermediate which absorbs in the 560 nm region.

The photoreactions of P_f are sensitive to the ionic composition of the medium. Fig. 4f shows absorbance changes generated by 590 nm light in SP1W membranes pelleted from Cl^--free medium (NaCl $<10^{-6}$ M) at pH 7.0. There is an absorbance decrease in the 540 nm region and an absorbance increase in the 640 nm region. The halftime for the return to the original state is ~110 ms. When SP1W membranes are pelleted from suspension in 3M NaCl/50mM CAPS pH 9.5 and excited at 510 nm, the difference spectra are virtually identical to those shown in Figs. 4b and 4c (data not shown), whereas displacement of NaCl by Na_2SO_4 yields spectra identical to those in Fig. 4f. We conclude that high Na^+ concentrations and high pH do not seem to affect the photoreaction cycle strongly but Cl^- does. When membranes were dialysed against 50 mM HEPES pH 7 until the NaCl

concentration was less than 10^{-5} M, and the fast absorbance increase at 500 nm was titrated with NaCl, we observed an apparent pK of about 12 mM for this effect (data not shown).

The cyclic absorbance changes of P_f and its Cl^--dependence are virtually identical to those of hR from H. halobium except for the slower kinetics in Cl^--free medium. Halorhodopsin during extensive long wavelength illumination accumulates a photoproduct, which absorbs near 410 nm and is stabilized by high pH, but can be photoconverted back to hR_{578}. The same holds true for P_f. Fig. 5 shows steady-state absorbance difference spectra of a SP1W membrane suspension at pH 9.0. For each spectrum shown, the spectrum of the sample after each successive illumination was subtracted from the spectrum obtained after an initial illumination with blue light. The sample, after the first illumination with blue light (2 min), was illuminated for 1.5 min (A) and 6 min (B) with yellow light; then the sample was illuminated with blue light for 2 min (C). Initial illumination with blue light ensured that the pigment molecules in the sample were in the same photostationary state. The first illumination with orange light induced an absorbance decrease in the 580 nm region and an absorbance increase in the 410 nm region, and the second illumination with orange induced an even greater absorbance decrease in the 580 nm region together with a greater absorbance in the 410 nm region, additional illumination with orange light produced no further absorbance changes. Evidently, illumination with orange light had driven pigment molecules in the sample from a form which absorbs maximally near 580 nm to a form which absorbs maximally near 410 nm and the final illumination with blue light returned the pigment to its original state (trace C). The small deviation from the baseline between 420 and 470 nm in trace C is probably due to irreversible light-induced absorbance changes in cytochromes and

carotenoids. The same experiment done on membranes bleached in the dark gave essentially identical results indicating that we were observing only P_f (see below).

P_s and P_f are apparently retinal pigments. Subjecting the membranes of strain SP1W to NH_2OH for two h in the dark bleaches P_s but not P_f. Fig. 6 shows the difference between spectra taken before and after the addition of retinal to a suspension of membranes bleached in the dark. A broad absorbance band with its maximum at approximately 500 nm is generated; the shoulder in the 465 nm region separated by approximately 1,600 cm^{-1} from the main peak probably indicates fine structure which is characteristic for retinal pigments (Christensen & Kohler, 1973). The inset shows that the long-lived light-induced absorbance decrease at 500 nm characteristic for P_s was also regenerated. The transient absorbance spectrum at 580 nm remained unchanged, indicating that P_f was not bleached in the dark by NH_2OH. Fig. 7 shows the same experiment done with membranes bleached with NH_2OH under intense illumination. The main peak in the difference spectrum now is at approximately 575 nm with a shoulder near 500 nm. The peak in the 380 nm region is due to excess retinal. The time-resolved absorbance changes (inset) show a fourfold increase in the fast response and reappearance of the slow response after addition of retinal. We conclude that P_f and P_s, like the H. halobium pigments bR, hR, and sR, can be bleached with NH_2OH and regenerated by addition of retinal, but they differ in their susceptibility to NH_2OH bleaching. Membranes bleached in the dark and therefore containing only P_f were also reconstituted with retinal A2 (3,4-dehydroretinal). In the difference spectra between bleached and reconstituted preparations, a peak appeared near 525 nm with a shoulder near 500 nm. When membranes were bleached in intense white light and then reconstituted with retinal A2, a peak

appeared near 600 nm with a broad shoulder in the 520 nm region (data not shown). This is the expected red shift for pigments in which the retinal is replaced by retinal A2 in the chromophore (Sperling & Schimz, 1980).

Membrane preparations from SP1W grown in the presence of 2mM nicotine, which blocks retinal synthesis (Dencher & Hildebrand, 1979), show no detectable absorbance changes when assayed by flash photolysis. Addition of all-trans retinal generated the transient absorbance changes characteristic of both the fast and slow photocycles (Fig. 8). This again shows that both the fast and slow pigment require retinal to form a chromophore. Their properties are so similar to other rhodopsins that they are most likely retinyledene proteins. The apoproteins are apparently synthesized also in the absence of retinal.

DISCUSSION

We have characterized spectroscopically two pigments undergoing cyclic photoreactions in whole cells and membrane preparations of haloalkaliphiles. One pigment, P_f, has a cycle time of approximately 2 ms and an absorbance maximum at approximately 580 nm. The second pigment, P_s, cycles in approximately 500 ms and has an absorbance maximum near 500 nm.

By the criteria used here, P_f is very similar to the retinylidene pigment hR found in H. halobium (Stoeckenius & Bogomolni, 1982) and other halophiles which grow at or near neutral pH (Stoeckenius et al., 1985). The absorbance maximum of P_f is between 575 and 580 nm. In 3 M NaCl at neutral pH the pigment cycles in ~2 ms with a 500 nm intermediate whose rise time is less than 1μ s and a 640 nm intermediate with a rise time of ~0.5 ms. The difference spectrum of SP1W membranes excited with 590 nm light or that generated on excitation with 510 nm light in preparations in which P_s has been bleached by NH_2OH are indistinguishable from the difference spectra of membrane preparations from H. halobium strain JW-12, which contain no bR and in which sR had been bleached selectively. The absorbance changes induced in SP1W membranes upon illumination with blue and then orange light are essentially identical to those induced in hR from the H. halobium strain JW-12 (Taylor et al., 1983). Halorhodopsin shows a Cl^- effect similar to that of P_f but its apparent pK is 40mM (Schobert et al., 1983) instead of 12mM. Finally, membrane suspensions of both H. halobium JW-12 and of SP1W when solubilized in octylglucoside and high ionic strength retain the fast photoresponse whereas it disappears when membranes are solubilized at low ionic strength. Thus hR and P_f have almost identical properties. The only distinct difference we have found is a much slower photocycle in Cl^--free medium; the halftime for hR is 1 ms and for P_f 110 ms which could be due to differences in

the lipid or protein environment of the pigments in the two membranes. It is known that the lipid composition of the haloalkaliphiles is different from that of H. halobium (Tindall et al., 1984). The striking similarity of P_f and hR especially in their Cl^- dependence suggests that P_f may also be a light-driven Cl^- pump. We have found pH changes induced upon illumination of cell suspensions with yellow light. The pH of the medium is increased, indicating that the cells or cell membranes take up protons upon illumination.

The slow pigment, P_s, in some respects resembles the pigment sR found in H. halobium (Bogomolni & Spudich, 1982). The halftimes for their photocycles are nearly the same, approximately 500 ms for P_s and approximately 800 ms for sR. Both have spectral intermediates which absorb in the near UV: at 373 nm for sR and near 400 nm for P_s. Both pigments show little change in photocycle activity under low ionic strength conditions. The absorbance maxima of the pigments in their initial (resting) states are quite different, however: approximately 500 nm for P_s vs. 589 nm for sR, and sR loses photocycle activity and is presumably denatured by octylglucoside under both high and low salt conditions, whereas P_s retains photocycling activity under these conditions. The sR of H. halobium is thought to mediate phototactic responses (Spudich & Bogomolni, 1984); whether a similar function may be attributed to P_s remains to be seen.

Assuming molar absorption coefficients similar to those of H. halobium pigments we estimate that there are approximately 1,500 molecules of P_s and 6,000 molecules of P_f present in each haloalkaliphilic cell. This compares with approximately 20,000 molecules of hR and 5,000 molecules of sR in the bR^- strain of H. halobium JW-12 and more than 10^5 molecules of bR in most H. halobium strains. Retinal pigments function as both light energy transducers and signal transducers in H. halobium (Stoeckenius & Bogomolni, 1982; Spudich & Bogomolni,

1984). The relatively low concentration of P_s in the cells would be consistent with a sensory rather than a light energy-transducing function. The cells show no indication of containing a bacteriorhodopsin-like pigment, which can readily be distinguished spectroscopically from hR and sR (Spudich & Bogomolni, 1983), and we have not detected any indication of light-driven photophosphorylation (data not shown). The lack of bR is not too surprising if these cells, like other alkaliphiles, maintain their internal pH approximately constant near 9.0 (Guffanti et al., 1978), i.e. well below the high outside pH, because in such cells a light-driven outward-directed proton pump would obviously be counterproductive. The utility of an inward-directed chloride pump, however, would be obvious if these cells, like other halophiles, maintain an outside-positive membrane potential and a high internal salt concentration to balance the outside salt concentration. Whether they substitute sodium gradients for proton gradients in energy transduction, as some other alkaliphiles do (Hirota & Imae, 1983), is not known. Clearly, the physiology of the haloalkaliphiles poses many interesting problems that should stimulate further work.

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REFERENCES

Bogomolni, R. A. & Spudich, J. L. (1982). Identification of a third rhodopsin-like pigment in phototactic Halobacterium halobium. Proceedings of the National Academy of Sciences of the United States of America 79, 6250-6254.

Christensen, R. L. & Kohler, B. E. (1973). Low resolution optical spectroscopy of retinyl polyenes: low lying electronic levels and spectral broadness. Photochem. Photobiol. 18, 293-301.

Danon, A., Brith-Lindner M. & Caplan, S. R. (1977). Biogenesis of the purple membrane of Halobacterium halobium. Biophysics of Structure and Mechanism 3, 1-17.

Dencher, N. A. & Hildebrand E. (1979). Sensory transduction in Halobacterium halobium: retinal protein pigment controls UV-induced behavioral response. Zeitschrift für Naturforschung 34c, 841-847.

Guffanti, A. A., Susman, P., Blanco, R. & Krulwich, T. A. (1978). The protonmotive force and α-aminoisobutyric acid transport in an obligately alkalophilic bacterium. Journal of Biological Chemistry 253, 708-715.

Hirota, N. & Imae, Y. (1983). Na^+-driven flagellar motors of an alkaphilic Bacillus strain YN-1. Journal of Biological Chemistry 258, 10577-10581.

Hwang, S. B., Tseng, Y. W. & Stoeckenius, W. (1981). Spontaneous aggregation of bacteriorhodopsin in brown membrane. Photochemistry and Photobiology 33, 419-427.

Javor, B., Requadt, C. & Stoeckenius, W. (1982). Box-shaped halophilic bacteria. Journal of Bacteriology 151, 1532-1542.

Lanyi, J. K. (1986). Halorhodopsin. A light-driven chloride ion pump. Annual Review of Biochemistry and Biophysical Chemistry 15, 11-28.

Lozier, R. H. (1982). Rapid kinetic optical absorption spectroscopy of

bacteriorhodopsin photocycles. Methods in Enzymology 88, 133-162.

Oesterhelt, D. & Schuhmann, L. (1974). Reconstitution of bacteriorhodopsin. FEBS Letters 44, 262-265.

Oesterhelt, D., Schuhmann, L. & Gruber, H. (1974). Light-dependent reaction of bacteriorhodopsin with hydroxylamine in cell suspensions of Halobacterium halobium: demonstration of an apomembrane. FEBS Letters 44, 257-261.

Ross, H. N. M., Collins M. D., Tindall B. J. & Grant, W. D. (1981). A rapid procedure for the detection of archaebacterial lipids in halophilic bacteria. Journal of General Microbiology 123, 75-80.

Schobert, B., Lanyi, J. & Cragoe Jr., E. J. (1983). Evidence for halide-binding site in halorhodopsin. Journal of Biological Chemistry 258, 15158-15164.

Soliman, G. S. & Trüper, H. G. (1981). Halobacterium pharaonis sp. nov., a new, extremely haloalkaliphilic archaebacterium with low magnesium requirement. Zentralblatt für Bakteriologische Hygiene, Abteilung Originale C 3, 318-329.

Sperling, W. & Schimz, A. (1980). Photosensory retinal pigments in Halobacterium halobium. Biophysics of Structure and Mechanism 6, 165-169.

Spudich, J. L. & Bogomolni, R. A. (1983). Spectroscopic discrimination of the three rhodopsinlike pigments in Halobacterium halobium membranes. Biophysical Journal 43, 243-246.

Spudich, J. L. & Bogomolni, R. A. (1984). The mechanism of colour discrimination by a bacterial sensory rhodopsin. Nature, London 312, 509-513.

Stoeckenius, W. (1981). Walsby's square bacterium: fine structure of an orthogonal procaryote. Journal of Bacteriology 148, 352-360.

Stoeckenius, W., Bivin, D. & McGinnis, K. (1985). Photoactive pigments in halobacteria from the Gavish Sabkha. In Ecological Studies 53, Hypersaline Ecosystems: The Gavish Sabkha. pp. 288-295. Edited by G. M. Friedman & W. E.

Krumbein. Heidelberg: Springer-Verlag.

Stoeckenius, W. & Bogomolni, R. A. (1982). Bacteriorhodopsin and related pigments of halobacteria. Annual Review of Biochemistry 52, 587-615.

Sumper, M., Reitmeier, H. & Oesterhelt, D. (1976). Biosynthesis of the purple membrane of halobacteria. Angewandte Chemie-International Edition in English 15, 187-194.

Taylor, M. E., Bogommolni, R. A. & Weber, H. J. (1983). Purification of photochemically active halorhodopsin. Proceedings of the National Academy of Sciences of the United States of America 80, 6172-6176.

Tindall, B. J., Mills, A. A. & Grant, W. D. (1980). An alkalophilic red halophilic bacterium with a low magnesium requirement from a Kenyan soda lake. Journal of General Microbiology 116, 257-260.

Tindall, B. J., Ross H. N. M. & Grant, W. D. (1984). Natronobacterium gen. nov. and Natronococcus gen. nov., two new genera of haloalkaliphilic archaebacteria. Systematic and Applied Microbiology 5, 41-57.

Weber, H. J. & Bogomolni, R. A. (1982). The isolation of halobacterium mutant strains with defects in pigment synthesis. Methods in Enzymology 88, 379-390.

Table 1. Slow and fast responses in 51 strains of alkaliphilic halophiles examined by flash photolysis of colonies.

Grant strains*	Slow	Fast	Grant strains	Slow	Fast
SP1	+	+	SP2(5)	+	+
SP2	+	+	SP2(26)	+	-
SP1(2)	+	-	SP2(27)	+	-
SP1(8)	+	+	SP4(1)	-	-
SP1(10)	+	+	SP4(2)	+	-
SP1(11)	-	-	SP4(3)	+	+
SP1(12)	+	+	SP4(4)	+	+
SP1(13a)	+	+	SP4(5)	-	-
SP1(13b)	-	-	SP8	+	-
SP1(14)	+	+	SP10	+	+
SP1(17)	+	+	SP12	+	+
SP1(18)	+	+	SP18	+	+
SP1(20)	+	+	SP19	+	+
SP1(24)	+	-	F34	+	+
SP1(25)	+	-	MS3(16)	-	-
SP1(28a)	+	-	MS3(19)	-	-
SP1(28b)	-	-	MS3(21)	-	-
SP1(30)	+	+	MS3(22)	-	-
SP1(32)	+	+	MS3(23)	-	-
SP1(33)	+	+	MS3(31a)	-	-
SP1(36)	+	-	MS3(31b)	-	-
SP2(1)	-	-	MS3(33)	-	-
			H. pharaonis (Wadi Natrun, Egypt)	+	+
			Six isolates from brine collected by W. S. (Wadi Natrun)	+	+

*Described by Tindall et al. (1980).

FIGURE LEGENDS

Fig. 1. Absorbance changes from a single colony of strain F34 growing on an agar plate. Data were taken at 100μ s per point. Actinic light flashes were at 510 nm, duration 250 ns, repetition rate 0.3Hz. Data shown are the average of 256 separate transient absorbance spectra. The absorbance decrease at 560 nm is due to cycling of the hR-like pigment, P_f, and the absorbance decrease at 480 nm is due to the 500 nm pigment, P_s.

Fig. 2. Transient absorbance changes of SP1W membrane pellets in high salt. Responses to 64 flashes of 510 nm actinic light were averaged. Pulse rate was 0.1Hz. Data in (a) were taken at 20 μs per point. Data in (b) were taken at 1 ms per point. In (a) the transient absorbance decrease at 580 nm is due to the cycling of the fast pigment, the transient absorbance increase at 400 nm is due to the 400 nm intermediate of the slow pigment and the absorbance increase at 660 nm is due to the 620 nm intermediate of the fast pigment. In (b) the absorbance decrease at 480 nm is due to the slow pigment and the absorbance increases are due to its intermediates. In (a) the absorbance decrease at 480 nm due to the cycling of the slow pigment is superimposed on the absorbance increase of the 500 nm intermediate of the fast pigment.

Fig. 3. Transient absorbance changes for various preparations. Data were taken at 20 μs per point. The actinic light was at 510 nm except for (b). Repetition rate was 0.1 Hz. Each trace shown is the average of 64 traces. The upper trace of each pair is the absorbance at 480 nm;

the lower trace is the absorbance at 580 nm.

(a): SP1W membranes pelleted in 3 M NaCl/50 mM HEPES pH 7. Excitation at 510 nm. (b): As (a), but with actinic light at 590 nm. (c): SP1W membranes solubilized in octylglucoside at low Cl^- concentration (50 mM HEPES, $<10^{-6}$ M NaCl). (d): Membranes of strain SP1W bleached in the dark for 1 h in 3 M NaCl.50 mM HEPES/0.5 M NH_2OH pH 7. (e): SP1(24) membranes in 3 M NaCl/50 mM HEPES pH 7.

Fig. 4. (a): Difference spectra for SP1W membranes pelleted from high salt (3 M NaCl/50 mM HEPES) suspensions at pH 7. Actinic light at 590 nm with a repetition rate of 0.1Hz. 64 spectra were averaged to produce each point. The spectrum before excitation was subtracted from spectra obtained 1 (●), 3 (o), 6 (▲) and 15 (△) ms after excitation. (b): As (a) with 510 nm actinic light. (c) As (b) except that points shown are for 100 (●), 300 (o), 600 (▲) and 1000 (△) ms after excitation. (d) Difference spectra for the bR^- strain of H. halobium JW-12 from which sR has been bleached at 1 (●) and 15 (o) ms after excitation at 590 nm. Difference spectra for SP1W membranes from which P_s has been bleached at 1 (▲) and 15 (△) ms after 510 nm excitation. (e): Difference spectra for strain SP1(24) at 1 (●) and 100 (o) ms after 510 nm excitation. Difference spectra for SP1W membranes solubilized in octylglucoside at low ionic strength at 1 (▲) and 100 (△) ms after 510 nm excitation. (f): Difference spectra for SP1W membranes washed extensively in 50 mM HEPES pH 7 (NaCl $<10^{-6}$ M) at 40 (●), 120 (o) and 240 (▲) ms after 510 nm excitation. Difference spectrum for JN-2 membranes in 50 mM HEPES pH 7 from which sR has been bleached 1 ms after 590 nm excitation (△).

Fig. 5. Light-induced absorbance shifts of SP1W membranes suspended in 3 M NaCl, 50 mM CHES, pH 9, 2.5 mg(ml membrane protein)$^{-1}$. Difference spectra after subtraction from the spectrum of the sample illuminated 2 min with blue light (Corning 5-60 filter with a 250 W projector lamp) A, the spectrum after 1.5 min illumination with orange light (Corning 3-69 filter); B, the spectrum after 6 min illumination with orange light. C, the spectrum after 6 min illumination with orange light followed by 2 min illumination with blue light.

Fig. 6. Reconstitution of P_s by the addition of retinal to SP1W membranes bleached in the dark with hydroxylamine (pH 7). Protein concentration 2.5mg per ml^{-1}; all-trans retinal 0.33 mM in ethanol added to final concentration of 1μM. The spectrum 3 h after addition of retinal was subtracted from the spectrum before addition of retinal. Average of eight spectra. The inset shows time-resolved absorbance changes of the same sample flashed with 510 nm actinic light. Curves shown are the averages of 64 transients. (a) membranes bleached in the dark for 2 h in 0.5 M NH_2OH, pH 7; (b) 3 h after reconstitution of bleached membranes with all-trans retinal. The upper and lower traces of each pair are the time-resolved absorbance changes at 500 nm and 580 nm, respectively.

Fig. 7. Reconstitution of membranes bleached under intense white light for 30 h with hydroxylamine. Shown is the spectrum of the sample before, subtracted from the spectrum 4 h after addition of retinal (0.33 mM) to a final concentration of 2.5 μM; membrane protein concentration 2.5 mg per ml^{-1}. Average of four spectra. The inset shows time-resolved absorbance changes in the same sample. All other conditions

as in Fig. 6.

Fig. 8. (a) Transient absorbance changes in membrane pellets from cells of strain SP1W grown in the presence of nicotine. Actinic light at 510 nm. The upper trace of each pair shows the absorbance change at 580 nm; the lower at 480 nm. Path length was 0.1 mm. Each trace is the average of 256 recordings. (b) As in (a), except that membranes were incubated for 2 h with all-trans retinal at a concentration of 2.5 μM before they were pelleted 2 h after addition of all-trans retinal. (c) Control-membranes from cells grown without nicotine in the medium.

INDEX ENTRIES

Rhodopsin-like pigments in haloalkaliphilic bacteria.

Retinal pigments as possible photoenergy and photosignal transducers in haloalkaliphilic bacteria.

Flash spectroscopy for the sensitive detection of photoactive pigments.

Halorhodopsin-like pigment in haloalkaliphiles.

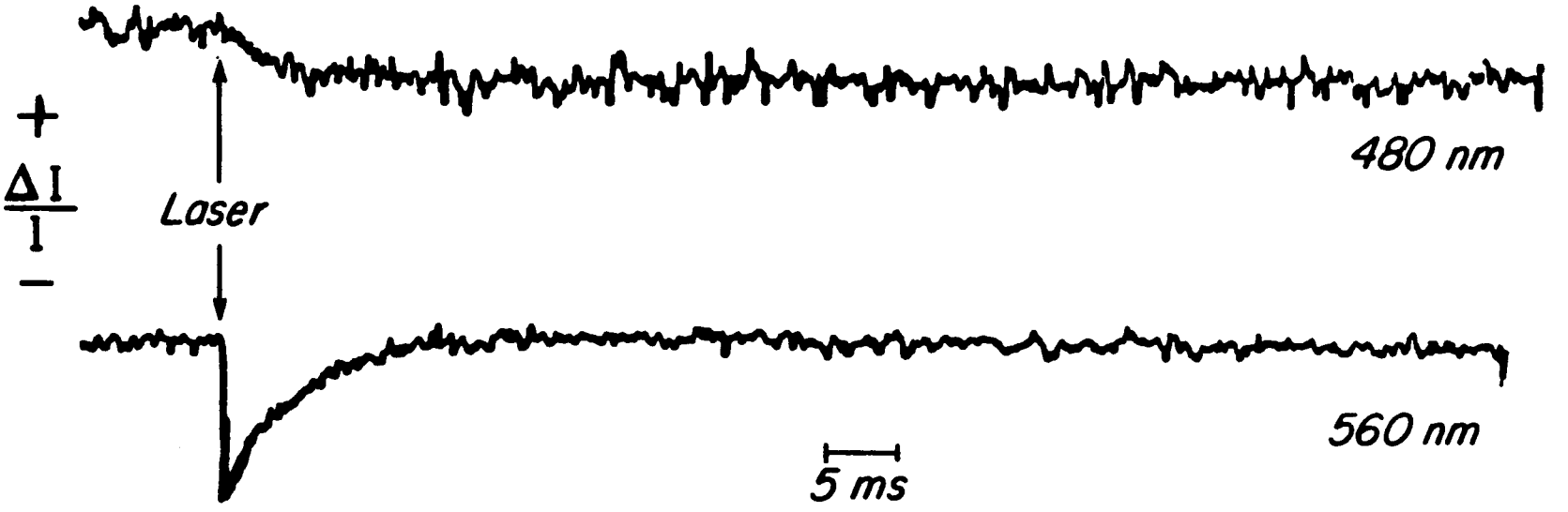

Fig. 1

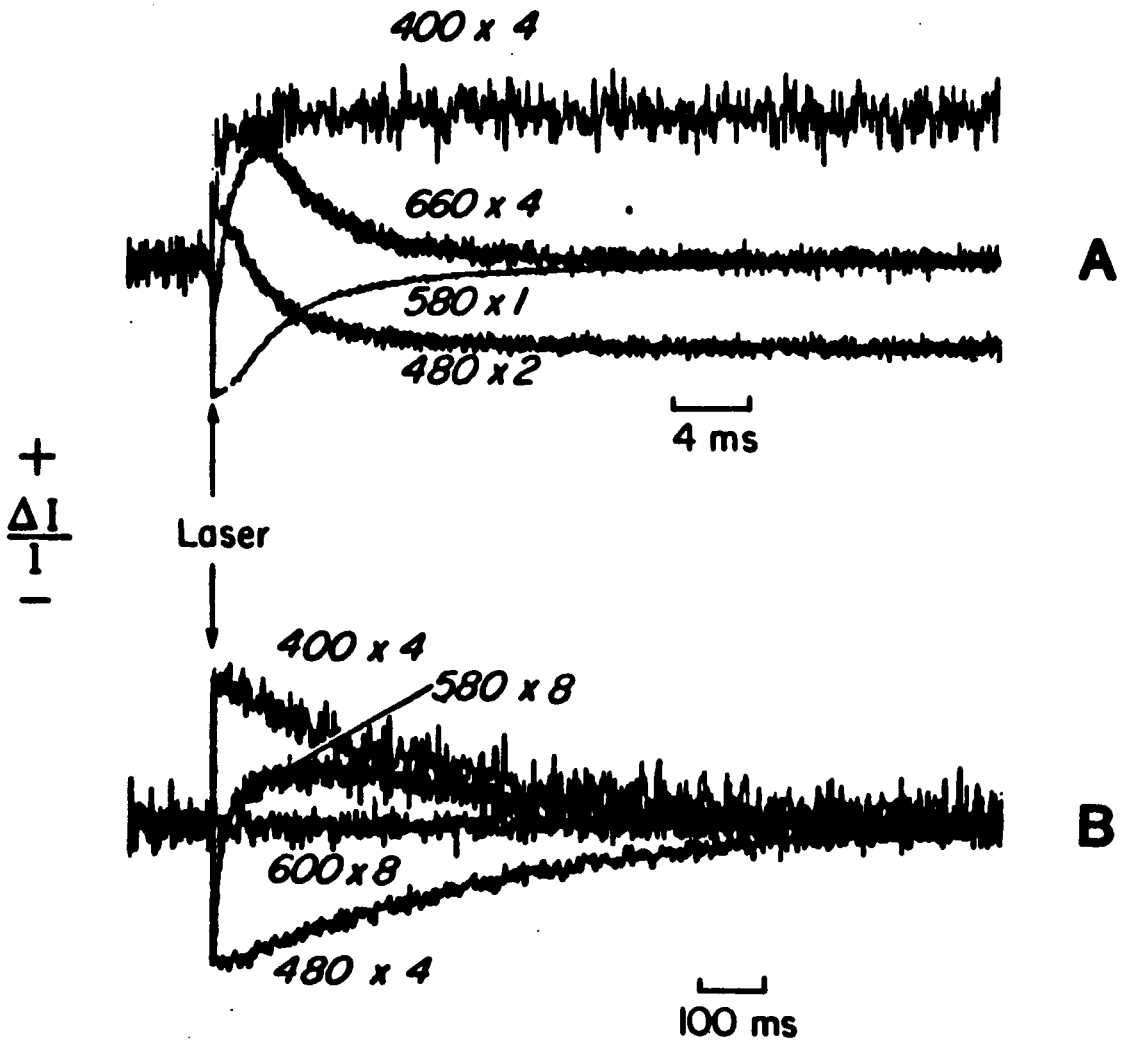
400 x 4
660 x 4
580 x 1
480 x 2
4 ms
A
+
ΔI/I
−
Laser
400 x 4
580 x 8
600 x 8
480 x 4
100 ms
B

Figure 2

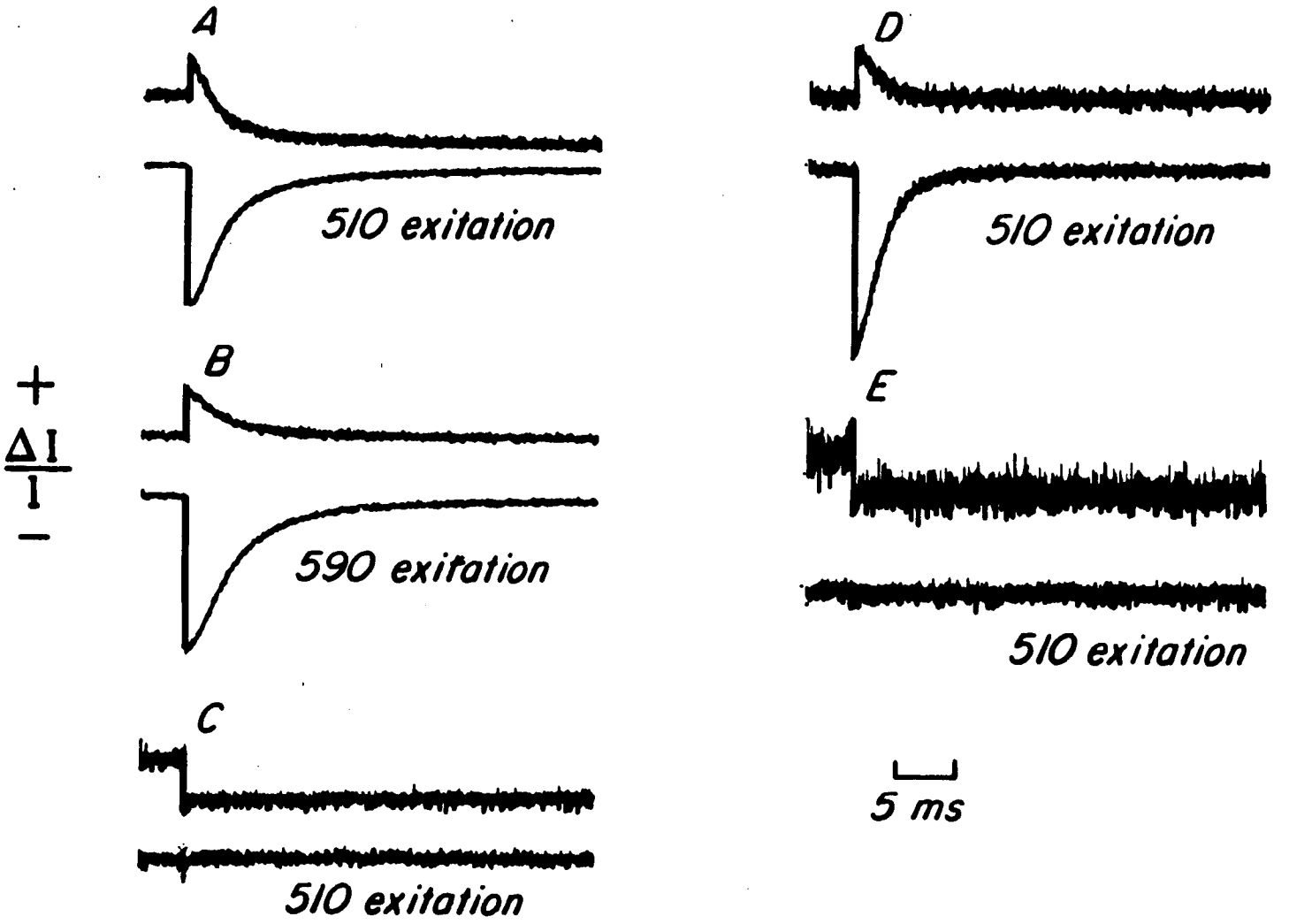
A
510 exitation
B
590 exitation
C
510 exitation
D
510 exitation
E
510 exitation
+
ΔI
I
−
5 ms

Figure 3.

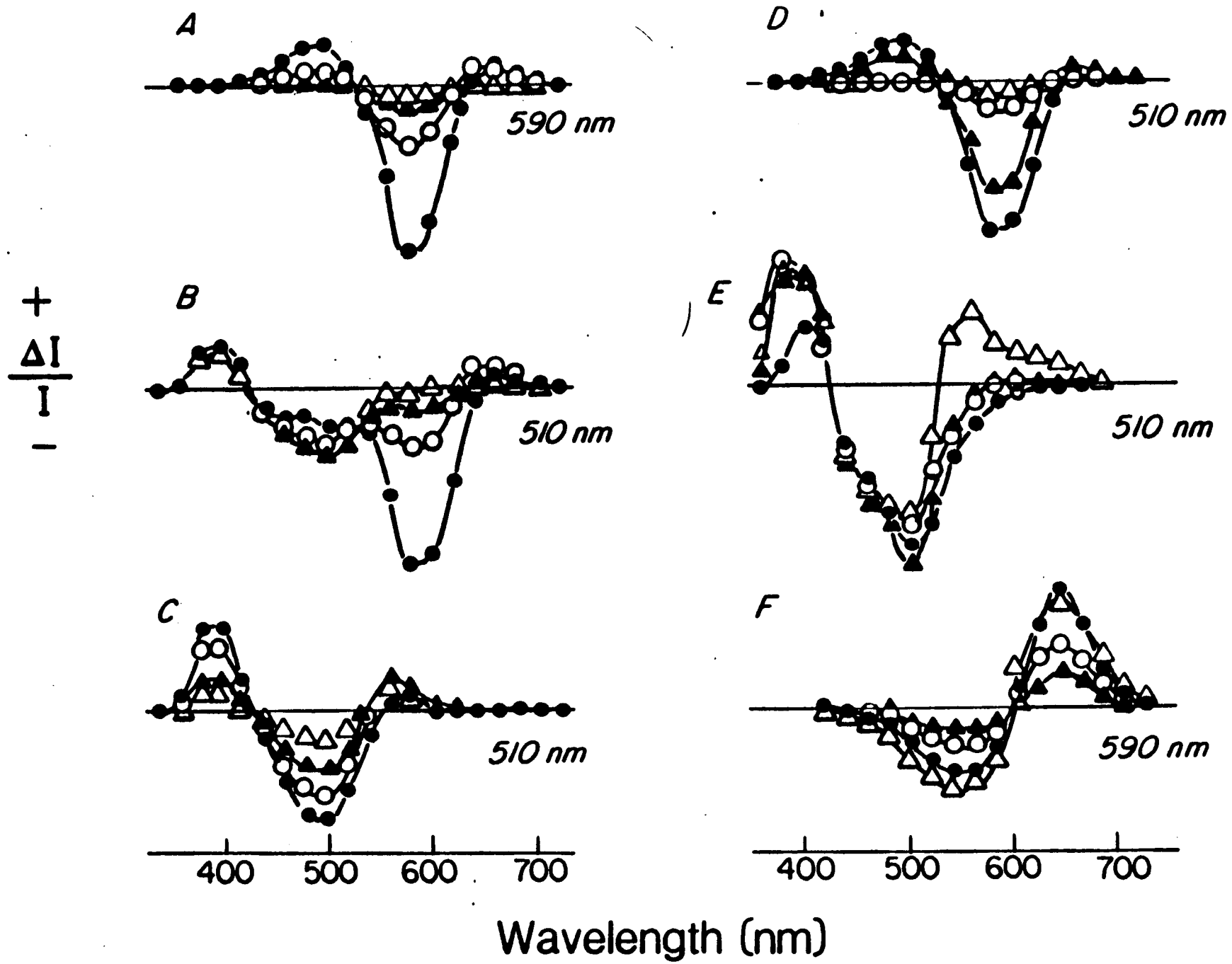
A
590 nm
B
510 nm
C
510 nm
D
510 nm
E
510 nm
F
590 nm
+
ΔI
I
−
400
500
600
700
Wavelength (nm)

Figure 4.

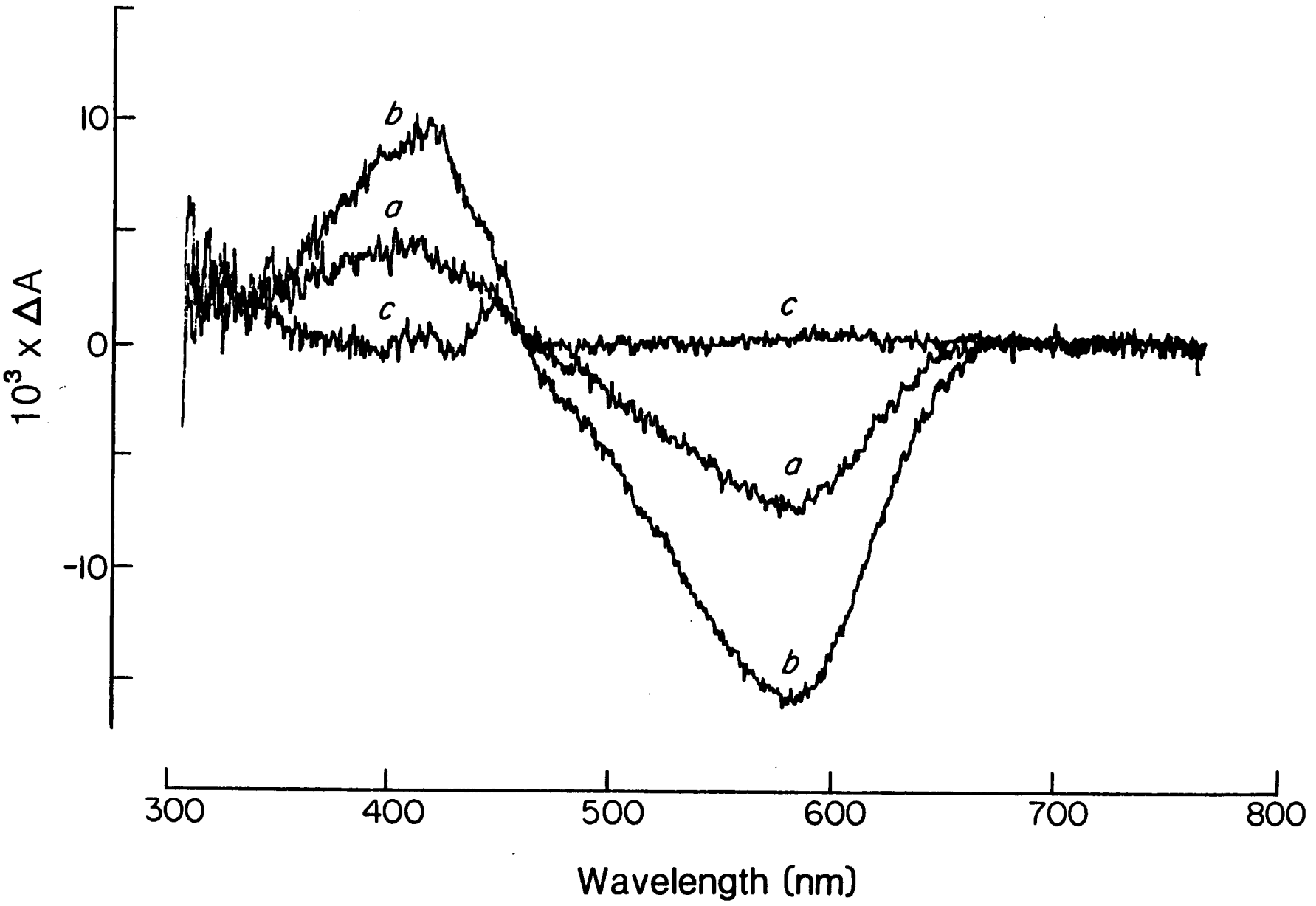
10
0
-10
$10^3 \times \Delta A$
b
a
c
c
a
b
300
400
500
600
700
800
Wavelength (nm)

Fig. 5

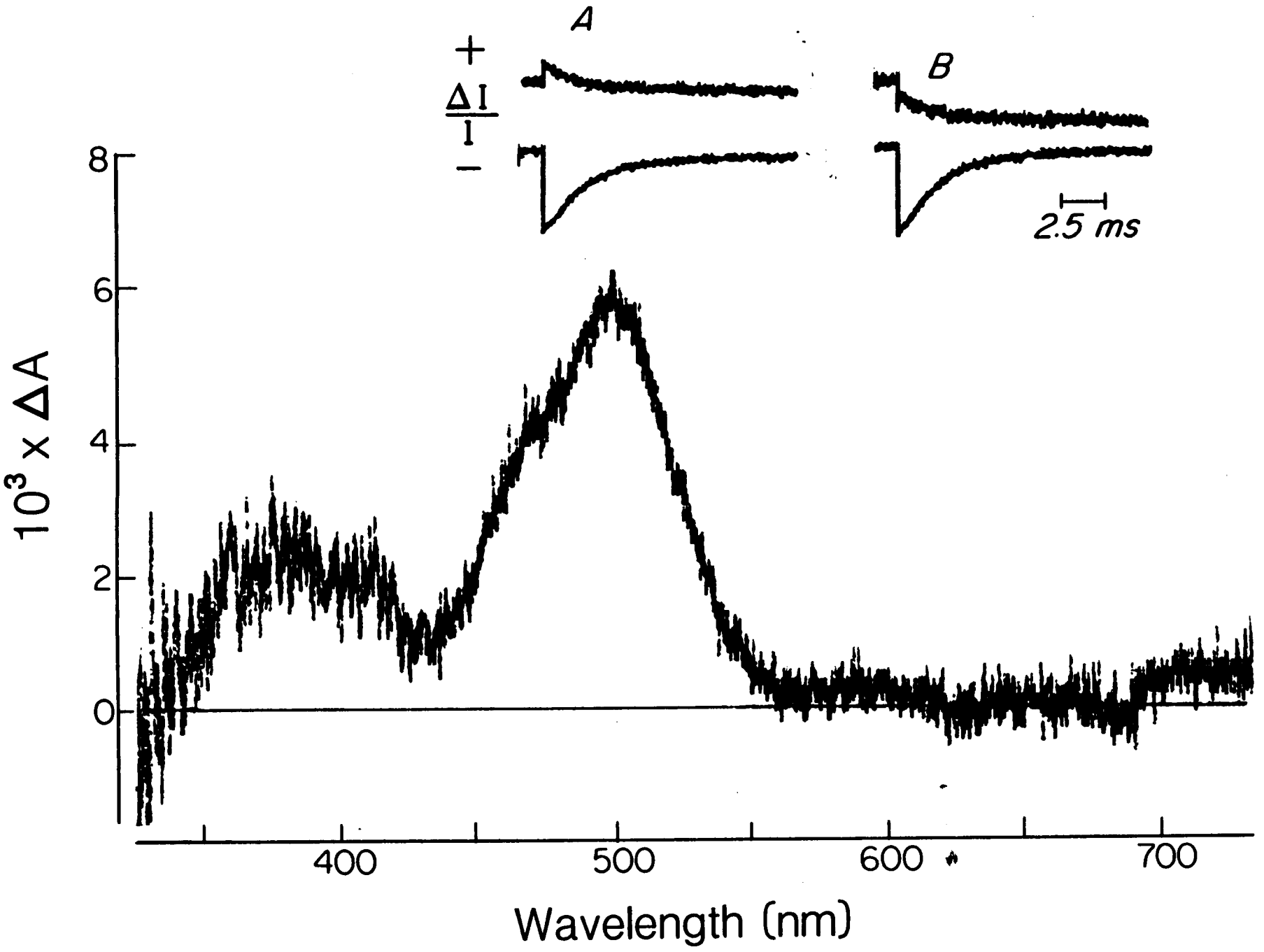
A
B
+
ΔI
I
−
2.5 ms
8
6
4
2
0
$10^3 \times \Delta A$
400
500
600
700
Wavelength (nm)

Fig. 6

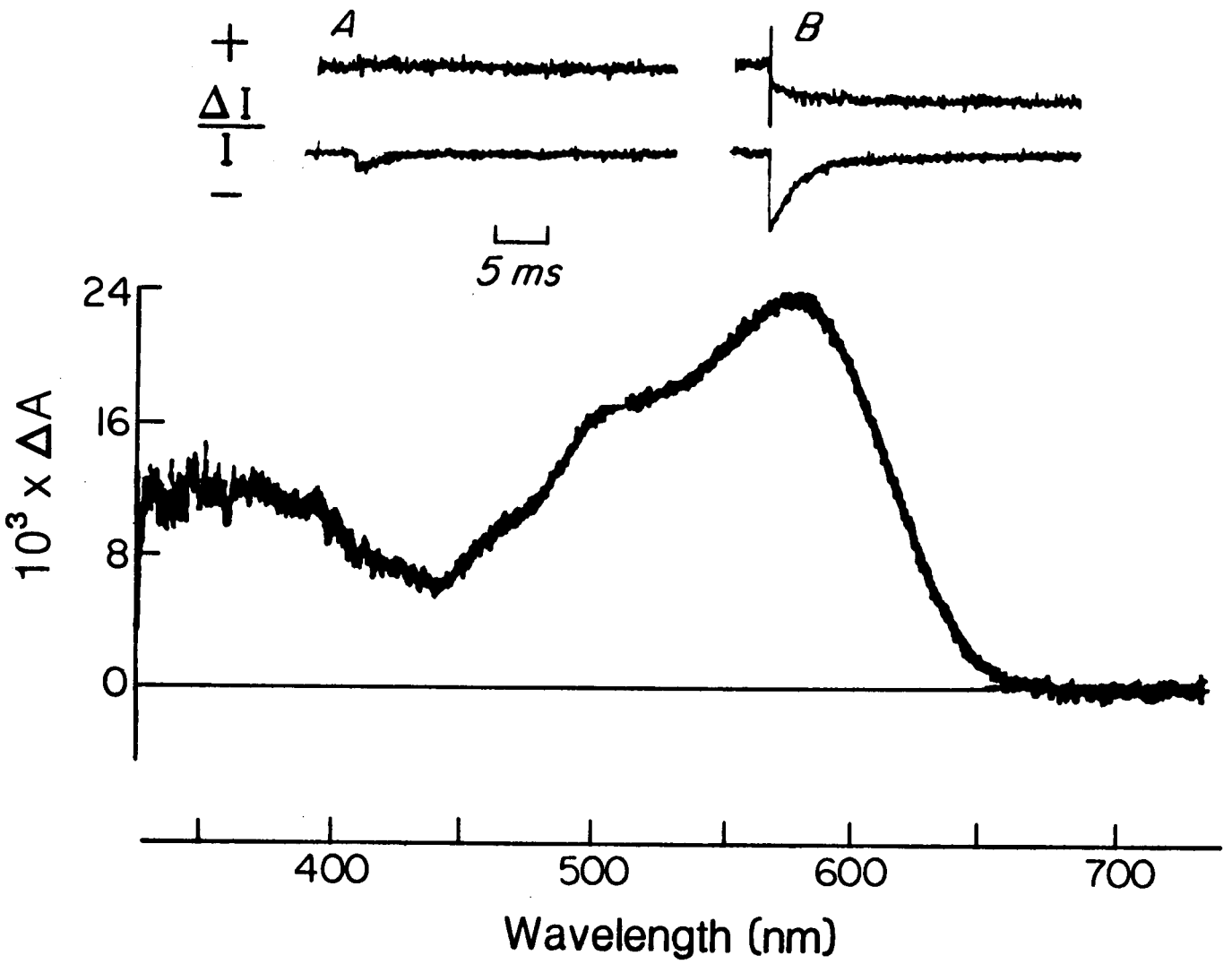
A
B
+
ΔI
I
−
5 ms
24
16
8
0
$10^3 \times \Delta A$
400
500
600
700
Wavelength (nm)

Fig. 7

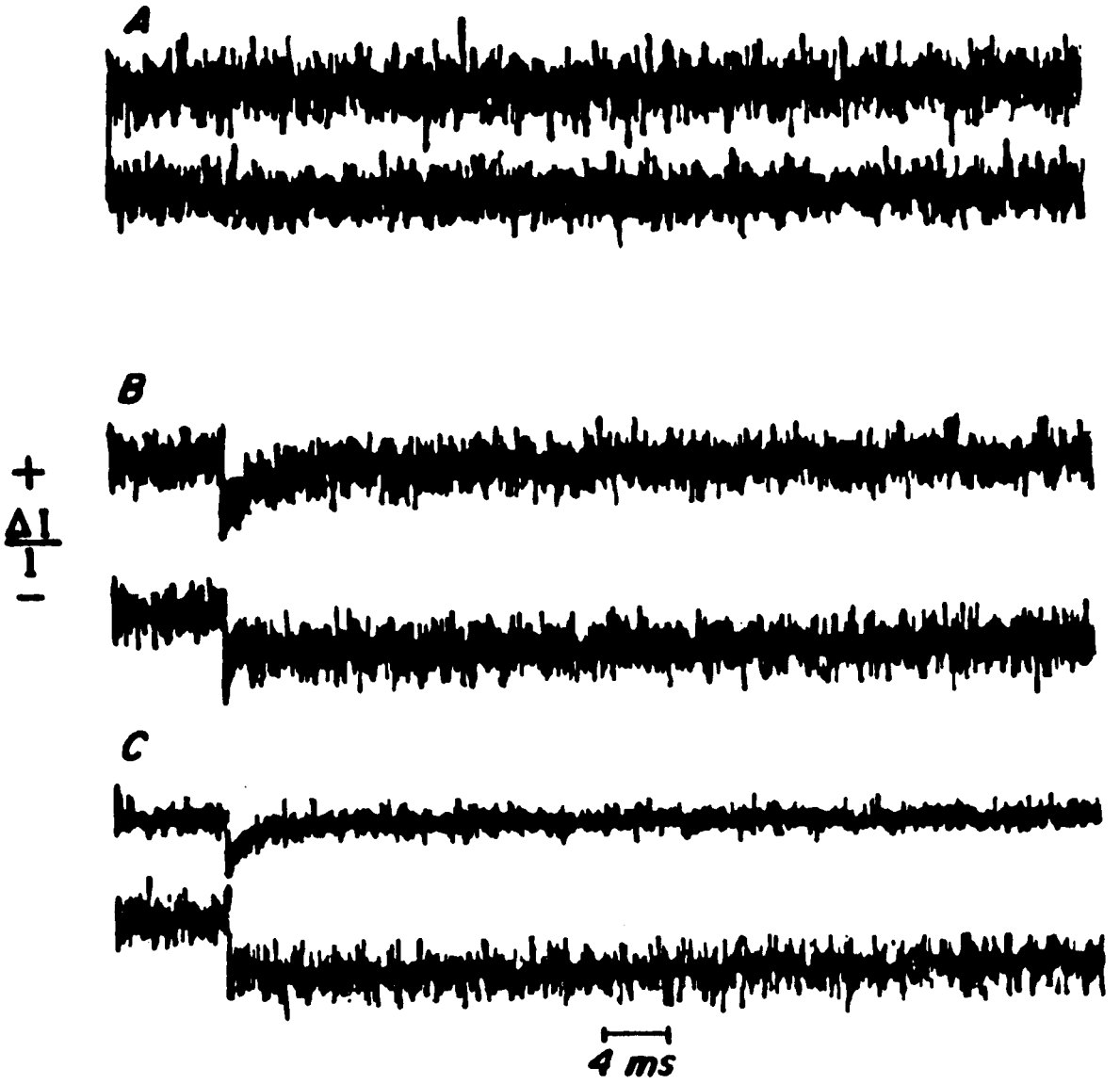

Fig #8