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SPECTRAL ANALYSES OF ABSORPTION CHANGES ASSOCIATED WITH NERVE EXCITATION IN DYE-STAINED CRAB NERVE

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Summary

Spectral characteristics of absorption changes associated with nerve excitation were studied with crab nerves stained with a homologous series of dyes, merocyanine-rhodanines and rhodanine oxonols. In these classes of dyes, the absorption changes which followed approximately the same time course as that of the action potential (fast responses) depended in a similar fashion on the wavelength and polarization of the incident light. In order to interpret those commonly observed dependencies, a mode of reorientation of the absorption oscillators of the dye molecules in the membrane matrix during nerve excitation was proposed. In addition to the fast changes mentioned above, slow responses which developed during and after the action potential were commonly observed with oxonols. The spectra of the slow changes differed from those of the fast ones, indicating a distinct mechanism on the response production. A possible mechanism of the production of fast responses was also discussed based on the proposed mode of reorientation of the absorption oscillators.

Introduction

It has been found that dye-stained nerve membranes produce changes in fluorescence emission and light absorption (fluorescence and absorption responses) associated with nerve excitation [1—3]. The phenomena interest us in two different aspects. In one way, one might expect that analyses of the optical responses can subserve for disclosing the physico-chemical nature and

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the structure of excitable membranes. This, however, may be possible only after the mechanism of the response production is clarified in each case. In this connection, dependencies of the responses on the wavelength (response spectra) and membrane potential have been measured by various investigators [4—11]. In some cases analyses of the response spectra provided important clues to discuss the mechanism [2,7,8,12]. On the other hand, as has been stressed by Salzberg and coworkers [13—15], these responses may be utilized as a new method for monitoring membrane potentials. For this purpose, they recently found a very effective dye, merocyanine-rhodanine, which gave an outstanding signal to noise ratio (30 : 1) in the absorption response and no practical toxicity to the membrane when it was applied to squid giant axons [11].

In this study, the spectral analyses of the absorption responses were carried out using the homologous series of dyes in which rhodanine ring occupies either one end (merocyanine-rhodanine) or both ends of a polymethine chain (rhodanine oxonol). In order to interpret the obtained response spectra, a particular mode of reorientation of the absorption oscillators in the membrane matrix during the action potential is proposed. The data described in this paper may be utilized for effective applications of these classes of dyes to excitable membranes for either purpose mentioned above.

Materials and Methods

The nerves were taken out from the walking legs of the three different species of crabs, *Ovalipes punctatus*, *Porturnus trituberculatus*, *Chinoectes opilo*. As far as the characteristics of the absorption responses were concerned, no appreciable difference arose from these three. Staining was carried out by immersing the nerve for 10—20 min in artificial seawater which contained the dye in a range from 10 to 100 μM. The stained nerve was mounted in a lucite chamber in which Ag-AgCl electrodes were set for the extracellular stimulation and recording. All the dyes used in this study were provided from Nippon Kankoh Shikiso Kenkyusho, Okayama, Japan.

The optical arrangement used here was similar to that used in the previous studies [10]. A 100 W quartz-iodine lamp was used as light source. In order to analyze the wavelength dependence of the absorption response, narrow band interference filters (about 20 nm bandwidth at half-height; Nippon Shinku Co., Tokyo) were used. The incident light was linearly polarized by the insertion of a dichroic film (Polaroid NH 32).

Results

Responses with merocyanine-rhodanine dyes

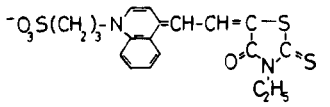
Large absorption responses associated with action potentials were observed from crab nerves stained with the dyes shown in Table I. The responses were large enough to be seen on the oscilloscope in a single sweep. Usually, however, 10—50 sweeps were summed with a transient signal processor to improve the signal to noise ratio. The wavelength dependence of the response obtained from NK-1936 is presented in the records at the top of Fig. 1. At the wavelength of

TABLE I

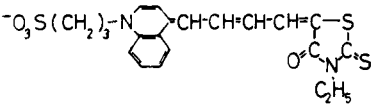
DYE USED TO STUDY CHANGES IN ABSORPTION

The number of each dye was taken from the organic chemical list of Nippon Kankoh Shikiso Kenkyusho.

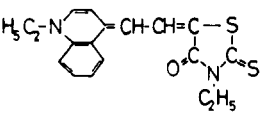
NK-1936



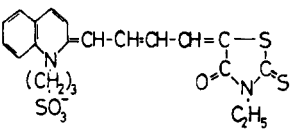
NK-2495



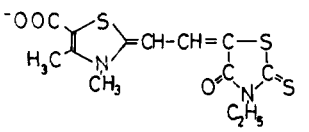
NK-1069



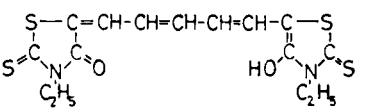
NK-1935



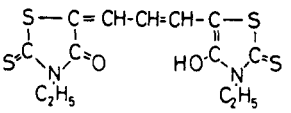
NK-1462



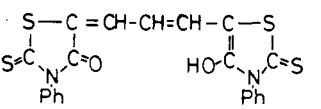
NK-2095



NK-1451



NK-1746



610 nm, the response measured with the polarized light in the parallel direction to the long axis of the nerve (parallel component) is positive which indicates a transient increase in the intensity of the transmitted light (decrease in absorption), whereas the response with the polarized light in the perpendicular direction (perpendicular component) is negative. The response spectra drawn in Fig. 1 were obtained by plotting the size of each response at its peak height. The spectrum of the parallel component stays on the positive side in the entire region of wavelength in which substantial size of the response was observed. On the other hand, the spectrum of the perpendicular component is more intracte, changing many times the direction of the response depending on the wavelength.

These essential features of the response spectra were observed also with the other merocyanine-rhodanine dyes in Table I. A brief comment on each dye is as follows: NK-2495 is a longer methine chain analogue of NK-1936. This dye was first found by Ross et al. [11] and Salzberg et al. [16] to be very effective for measuring the absorption response. The obtained response spectra with crab nerves were quite similar to those reported for squid axons by Ross et al. [11]. NK-1935 is a positional isomer of NK-2495. The response spectra were merely blue-shifted as a whole by approximately 30 nm from those with NK-2495.

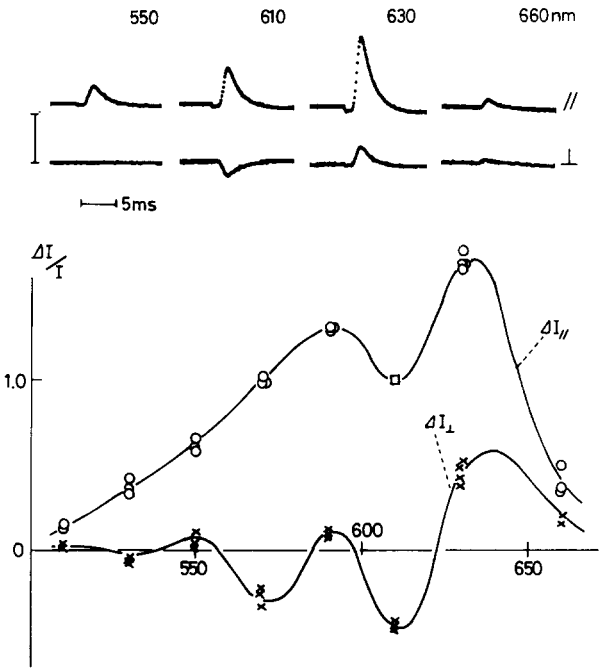


Fig. 1. Spectra of the absorption response obtained from a crab nerve stained with merocyanine-rhodanine, NK-1936. The response amplitudes measured by the light polarized in the parallel and perpendicular direction to the nerve are indicated by circles and crosses, respectively (normalized to the value represented by the square). Records of the responses are shown on top of the figure. Unity on the ordinate indicates a change of $1.2 \cdot 10^{-3}$ times the transmitted light intensity. Staining solution contained the dye at a concentration of 50 μM.

In NK-1069, the localized negative charge due to a sulfonate group is absent. The response spectra are shown in Fig. 2. Finally, in NK-1462, quinoline ring is substituted by thiazole ring. The fundamental features mentioned above were not altered by the substitution.

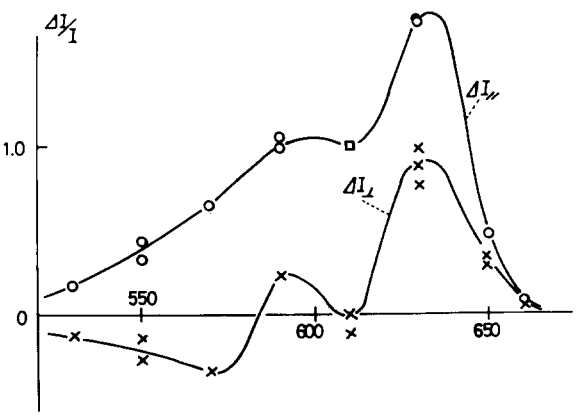


Fig. 2. Spectra of the absorption response obtained from a crab nerve stained with merocyanine-rhodanine, NK-1069. The polarization of the light is indicated. Unity on the ordinate indicates a change of $1.4 \cdot 10^{-4}$ times the transmitted light intensity.

Reconstruction of response spectra

The simplest way to interpret the commonly observed features is the following. If the perpendicular component (curve 2 in Fig. 3) of the response spectrum obtained from NK-1936 is subtracted from the parallel, the resultant constitutes curve 3. This inversely means that when the response is detected with the polarizer in the perpendicular direction, the absorption spectrum characterized by curve 1 is converted into that characterized by curve 3 during nerve excitation. Thus, we detected the resultant different spectrum (curve 2) which is complicated by a spectral shift involved in the conversion. On the other hand, with the parallel polarizer the absorption spectrum (curve 1) is converted into a very small one, so that the former solely determines the main feature of the response spectrum. This spectral conversion may occur if we assume a particular mode of the reorientation of the dye molecules in the membrane matrix during the nerve excitation. Namely, a small fraction of the dye molecules whose absorption oscillators into the normal direction to the membrane surface. The randomly distributed absorption oscillators result in an identical absorption spectrum in either direction of the polarizer, which may be approximated by curve 1. On the other hand, those distributed in the nor-

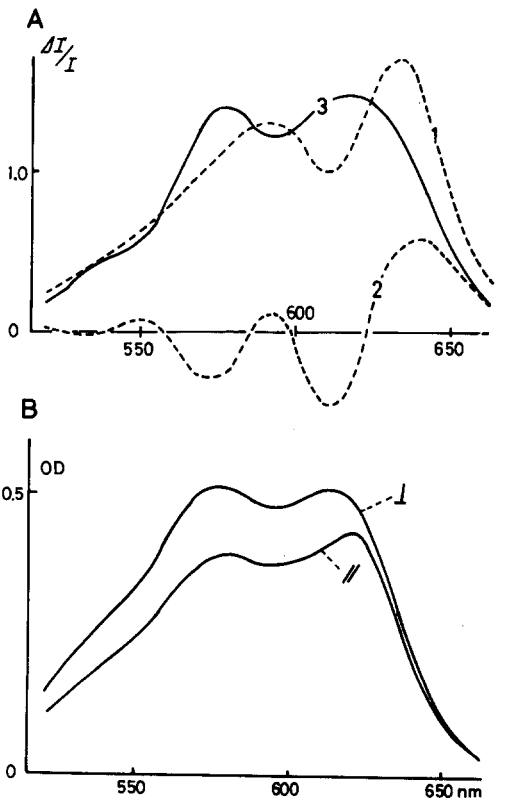


Fig. 3. (A) ------, transcriptions of the response spectra for NK-1936 shown in Fig. 1. Curve 3 (———) was composed by subtracting curve 2 (perpendicular component of the spectra) from curve 1 (parallel component). (B) Total light absorption of a resting crab nerve stained with NK-1936.

mal direction to the membrane are presumably related to a blue-shifted absorption spectrum with perpendicular direction of the polarizer, which may be approximated by curve 3, but no absorption is expected with the parallel polarizer due to angular relationship between the absorption oscillators and the electric vector of light. These can be handled mathematically with the geometric factors which were introduced in previous papers [10,12].

By measuring the transmitted light through the stained nerve with NK-1936 under the resting condition of the nerve with a monochrometer, the occurrence of the spectral shift correlated to the preferential orientation of the absorption oscillators is detected as shown in Fig. 3B. It is noted that the regions in wavelength where the two main peaks in the absorption spectrum were measured with perpendicularly polarized light (denoted by ⊥) roughly correspond to those in the reconstituted spectrum (curve 3). The spectrum of the total absorption measured with the parallel polarizer is relatively similar to the response spectrum (curve 1) detected with the corresponding direction of the polarizer. The two large absorption bands were also seen with NK-1936 freely dissolved either in ethanol or artificial sea water. The absorption band at shorter wavelength is most likely related to dimer (or higher aggregates) absorption and the band at longer wavelength to monomer since an increase in dye concentration in sea water enhanced the former at the expense of the latter.

Responses with rhodanine oxonols

The response obtained from the crab nerve stained with the dye classified as rhodanine oxonol (NK-2095, NK-1451, NK-1746) was commonly composed by at least two different components (fast and slow responses) which were distinguished by their time courses as seen in Fig. 4A. The slow responses developed even after the extracellularly recorded action potential was terminated. Moreover, when a train of pulses were applied to stimulate the nerve, the summation of the slow responses took place as seen in Fig. 4B. The response

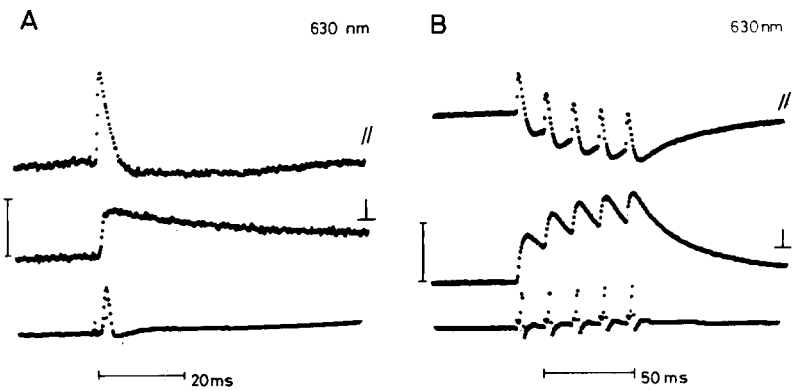


Fig. 4. (A) Absorption responses obtained from a crab nerve stained with rhodanine oxonol, NK-2095. Following the fast responses, the slowly developing responses are seen. The polarization and wavelength of the light used are indicated. The vertical bar indicates a change of $2.6 \cdot 10^{-4}$ times the transmitted light intensity. (B) The slow responses are summated during respective stimulation. The vertical bar indicates $1.4 \cdot 10^{-4}$ times the transmitted light intensity. The bottom traces in both figures are the extracellularly recorded action potential.

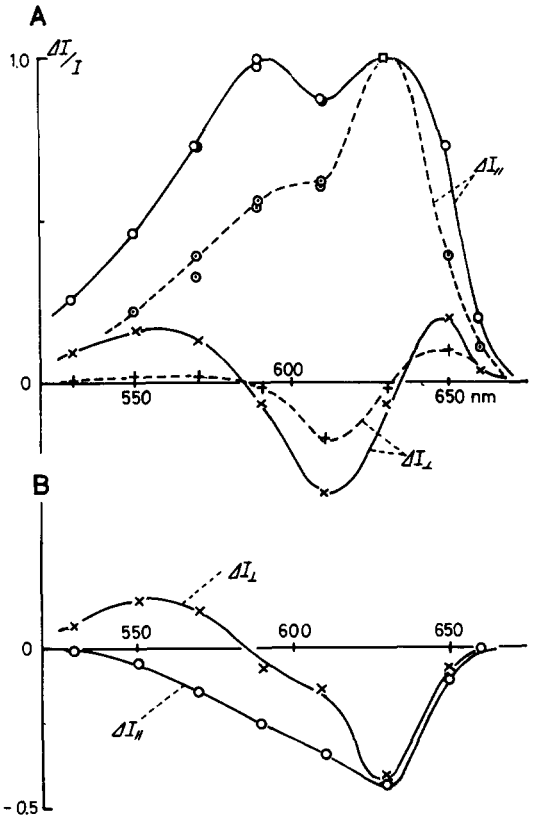


Fig. 5. (A) Spectra of the absorption response obtained from crab nerves stained with rhodanine oxonol, NK-1746, for 10 min. Staining solution contained different concentration of the dye: ———, 70 μM; - - - - - -, 2 μM. Unity on the ordinate indicates a change of $8.0 \cdot 10^{-4}$ or $5.5 \cdot 10^{-4}$ times the transmitted light intensity either for the continuous or for the broken lines. (B) Relative amplitudes of the slow responses which were separated from the fast ones by using the same records as those employed for the continuous lines in the upper figure.

spectra of the fast component with NK-1746 are composed in Fig. 5A by plotting the response amplitudes at the peak of the action potential without correcting for the small contribution of the slow component (maximum error was estimated at 15%). Two sets of spectra are presented in the figure. The continuous or broken lines connect the data obtained from the nerve stained in the solution containing either 70 μM or 2 μM of the dye, respectively. The large alternation of the response spectra with increased dye concentration can be noticed in the figure. A relative enhancement in the region centered around the peak at shorter wavelength is most likely related to an increased dimer (or aggregates) formation of the dye molecules in the membrane matrix when the nerve is immersed in the solution with an increased dye concentration.

It is noted that the basic features of the response spectra mentioned before for the merocyanine-rhodanine dyes were still commonly seen in the fast response of the rhodanine oxonols listed in Table I. On the other hand, the spectra of the slow response were very much different from those of the fast one as exemplified by the spectra shown in Fig. 5B. In the figure, the response

size measured 15 ms after the electrical stimulation were plotted against wavelength. At that time the slow responses still remained near their maximum levels but the fast ones were completely decayed.

Discussion

General aspects on the production of fast responses

The dyes, merocyanine-rhodanines and rhodanine oxonols, applied to the crab nerve produced large absorption responses with little toxicity for the nerve due to photodynamic effect [17,18] as first demonstrated with the squid axon [11,16]. In either class of dye, the absorption responses, which followed approximately the same time course of the action potential, showed a common feature in their wavelength and light polarization dependencies (response spectra). This suggests the existence of a common basic mechanism involved in the production of the response. Judged from the experimental facts, the length of the polymechine chain, the presence of a quinoline ring and a localized negative charge on the dye molecule do not seem to primarily determine the basic mechanism. Since the rhodanine ring is the common constituent of the dyes used, it may play an important role to determine the common feature through its physico-chemical nature.

The statistical reorientation of absorption oscillators from random to normal direction to the membrane surface during the nerve excitation was proposed in the preceding section. The scheme seems adequate to interpret the dichroic nature of the response and the complex feature of the perpendicular component of the response spectra, although it cannot exclude other way of interpretation. Moreover, assuming that the proposed scheme is correct, the cause of the reorientation of the absorption oscillators still remains a puzzling question. In order to discuss this point, experimental facts other than those described in the preceding section should be taken into account.

According to the studies done by Ross et al. [11], the absorption change with the merocyanine-rhodaine dye was linearly related to the membrane potential when it was examined under voltage-clamped steps. They also reported that the sign of the response spectra with an externally stained axon was reversed by introducing the same dye inside the squid axon (similar observation with other dye was reported by Tasaki and Warashina [10]). In these cases, the responding dye molecules are thought to occupy the sites rather directly exposed to the membrane potential but not to permeate through the membrane.

The following facts obtained by the author's preliminary experiments may give additional support for this idea. When the stained crab nerve with NK-2495 was kept at 6°C for 20 h, it still produced good response and no significant change in the basic features of the response spectra. The situation did not altered even when the stained nerve was stored in the artificial sea water that contained different kinds of enzymes, such as protease, collagenease, neuraminidase and hyaluronidase. Since the dye used above is rapidly decomposed under the condition freely dissolved in the artificial sea water (about 220 min for half-decay at 6°C), the dye is probably embedded in the membrane matrix where the external medium cannot easily reach it.

Origin of the fast responses

The experimental results mentioned above offer important clues to conceive possible mechanisms on the response production.

The electrophoretic movement of the dye molecules in or near the membrane seems to be ruled out from the central mechanism of the response production since either a deprivation of a negative sulfonate group (in this study) or replacement of the same group with a positively charged quarternary ammonium (by Cohen et al. [19]) did not give essential modification on the response spectrum.

The critical involvement of dimer (or aggregate) to monomer conversion of dye molecules to the production of absorption response has been revealed in merocyanine 540 [11,12,20], which is one of the extensively studied dyes. In the present case this type of conversion during nerve excitation is not occurring to the significant extent for determining the spectral feature. Instead, the reconstructed response spectra suggested that the randomly oriented absorption oscillators of both species reoriented together into the normal direction of the membrane during nerve excitation.

Conti et al. [8] and Carbone et al. [21] have thoroughly investigated the origin of the responses which arose in squid axons and lipid bilayers labelled with fluorescent probes. As analysed by them, changes in the electric field across the membrane possibly causes reorientations and changes in partition coefficient of bound probe molecules through the electrical interaction worked on the electric dipole of the probes. However, the picture which comprise a continuous, small reorientation of the dye molecules depending on the change in the membrane potential seems inadequate for the present case since rather large reorientation, random to normal of the membrane surface, was proposed in the preceding section. Alternatively, the second picture, changes in partition coefficient of membrane-bound dye molecules are attractive here by assuming two classes of adsorbing site (N and R) in the membrane matrix which are characterized as follows: site N consists of highly ordered structures, such as the lipid layer of axonal membrane, and is directly exposed to the electric field. Thus, binding modes are restricted, so that the absorption oscillators are aligned in the normal direction to the membrane and electric energy, $\mu\Delta E$, is assigned to each dye molecules, where μ and ΔE denote an electric dipole moment of dye molecule and a change in the electric field in the normal direction, respectively. In site R, on the other hand, dyes are adsorbed in the random orientation and not directly exposed to the electric field. The population ratio of the dye molecules in these two sites is governed by the partition coefficient, K_p, that is

$$\frac{(N)}{(R)} = K_p \tag{1}$$

As derived by Conti et al. [8], the change in the electric field, ΔE, shifts the value of coefficient by the following relation with the first-order approximation.

$$\Delta K_p = K_p \mu \Delta E / kT \tag{2}$$

In consequence, the change in the number of adsorbed dye molecules in site N

due to the shift in the partition coefficient linearly depends on the change in the applied electric field, and is given by

$$\Delta N = \frac{(N)(R)}{C} \mu \Delta E / kT \tag{3}$$

where C is the total number of the dye molecules. The absorption response expected from Eqn. 3 also satisfies the spectral characteristics described in the preceding section by virtue of the assumed nature of binding. It is evident that the scheme may be applicable to the underlying mechanism of dimer to monomer conversion during nerve excitation as seen in merocyanine 540 by assuming that site N adsorbs only monomeric form of the dye.

The interpretation mentioned above, however, is still at a speculative stage and may have various shortcomings in the present form. According to analyses done by Conti et al. [8], the time course of the change in the number of bound dye is too slow to account for the fast responses if site R is diffusively distributed in the space adjacent to the membrane or separated across the Schwann cell layer. This difficulty, however, might be avoided by localizing site R in very vicinity of site N. The question how monomers and dimers (aggregates) can behave similarly, as suggested from the spectral analysis in merocyanine-rhodanine dye, still remains. Moreover, physico-chemical quantities such as values of the dipole moment of the dye molecules and the partition coefficient have to be obtained to evaluate the scheme properly.

Origin of the slow responses

In addition of the fast response, the slow one was commonly observed in the rhodanine oxonols. The distinct mechanism of the slow from the fast response was suggested not only from their time courses but also from their spectral aspects. The summation of the slow responses with repetitive stimulation (Fig. 4B) is phenomenologically similar to that found by Watanabe and Terakawa [22,23] in birefringence signals. They stated that the summated component which was affected by calcium salt or colchicine was determined to have an axoplasmic origin. The rhodanine oxonols which do not have a localized charged group might penetrate across the membrane into the region where the summated birefringence signal was produced. In the slow absorption response, however, no significant effects were found with those reagents. A slow conversion from the aggregates to monomers of the dye may offer alternative explanation.

Acknowledgments

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