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Further Studies on Absorption Changes Arising in Dye-Stained Nerves during Excitation

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Summary. Changes in light absorption during nerve excitation (absorption responses) were detected from the crab leg nerve, the rabbit vagus, and the rat superior cervical ganglion (SCG) stained with a merocyanine-rhodanine. Dependences of the responses on the wavelength and polarization of the incident light (absorption spectra) showed characteristic features with the respective nerves. In the crab nerve, the pattern of response spectra was precisely analyzed based on the previously proposed scheme, which included the shift of absorption bands and the statistical reorientation of absorption oscillators of the dye molecules in the membrane matrix during nerve excitation. Different patterns of the response spectra between the crab nerve and the rabbit vagus suggested that distinct physicochemical environments of the dye occurred in these two classes of membranes. On the other hand, the characteristic pattern that arose in the rat SCG was explained by its morphological form, that is, unlike those in a bundle of axons, the membrane elements in the ganglion were randomly oriented with respect to the direction of the light polarization.

Key words: absorption change, merocyanine-rhodanine, rat vagus, crab nerve

Since changes in light absorption (absorption responses) were found in dye-stained nerve membranes associated with their electrical activities [8, 13], methods for measuring and analyzing the responses have been developed using various kinds of dyes [3, 15]. Among the dyes examined, it was demonstrated by Salzberg et al. [10] that merocyanine-rhodanines and a merocyanine-oxazolone gave remarkable signal to noise ratios in the absorption response and no practical toxicity to excitabilities when they were applied to squid axons. Thereafter, this class of dyes has been exploited with various kinds of nerve tissues for monitoring nerve activities and investigating the physicochemical nature of nerve membranes [6, 7, 9, 11, 16]. The origin of absorption response with these dyes, however, is not yet fully understood, although possible mechanisms have been discussed by Ross et al. [9] and Warashina [16].

For a further study in this respect, the responses from various nerve membranes, including those from mammalia, stained with the merocyanine-rhodanine were measured and analyzed. The dependence of the responses on the wavelength and polarization of the light was found to be different in different nerve elements, as was reported by Ross and Reichart [7]. The observed dependencies examined in the following section are based on a previously proposed scheme for the response production [16] and other factors which may affect the characteristic feature of the response.

Materials and Methods

The cervical portion of vagus and the superior cervical ganglion were dissected from the wister rat or the rabbit under urethane anesthesia. Nerve bundles were also taken out of the walking legs of the sea crab, *Chionoecetes oplio*. The nerves were stained at a level of 100–50 μM of the merocyanine-rhodanine, 5-[(1-γ-sodium sulfopropyl-4(1 H)-quinolylidene)-2-butenyliden]-3-ethylrhodanine (NK-2495 in a chemical list of Nippon Kankoh Shikiso Kenkyusho, Okayama, Japan), in the Krebs solution, or in artificial sea water. A chamber loaded with the rat superior cervical ganglion was perfused with oxygenated Krebs solution except during the optical measurement. Measurements were done at room temperature, 20–25 °C. Optical and electrical systems employed were similar to those used in a previous study [15].

The absorbance of the dye with liposomes was measured using a Nikon monochrometer G-250. The liposomes were prepared by a rapid injection of egg lecithin dissolved in ethanol into the Krebs solution through a syringe [1].

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Results

Absorption Changes Arising in Stained Nerve Fibers

When a rabbit vagus stained with the merocyanine-rhodanine (NK-2495) was examined using 660 nm light polarized in the direction of the long axis of nerve, two distinguishable deflections were observed during the nerve activity as shown in the middle trace in Fig. 1. A negative response here denotes a transient decrease in the intensity of transmitted light or an increase of the absorbance. The responses are associated with action potentials (bottom trace) which also consist of two peaks. The first peak represents the electrical activity of myelinated B fibers while the second one that of unmyelinated C fibers. The occurrence of the response with the B fibers is more clearly seen in the top trace, in which only the B fiber activities are evoked by weaker stimuli.

The wavelength dependence of the response (response spectrum) in C fibers of the vagus is drawn in Fig. 2*A* by plotting the relative size of each response at its peak height. The response spectrum is highly dependent on the polarization of the light. The response measured with the light polarized in the perpendicular direction to the long axis of nerve (perpendicular component of the response) is much larger than that with the light polarized in the parallel direction (parallel component), except at a small region around 640 nm where the polarity of the response reverses. A similar pattern of response spectra was obtained from B fibers of the vagus. In the parallel component of the latter case, however, a trough (approximately one-third of the other component in depth) appeared between 650 and 730 nm. The absorption responses from unmyelinated C fibers in the rat vagus and the rat sciatic nerve also had essentially the same response spectra as those seen in Fig. 2*A*. The absorption response was not detected from myelinated A fibers in the rat sciatic nerve (if any, the magnitude of the response was below $5 \cdot 10^{-6}$ times the transmitted light intensity).

In sharp contrast to these mammalian nerves, the response spectra from the crab nerve stained with the same dye were different, as seen in Fig. 2*B*. The parallel component with a simpler shape dominates the perpendicular component with an intricate wavy shape. These spectra resemble those found by Ross et al. [9] in squid axons with the same dye.

The different patterns of response spectra between the mammalian and invertebrate nerves were not commonly seen with other dyes. When a shorter polymethine analogue (NK-1936) of the present dye, or a triphenyl dye, crystal violet, was used, the pattern of response spectra with the mammalian nerve was not much different from that with the crab nerve (the response spectra with these dyes were presented in previous articles [14, 16]).

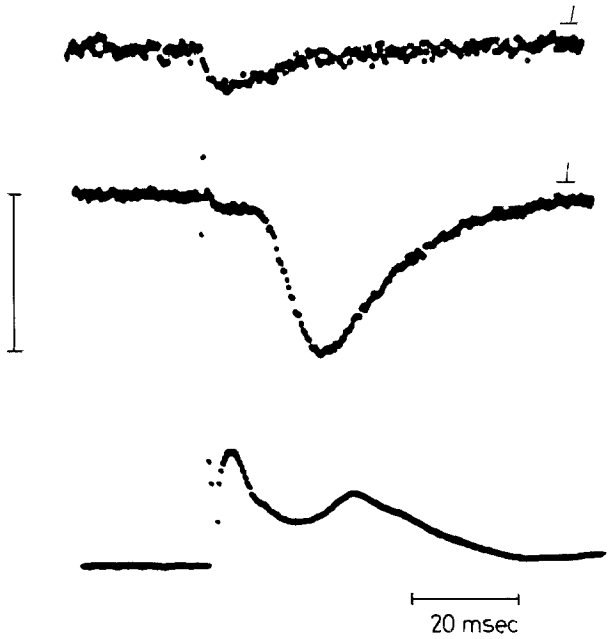


Fig. 1. Absorption response obtained from the rabbit vagus stained with NK-2495. The light used was 660 nm and linearly polarized in the perpendicular direction of the nerve. Top: the response associated with fast component of the electrical activities; 120 sweeps averaged with a signal processor. Middle: the responses with fast and slow components of the electrical activities; 40 sweeps averaged; the vertical bar indicates a change of $1.1 \cdot 10^{-4}$ times the transmitted light intensity. Bottom: extracellularly recorded electrical activities of the vagus nerve

Absorption Response Arising in the Rat Superior Cervical Ganglion (SCG)

By a presynaptic volley, roughly speaking, two groups of electrical activities were evoked in postganglionic elements of the rat SCG. The absorption responses observed (inset of Fig. 3) were associated with the two groups of activities. The response spectra obtained from the early group of the responses are shown in Fig. 3, in which the parallel and perpendicular components are assigned in the conventional way, as illustrated in the figure. The spectra from the ganglia were much less dichroic than those from the axons.

Reconstruction of the Response Spectra and Physicochemical Properties of the Dye

A particular scheme of the reconstruction of the response spectra has been proposed for cases of crab

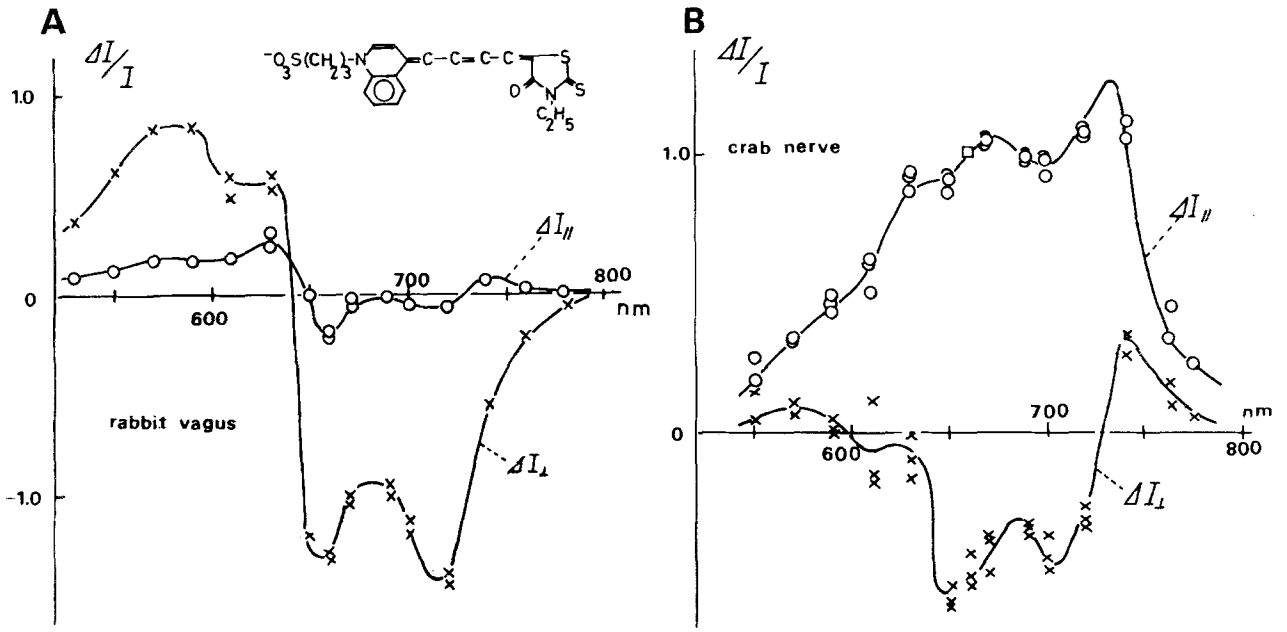


Fig. 2. Wavelength dependence of the absorption response obtained from (*A*) the rabbit vagus and (*B*) the crab nerve stained with NK-2495. Relative amplitudes of the response measured with the light polarized in the parallel direction and perpendicular direction to the nerve were indicated by circles and crosses, respectively. Unities on the ordinates indicates changes of $9.2 \cdot 10^{-5}$ (for *A*) and $1.7 \cdot 10^{-3}$ (for *B*) times the transmitted light intensity

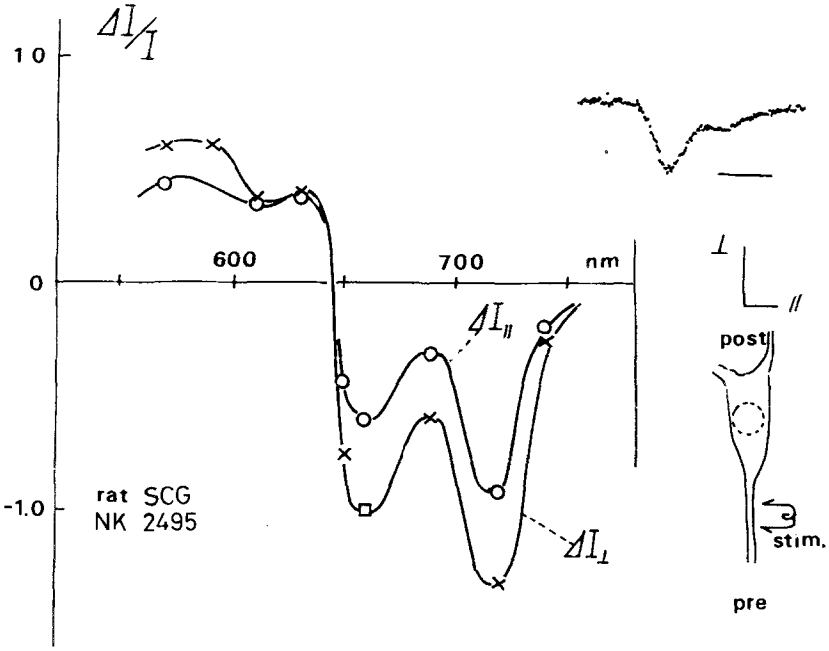


Fig. 3. Spectra of the absorption response obtained from the rat superior cervical ganglion stained with NK-2495. Unity on the ordinate indicates a change of $4.2 \cdot 10^{-5}$ times the transmitted light intensity. Insets: the absorption response obtained after presynaptic volleys. The horizontal bar indicates 40 msec. The parallel and perpendicular directions are shown. The light passed at the middle portion of the ganglion

nerves stained with various kinds of merocyanine-rhodanines [16]. As illustrated in Fig. 4*A*, the subtraction of the perpendicular component (line *2*) from the parallel component (line *1*) constitutes a relatively simple spectrum (line *3*). Inversely, we may explain the intricate spectrum of absorption response (line *2*) as a result of the alteration of the absorption spectrum from that characterized by line *1* to that by line *3*, which presumably takes place in the dye molecules in or near the membrane during the nerve excitation.

The question arises whether the dye bound to the macromolecules which occur in or near the nerve membrane may give rise to spectra similar to lines *1* and *2*. In order to get an insight into this point, the absorbance of the dye dissolved with various kinds

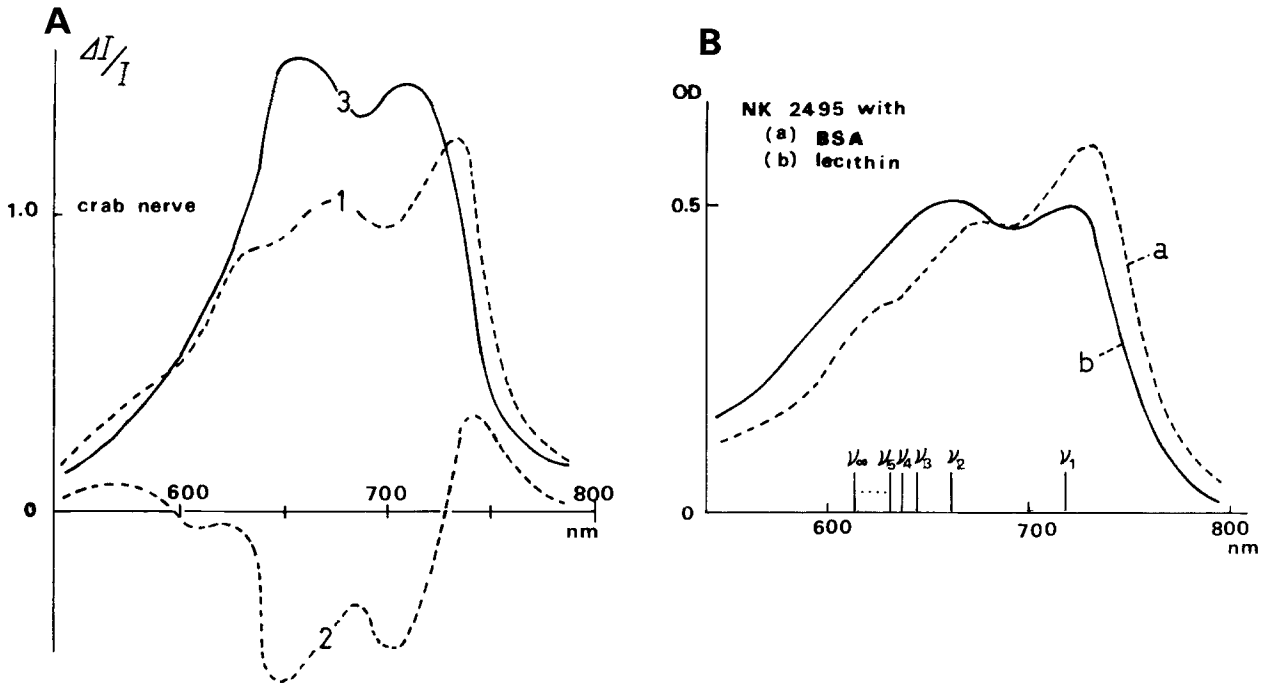


Fig. 4. (*A*): Reconstruction of the absorption spectra obtained from the crab nerve stained with NK-2495. Lines *1* and *2* were transcribed from Fig. 2*A*. The subtraction of line *2* from line *1* leaves line *3*. The significance of this reconstruction is described in the text. (*B*): Optical density of NK-2495 (34 μM) with (*a*) 6.7 mg/ml of bovine serum albumin, (*b*) 1.6 mg/ml of lecithin liposomes in 10 nm cuvettes. Monomer (ν_1), dimer (ν_2)····· and *N*-mer (ν_N) bands are indicated on the abscissa

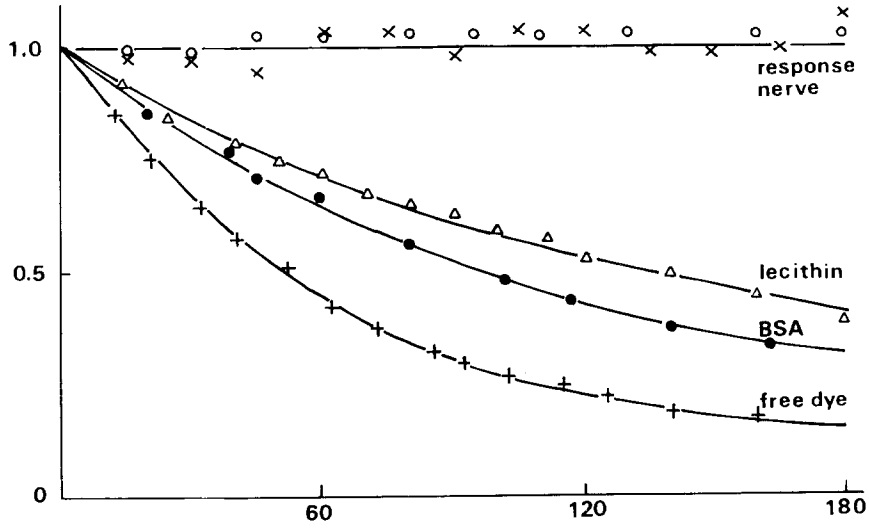


Fig. 5. Decomposition rate of merocyanine-rhodanine, NK-2495 in various environments. Absorbances of the dye (34 μM) dissolved freely (+), with 6.7 mg/ml of bovine serum albumin (●), with 1.6 mg/ml of lecithin liposomes (△) in the Krebs solution. The total absorbance (○) and the response amplitude (×) with the rabbit vagus are also included. Quantities were normalized at initial values. Measurements were done with 720 nm light at 23 °

of biomolecules, bovine serum albumin (BSA), lecithin liposomes, inulin, and gelatin in the Krebs solution (6.7 mg/ml of lecithin, 1.6 mg/ml of the other biomolecules, 34 μM of the dye) was measured. Line *a* with BSA in Fig. 4*B* is in good accord with line *1* of the response spectra, showing three prominent peaks around 625, 670, and 730 nm. On the other hand, a resemblance can be recognized between line *b* with lecithin and line *3* (peaks of 718 and 660 nm for the former, 710 and 656 nm for the latter). The spectrum with inulin or gelatin differed little from that of the free dye in the Krebs solution. The multi-bands structure in the absorption spectra either with the nerve or biomolecules is ascribed to the formation of aggregates of the dye molecules [12]. The location of blue-shifted absorption bands due to aggregates of cyanine dyes has been described by a relationship, $\Delta\nu_N=(N-1)/N\Delta\nu_\infty$, where $\Delta\nu_N$ denotes the frequency

shift from monomer to N-mer band and $\Delta\nu_\infty$ denotes that for infinitely large aggregation [5]. By applying the relationship to the absorption spectrum with lecithin liposomes (line (*b*)), the monomer (ν_1), dimer (ν_2)·····N-mer (ν_N) bands are calculated and represented by the vertical bars on the abscissa in Fig. 4*B*.

The dye dissolved in the Ringer solution decolored with 50 min for a half decay time at 23 °C, while neither decoloring of the dye-stained rabbit vagus nor decline of the absorption response was detected to a significant degree within 180 min (Fig. 5). The presence of BSA or lecithin liposomes slowed the decomposition rate (95 or 115 min for a half-decay). With inulin or gelatin the rate of decomposition was not significantly altered from that of the free dye.

Discussion

Absorption Responses from Various Nerve Fibers

Absorption responses were detected from both myelinated B fibers and unmyelinated C fibers in the rabbit vagus (as well as the rat vagus) (Fig. 1). On the other hand, myelinated A fibers in the rat sciatic nerve did not produce a response above the noise level. The absence of the response in A fibers may be caused by the fact that the heavy myelination of A fibers obstructs the penetration of the dye into the internodal portion of the axonal membrane and weakens a change in the electrotonically generated potential across the membrane. The nodal parts of A fibers might be stained and generating the response during the excitation, which, however, may not be detected by the method used here since the fractional area of the nodal membrane is too small.

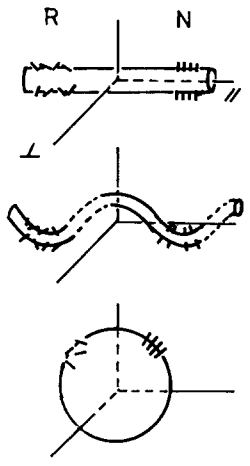
Factors Which Affect the Pattern of Response Spectra

To discuss different spectral features obtained from the various nerve elements stained with the same dye (NK-2495), one should take account of the following two processes, which are directly related to the pattern of response spectra: (1) the spectral shift in dye absorption and the statistical reorientation of absorption oscillators of dye molecules in the membrane matrix, and (2) a morphological arrangement of the membrane in the nerve. The first has been adequately analyzed by means of the reconstruction of response spectra, while the second one by the "geometric factors" introduced by Warashina and Tasaki [15, 17].

The geometric factors can be calculated by integrating square of cosines between the directions of absorption oscillators and that of light polarization over the entire surface of a certain morphological

TABLE I GEOMETRIC FACTORS

SHAPES	LIGHT POLARIZATION	
	PARALLEL ($A_{//}$)	PERPENDICULAR ($A_{\perp}$)
CYLINDER		
STRAIGHT		
R	1/3	1/3
N	0	1/2
SPIRAL		
R	1/3	1/3
N	$1/2\ \sin^2\alpha$	$1/2 - 1/4\sin^2\alpha$
SPHERE		
R	1/3	1/3
N	1/3	1/3



Values of calculated geometric factors which represent relative efficiencies on the light absorption of dye molecules distributed on the surfaces of three different forms. Straight and spiral cylinders, and a sphere, as shown in the right column of the figure. The angle between the central axis and the tangential direction of spiral is denoted by α. Only two particular modes on the angular distribution of absorption oscillators are listed, in which those completely random and all those normal to the tangential plane of the surfaces are indicated by (*R*) and (*N*), respectively.

form on which the oscillators are distributed with a particular mode of the angular orientation relative to the membrane [15]. The numerical value of the factor represents the efficiency of absorption oscillators in respective cases. Unity of the geometric factor represents the case where all the oscillators involved are parallel to the direction of the light polarization (maximum absorption). Some of the values are presented in Table 1, taking straight and spiral cylinders as the axonal models and a sphere as the model of a ganglionic cell body. Only two modes of the angular distribution of the absorption oscillators, completely random (R) and all normal (N) to the membrane surface, were used for these purposes.

Different Response Spectra Arising in Various Nerve Elements

The various patterns of the response spectra obtained from the various nerve elements can be analyzed based on the aforementioned factors.

Crab Nerve

It was assumed in the reconstruction of the response spectra in Fig. 4*A* that a small fraction of the absorption oscillators were converted from the random to the normal direction to the membrane during the nerve excitation. This process alters the geometric factor of the perpendicular component from 1/3 to 1/2 and leaves line *2* as the difference spectrum between lines *1* and *3*. The resultant spectrum is intricate because of the multiband absorption of the dye and the spectral shift involved. In the parallel component, a conversion from 1/3 to zero takes place during the same process and leaves line *1* as it was before the conversion.

The absorption spectra obtained with bovine serum albumin (line *a*) and lecithin (line *b*) were found to be similar to lines *1* and *3*, respectively, as shown in Fig. 4. This finding provides further support for the assumed model, although albumin is not related to the membrane ingredients. The enhanced absorption line *3* relative to line *1*, when compared with lines *a* and *b*, may be explained by an increase of the geometric factor from 1/3 to 1/2 during the conversion. The absorption oscillators oriented in the normal direction to the membrane appear to occur favorably with lipid layers since the long axis of the merocyanine-rhodanine may be inserted into the narrow cleft of the side-by-side packing of lipid molecules. Thus, the resemblance between the spectrum *3* and that from lecithin liposomes might not be accidental. Furthermore, a possible mechanism on the physicochemical basis of the statistical reorientation of the absorption oscillators has been proposed [16]. The mechanism comprised an interaction between the electric dipole moment of dye molecule and the membrane potential. The interaction mentioned above has been invoked by Conti et al. [2] and Dragsten and Webb [4] to explain changes in the fluorescence intensity associated with the potential change across lipid bilayers stained with fluorescent dyes.

The axon in the vagus appears to run along its bundle in a wavy or spiral fashion. With a spiral model the geometric factors are listed in Fig. 6. When the reorientation of absorption oscillators from the random to the normal takes place in this model axon, the amplitude ratio of the parallel to the perpendicular component of the response is given by $[1/3–1/2 \sin^2\alpha]/[1/3–(1/2–1/4 \sin^2\alpha)]$, where α is an angle between the long axis of the bundle (center axis of the spiral) and the tangential direction of the spiral. The above ratio yields a constant value of -2 irrespective of α, suggesting that the spiral itself does not affect the pattern of the response spectra.

The response spectra obtained from the rabbit vagus showed a diphasic nature in the perpendicular component and a small response in the parallel component. It may be possible to infer the following scheme from these characteristics. Monomer and dimer species, of which absorption oscillators are in the normal direction, increased during the nerve excitation, as assumed in the crab nerve. They, however, seem not to be supplied from the randomly oriented monomers and dimers but to be converted from higher aggregates, whose absorptions arise in wavelengths shorter than 650 nm. A precise physicochemical basis, which creates the different modes of conversion, remains unknown.

Rat Superior Cervical Ganglion

Many membrane elements, presynaptically and postsynaptically, could produce the absorption response in the SCG. In this study, no attempt was made to break the response down into its components. Without knowing the exact sites of the response production, it seems evident that a large loss of dichroic nature in the response spectra (Fig. 3) is related to the membrane organization of the ganglion. The membrane elements of both cell somata and complex networks of axons and dendrites are, in a statistical view, considered to be oriented randomly. The random orientation of the membrane results in an isotropic absorption, even if angular distribution of the absorption oscillators is highly anisotropic in each membrane element. The corresponding geometric fac-

tor is 1/3 in any direction, as exemplified by a calculation on a sphere (*see* Table 1). (No particular weight arises in membrane elements on a sphere because of its complete symmetric arrangement. In that sense, they are in random orientation). When membrane elements are random, the statistical reorientation of absorption oscillators no longer causes the absorption response, but the spectral shift remains as a sole factor of the response production. Since there is no positive phase in the wavelength longer than 730 nm, the spectral shift is not the type similar to that seen with crab nerve. The spectral shift accompanied by the conversion of larger aggregates into monomers and dimers, which was already described with the rabbit vagus, may also be taken here.

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References

1. Batzri, S., Korn, E.D. 1973. Single bilayer liposomes prepared without sonication. *Biochim. Biophys. Acta* **298**:1015
2. Conti, F., Fioravanti, R., Malerba, F., Wanke, E. 1974. A comparative analysis of extrinsic fluorescence in nerve membranes and lipid bilayers. *Biophys. Struct. Mechan.* **1**:27
3. Cohen, L.V., Salzberg, V.M. 1978. Optical measurement of membrane potential. *Rev. Physiol. Biochem. Pharmacol.* **83**:35
4. Dragsten, P.R., Webb, W.W. 1978. Mechanism of the membrane potential sensitivity of the fluorescent membrane probe merocyanine 540. *Biochemistry* **17**:5228
5. Emerson, E.S., Conlin, M.A., Rosenoff, A.E., Norland, K.S., Rodriguez, H., Chin, D., Bird, G.R. 1967. The geometrical structure and absorption spectrum of a cyanine dye aggregate. *J. Phys. Chem.* **71**:2396
6. Grinvald, A., Salzberg, V.M., Cohen, L.B., Kamino, K., Waggoner, A.S., Wang, C.H., Ti, D. 1976. Simultaneous recording from twelve neurons in the supraesophageal ganglion of *Balanus nubilus* using a new potential sensitive dye. *Biol. Bull. Woods Hole* **151**:411
7. Ross, W.N., Reichart, L.F. 1977. Species-specific effects on the optical signal of voltage-sensitive dyes. *J. Gen. Physiol.* **70**:15*a*
8. Ross, W.N., Salzberg, B.M., Cohen, L.B., Davila, H.V. 1974. A large change in axon absorption during the action potential. *Biophys. J.* **14**:983
9. Ross, W.N., Salzberg, V.M., Cohen, L.B., Grinvald, A., Davila, H.V., Waggoner, A.S., Wang, C.H. 1977. Changes in absorption, fluorescence, dichroism, and birefringence in stained giant axons: Optical measurement of membrane potential. *J. Membrane Biol.* **33**:141
10. Salzberg, V.M., Cohen, L.B., Ross, W.N. Waggoner, A.S., Wang, C.H. 1976. New and more sensitive molecular probes of membrane potential: Simultaneous optical recordings from several cells in the central nervous system of the leech. *Biophys. J.* **16**:23*a*
11. Salzberg, V.M., Grinvald, A., Cohen, L.B., Davila, H.V., Ross, W.N. 1977. Optical recording of neuronal activity in an invertebrate central nervous system: Monitoring of several neurons. *J. Neurophysiol.* **40**:1281
12. Sheppard, S.E. 1942. The effects of environment and aggregation on the absorption spectra of dyes. *Rev. Mod. Phys.* **14**:303
13. Tasaki, I., Warashina, A. 1975. Changes in light absorption, emission and energy transfer produced by nerves labeled with fluorescent probes. *Proc. Jpn. Acad.* **51**:604
14. Tasaki, I., Warashina, A. 1976*a*. Fast and slow rotation of dye molecules in squid axon membrane during excitation. *Proc. Jpn. Acad.* **52**:37
15. Tasaki, I., Warashina, A. 1976*b*. Dye-membrane interaction and its changes during nerve excitation. *Photochem. Photobiol.* **24**:191
16. Warashina, A. 1979. Spectral analyses of absorption changes associated with nerve excitation in dye-stained crab nerve. *Biochim. Biophys. Acta* **554**:51
17. Warashina, A., Tasaki, I. 1975. Evidence for rotation of dye molecules in membrane macromolecules associated with nerve excitation. *Proc. Jpn. Acad.* **51**:610

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