J. Membrane Biol. 109, 201–207 (1989)

The Journal of
Membrane Biology

Species Specificity in the Extrinsic Absorption Changes Exhibited by Some Invertebrates Stained with Voltage-Sensitive Dyes

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Summary. The absorption changes of several invertebrate neuronal preparations stained by the potentiometric dyes (WW 375, WW 433, WW 401 and RGA 84) in response to electrical nerve stimulation were examined. The dyes did not penetrate the connective sheath of insect preparations, but stained it. Only a decremental spreading of optical signals was seen on *Periplaneta americana, Gryllus bimaculatus* and *Gryllus campestris* ganglia and nerves. In contrast to insect preparations, pond snail and leech neurons were well stained by these dyes. The dye WW 375 behaved somewhat distinctly on insect and pond snail preparations than had been previously reported on other invertebrates. Like the signals from vertebrate neurons, they usually had triphasic action spectra. Therefore, this kind of action spectrum is not found only in membranes of vertebrate neurons. The main conclusion of this work is that the species-specific effects of the dye on different invertebrate preparations have a common feature: the existence of three peaks in the change of absorption (at 575, 675 and 750 nm) in both kinds of WW 375 action spectra (monophasic or triphasic). The wavelength dependence of the change in absorption was not affected by concentration, staining time, pH, osmolarity or ionic composition of physiological saline.

Key Words voltage-sensitive dyes · optical signals · neuronal membranes · nerve cells · nerve fibers · invertebrates

Introduction

Changes in absorption, fluorescence and birefringence that occur during action potentials in neurons and processes stained by voltage-sensitive dyes can be monitored in real time with high spatial resolution (Salzberg et al., 1977; Grinvald et al., 1982; Orbach & Cohen, 1983). Progress has been achieved both in the development of sensitive optical probes for measurement of rapid changes in membrane potentials and in the improvements in apparatus. In 1970, the largest signal-to-noise ratio obtained during a single action potential in a squid axon was about 1 : 1 (Cohen et al., 1970). During the past 15 years, Cohen, Salzberg, Waggoner, Grinvald and their collaborators have screened more than 1000 dyes in an effort to find optical probes for detecting the signals in excitable cells (Gupta et al., 1981). This testing has resulted in the discovery of dyes with a signal-to-noise ratio 100 times larger than was available in 1970.

The properties of potential-sensitive dyes differ when applied to different preparations. Some of the dyes were unable to penetrate the connective tissue or along intracellular spaces to the membrane of interest (Cohen & Lesher, 1986). In the best cases, the probe penetrates through the connective tissues and stains the neurons specifically. However, in some instances, well-stained neurons gave small optical signals. Optical signals are also affected by the kind of preparation. Ross and Krauthamer (1984) have reported that barnacle ganglia from different species of the same genus had optical signals with different spectra.

The fractional changes in light intensity transmitted through the preparation stained by a voltage-sensitive dye during an action potential have a strong wavelength dependence. The shape of this dependence is called the action spectrum of the dye. The kind of action spectrum is determined by the dye and the preparation. One of the best absorption dyes, WW 375, has typically monophasic action spectrum when applied to the squid giant axon. A decrease of absorption-accompanied action potential is seen at all wavelengths (Ross et al., 1977). But when this dye was applied to vertebrate preparations; there was a triphasic action spectrum. At some wavelengths, a decrease in absorption is obtained; at other wavelengths an increase in absorption occurs (Morad & Salama, 1979; Ross & Reichardt, 1979). Working on different preparations, Ross and Reichardt concluded that the dye WW 375 exists in two different states. On invertebrate neurons, the dye has a monophasic spectra; in contrast, in vertebrate preparations, there was a triphasic action spectra (Ross & Reichardt, 1979).

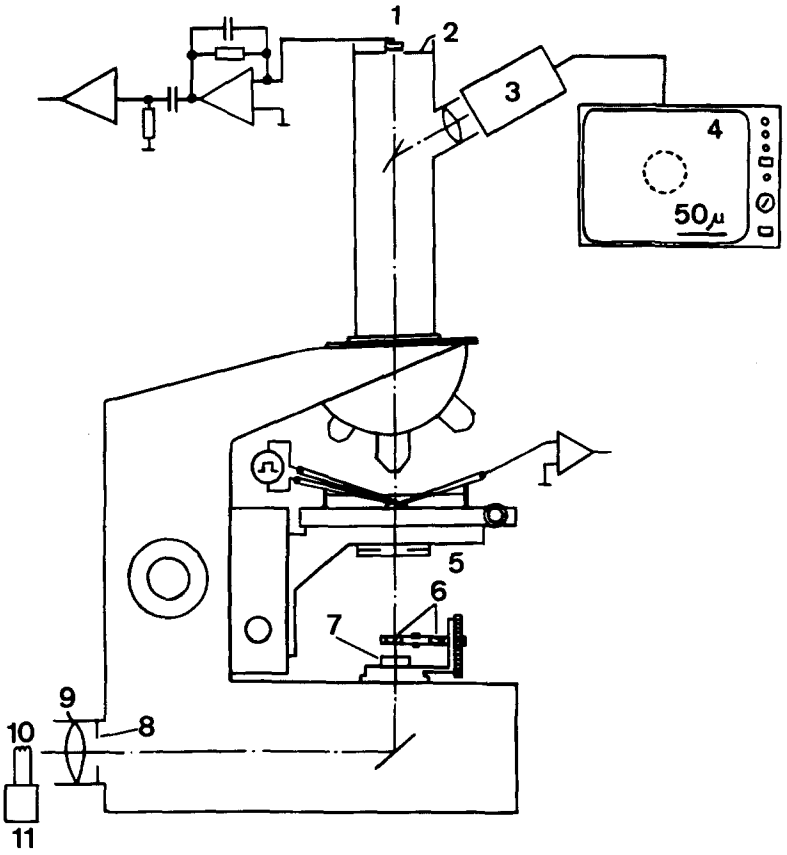

Fig. 1. Schematic diagram of the apparatus used for monitoring the absorption changes in invertebrate preparations. *1*–photodiode, *2*–aperture, *3*–vidicon, *4*–TV monitor, *5*–condenser iris, *6*–interference filters, *7*–heat filter, *8*–substage diaphragm, *9*–condenser, *10*–lamp, *11*–power supply

The conclusions of Ross and Reichardt regarding the monophasic character of the response of WW 375 applied to invertebrate was shown not to apply in the case of merocyanine-oxazolone dye, NK 2367, which stained the salivary gland of the pond snail *Helisoma trivolvis* (Senseman & Salzberg, 1980).

In our initial experiments with WW 375 applied to the nerves of the pond snail *Planorbis corneus*, we found that this dye behaved somewhat differently on this preparation than had previously been reported on other invertebrates. Consequently, we examined several invertebrate preparations to define the kinds of optical signals, their structure and wavelength dependence.

Some of these results have been reported in abstract form (Yagodin, Slutsky & Pushkarev, 1985).

Materials and Methods

Preparations

Six different preparations were examined. The pond snails *Planorbis corneus*, *Lymnaea stagnalis* and the leech *Hirudo medicinalis* were maintained in laboratory culture at room temperature. The insects *Periplaneta americana*, *Gryllus campestris* and *Gryllus bimaculatus* were obtained from Drs. O. Antonova and A. Knjazev (Sechenov Institute of Evolutionary Physiology and Biochemistry, Leningrad, USSR). The colonies of these insects were maintained in a 12 : 12 light-dark cycle at 25–27°C and a relative humidity of 50 ± 10%.

The nerves and ganglia of experimental animals were isolated from the body and pinned out on the Sylgard bottom of a simple chamber. No attempt was made to remove the connective tissue sheath surrounding the nerves and ganglia.

Because the somata of insect neurons, in most cases, are not electrically excitable, we tested 50–90 μm nerve regions in all preparations and also the nerve cells whenever possible.

Electrical Stimulation and Recording

For stimulation and measuring of the electrical signals, suction electrodes and microelectrodes were used. Current passage through the bipolar suction electrodes elicited the electrical events in the nerve fibers and cells, which were measured by suction electrodes and intracellular microelectrodes. In extracellular experiments, the time constant was limited from 0.3 msec (high-frequency response time constant) to 150 msec (low-frequency coupling time constant). Intracellular stimulation and recording were done with glass microelectrodes, which were filled with 3-M potassium chloride and had resistances between 30–60 MΩ. The microelectrode was connected to a bridge electrometer, which was used for simultaneous current passing and potential measurement.

Optical Recording

The main equipment used to measure the optical signals that accompanied the electrical activity are shown in Fig. 1 and are,

in principle, the same as that described by Ross and Reichardt (1979). All preparations were mounted in a chamber fixed to the stage of a Biolam microscope. Constant illumination was provided by a 12 V, 100 W tungsten-halogen lamp controlled by highly regulated DC power supply or batteries. The water-immersion, high aperture objective (30 ×; 0.9 NA) allowed us to eliminate the fluctuations in light intensity due to the vibrations of the air-water interface and to obtain improved signal-to-noise ratios. A photodiode (dark current 10^{-12} A) was positioned in the objective image plane above the microscope. Accurate positioning was accomplished by using the coordinate array of a TV monitor. The preparation image plane could be obtained at the photodiode or at a TV vidicon. Three apertures were used so that the photodiode received light passing through the region of nervous tissue under investigation and to reduce the amount of scattered light reaching the photodiode. The substage diaphragm was adjusted to just fill the 30 × field. The condenser iris was closed until a reduction of light intensity was just observed in photodiode. The aperture was selected to conform to the magnified diameter of the nerve region or the cell body under investigation.

The wavelength of illumination was determined by Zeiss interference filters. Several components of the apparatus are strongly wavelength dependent. In order to calculate the relative absorption changes at different wavelengths, we corrected the observed changes in photodiode output for the efficiency of the apparatus. For this purpose, the absorption spectrum of photodiode output was measured at all wavelengths. The experimental results were multiplied by the correction factor.

The output of the photodiode was fed to a low-noise current-to-voltage converter whose output was capacity coupled to the amplifier (× 1000). The high-frequency response was limited to 0.3 msec. The low-frequency coupling time constant was 150 msec. The coupling capacitor was removed for measurements of the resting light intensity. Signal averaging was not used; all of the results are from single trials.

Two kinds of noise were significant in our optical measurements: (i) statistical fluctuations in the arrival of photons at the photodiode surface (shot noise); and (ii) mechanical vibrations. The fractional shot noise in our experiments was typically 5×10^{-5}, while the vibrational noise increased this value to 10^{-4}.

Dyes and Staining Procedure

In most experiments, the merocyanide dye WW 375 (Ross & Reichardt, 1979) = dye XVII (Ross et al., 1977) was used. In some cases, the additional dyes: WW 433 = 644 = XXIII (Gupta et al., 1981), WW 401 (Grinvald, Ross & Farber, 1981) and RGA 84 were utilized. After incubation in the potentiometric probe with a concentration of 0.1–2 mg/ml, usually for 20–30 min, the dye solution was replaced with appropriate physiological solution. All the dye solutions were prepared just prior to the incubation. The staining procedure was carried out at room temperature. All the dyes used were obtained from Dr. A. S. Waggoner, Fluorescence Center, Carnegie-Mellon University, Pittsburgh, PA.

Results

Staining and the Types of Optical Signals

The neurons and nerve fibers of a leech and pond snails were well-stained by the dye WW 375. In *Gryllus bimaculatus* and *Gryllus campestris*, the connective tissue stained well, but in *Periplaneta americana*, it stained much less. This staining effect did not depend on the dye concentration and staining time. The use of additional dyes (WW 401, WW 433 and RGA 84) did not resolve the problem of staining the nervous tissue of insect preparations.

In leech and pond snail preparations, we obtained optical signals accompanying action potentials in nerve cell bodies and accompanying electrotonic potentials and action potentials in nerve fibers. In insect preparations, we obtained only decrementally spreading optical signals. Because none of the insect preparations gave optical signals during action potentials, we regard the decremental optical signals in insects as electrotonic potentials spreading along the connective sheath. Because the fractional changes in light intensity transmitted through the preparation have a strong wavelength dependence, we disregard them as the stimulus artifacts.

A Comparison of Electrical and Optical Signals

Simultaneous optical and intracellular microelectrode recordings show (Fig. 2*A*) that the time course of the absorption changes was similar to the time course of the depolarizations, suggesting that the absorption signals are potential dependent (Ross et al., 1977; Gupta et al., 1981). The same absorption signals were recorded from the neurons of *Planorbis* and *Hirudo* ganglia. However, in the optical measurements from 50 μm spots of nerves and connectives, there are differences in the optical and electrical signals even when the two recording sites are close together. This phenomenon is not due to the apparatus, since both channels had the same time constants. In most preparations, the optical signals were somewhat slower than the electrical signals (Fig. 2*B*). In some cases, the time course of the optical signal was similar to the time course of the electrical signal (Fig. 2*C*); in others, there were very slow components in the optical signal (Fig. 2*D*).

A similar relationship between electrical and optical recordings was obtained in measurements of birefringence and light scattering on crab nerves (Cohen & Keynes, 1968; Cohen, Keynes, & Hille, 1968). The slow components of the optical signals could well have been derived from glial depolarization. This has been demonstrated in the cerebellum of the scate with pyrazo-oxonol dye RH 155 (Konnerth, Obaid & Salzberg; 1985; Salzberg, Obaid & Konnerth, 1986).

The optical signals disappeared in white light. This is the evidence that these signals are changes

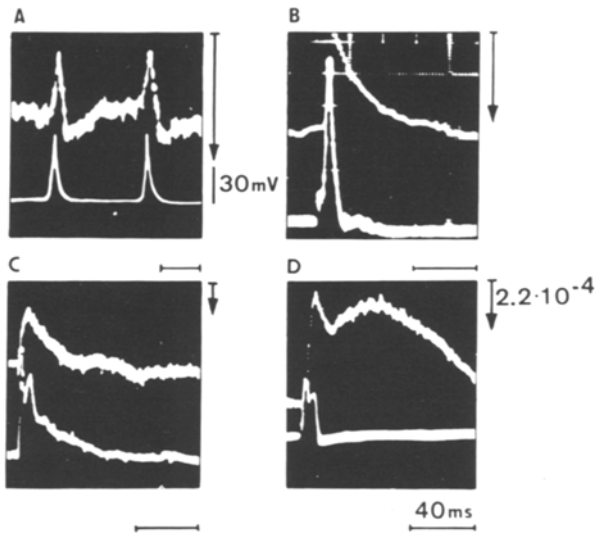

Fig. 2. Changes in light absorption by a stained 60 μm *Lymnaea* neuron (*A*) and 50 μm region of *Planorbis* nerves (*B-D*) during action potentials. (*A*) A cell from pedal ganglion was used. The absorption changes have the same time course as the changes of membrane potential. (*B-D*) The optical and suction electrode recordings were made from regions of the left pallial nerves during nerve stimulation. The absorption signals in most preparations were somewhat longer in duration than the electrical signals (*B*). In some cases, the shapes of both signals have the same time course (*C*), in others, a slow component occurs (*D*). A 750-nm interference filter was used in (*A-D*). Upper traces-absorption signals, lower traces-electrical signals. In this and subsequent figures, the direction of the arrows adjacent to the optical traces indicates an increase in absorption and the length of the arrows represents the stated values of the changes in absorption divided by the resting absorption

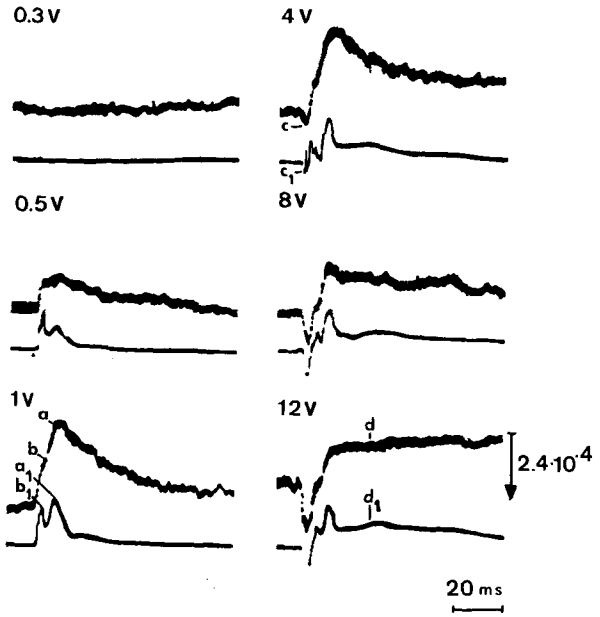

Fig. 3. The change of the structure of optical signal as a function of the intensity of electrical stimulation. Peaks a, b, c, d – the components of the optical signals; peaks a_1, b_1, c_1, d_1 – the corresponding components of the electrical signals. A 750-nm interference filter was used. The optical recordings were made from a 50 μm spot of *Planorbis* nerve

in absorption but not changes in light scattering. Only threshold and suprathreshold stimulation elicited the appearance of electrical and absorption responses in nerve fibers (Fig. 3). The largest optical signals (peaks a – traces of optical signals) were obtained after activation of nerve fibers with slow conduction velocity (peaks a_1 in the traces of electrical signals). Probably, these fibers occupied a large volume in nerve. The nerve fibers with fast conduction velocity in this nerve are not numerous and their contribution to the optical signal is small (peaks b – traces of optical signals, peaks b_1 – traces of electrical signals). With large stimuli, a negative (or positive after a change of stimulation polarity) electrotonic signal appears. Because all fibers are induced to respond with strong stimulation, the optical signals correlate well with the electrotonic potentials (peaks d and d_1). Changing the stimulation polarity, leads to the inversion of electrotonic signals (peaks c and c_1) and only a small alteration of peaks a, a_1 and b, b_1.

Wavelength Dependence of Optical Signals

In our experiments we found that the optical signals had different wavelength dependence in different preparations.

For action potentials in the pond snail *P. corneus* and *L. stagnalis*, and for the electrotonic potentials of the insects *G. bimaculatus* and *G. campestris*, there are typically triphasic action spectra (Fig. 4*A*). A decrease of absorption accompanied action potential or electrotonic potential in snail and insect preparations at both 500–625 nm and 750–800 nm wavelengths. At 625–725 nm wavelengths, there was an absorption increase (Fig. 4*A*). Maximal optical signals were seen in most cases at 700 nm, not at 750 or 575 nm. In some preparations of these animals, we could not measure any signal at 750 nm. In these cases only, wavelengths of 625 and 725 nm, could we find either the decrease or increase of absorption, which accompanied electrical activity. In Fig. 4*A*, there are two action spectra of *L. stagnalis* preparations, which have different signals at 725 nm. The case of this difference is unknown. However, it should be stressed that the spectra of WW 375 are approximately the same in these several species and with different electrical signals (action

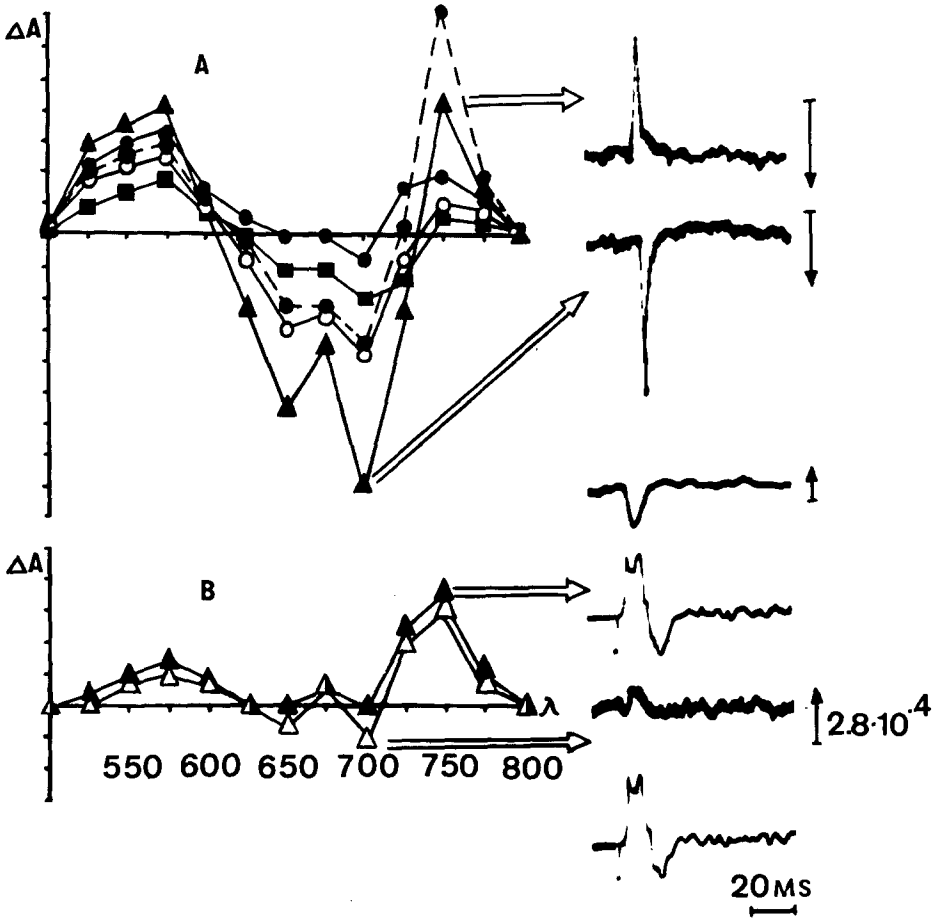

Fig. 4. Wavelength dependence of the absorption changes resulting from electrical stimulation of nerve trunks of several preparations. The peak transmission (in nm) of the interference filters is shown on the abscissa. (*A*) Triphasic action spectra of optical action potentials of nerve trunks of *Planorbis* (●—●), *Lymnaea* (○—○, ●– –●), and the optical signals of leg nerves of *Gryllus campestris* (▲—▲) and *Gryllus bimaculatus* (■—■). The *Gryllus* optical signals inverted with a change of polarity of the electrical stimulation. In most preparations of this group, we have measured an increase of absorption at 725 nm, but in some cases a decrease of absorption occurs at this wavelength (●—●, ●– –●). (*B*) Monophasic action spectrum of the optical action potentials in *Hirudo* connectives. In most preparations, there were decreases of absorption at all wavelengths (▲—▲). In some preparations, a slight increase of absorption at 650 and 700 nm was obtained (△—△). This may be a suggestion of triphasic action spectrum. The data on graphs are represented after a correction factor for the efficiency of the apparatus as a function of wavelength. On photo: upper traces-optical signals; lower traces-electrical signals. There appear to be three peaks both in the monophasic and triphasic action spectra: 575, 675 and 750 nm

potentials or electrotonic potentials). Virtually the same triphasic wavelength dependence (with approximately the same blue shift compared with squid) was reported in salivary gland acini from the pond snail *Helisoma trivolvis* (Senseman & Salzberg, 1980). They found the peak increase in absorption (with depolarization) at 675 nm, with a decrease at 540 and 720 nm.

A second type of action spectra was found on nerves, connectives and cells of the leech *H. medicinalis*. The typical monophasic action spectrum is represented in Fig. 4*B* (filled triangles). In most cases, there were decreases of absorption at all wavelengths. But, sometimes a slight increase in absorption at 650 and 700 nm was seen (open triangles). It should be noted that the monophasic spectrum has peaks at the same wavelength as the triphasic action spectra (575, 675 and 750 nm). This fact indicates a resemblance of the general shape of monophasic and triphasic action spectra.

The triphasic action spectra obtained from cell bodies of the pond snails is illustrated in Fig. 5. The largest optical signals were obtained, as in the case of nerve trunks, at 690–710 nm as an increase of absorption.

In *P. americana* preparations, we could find small optical signals at only 700 and 750 nm wavelength and they were of opposite polarity.

Could We Shift the Peaks of the Action Spectrum?

In order to find factors that determine the shape of the wavelength dependence, experiments with different physiological solutions, dye concentrations and staining time were performed.

Woolum and Strumwasser (1978) reported that at high concentrations of WW 375 and prolonged staining time they have obtained an inverted action spectrum in *Aplysia* neurons. In our experiments with concentrations of WW 375 0.1–2 mg/ml and staining time of 10 min–2 hr, we found no influence of these factors on the course of wavelength dependence of optical signals. Several experiments were performed to study the influence of pH on staining and action spectrum. Figure 6 represents the data of two groups of *Planorbis* preparations at pH 6.0 and pH 7.4. The shape of action spectrum and the optical signal amplitude were not affected by pH in this range.

Triphasic action spectra were obtained on invertebrates with low (*P. corneus* and *L. stagnalis*) and high ionic strength physiological solutions (*G. bimaculatus*, *G. campestris* and *P. americana*). Because the ionic concentration of the leech solution is intermediate between that of pond snails and insects, we think that the action spectra are not dependent on the ionic strength. In addition, the ionic strength of physiological solutions of vertebrate preparations where the triphasic spectra were obtained (Ross & Reichardt, 1979) were higher than in *H. medicinalis*.

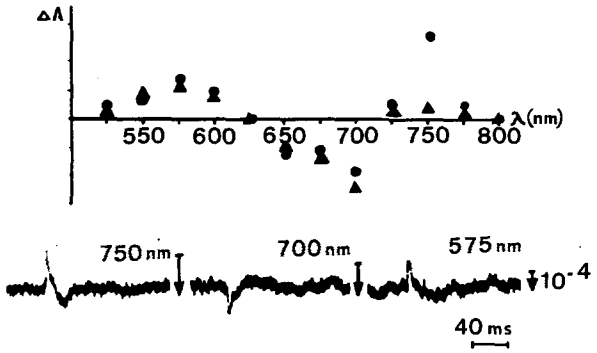

Fig. 5. The wavelength dependence of the changes in absorption of cell bodies of the snails *Planorbis* (▲) and *Lymnaea* (●) during action potentials. The peak transmission of the interference filter is shown on the abscissa. At the bottom, examples of optical recordings from a cell in the *Lymnaea* pedal ganglion. The cell diameter was 60 μm

Discussion

The existence of electrotonic and action potentials is a main feature of nerve cells and their processes.

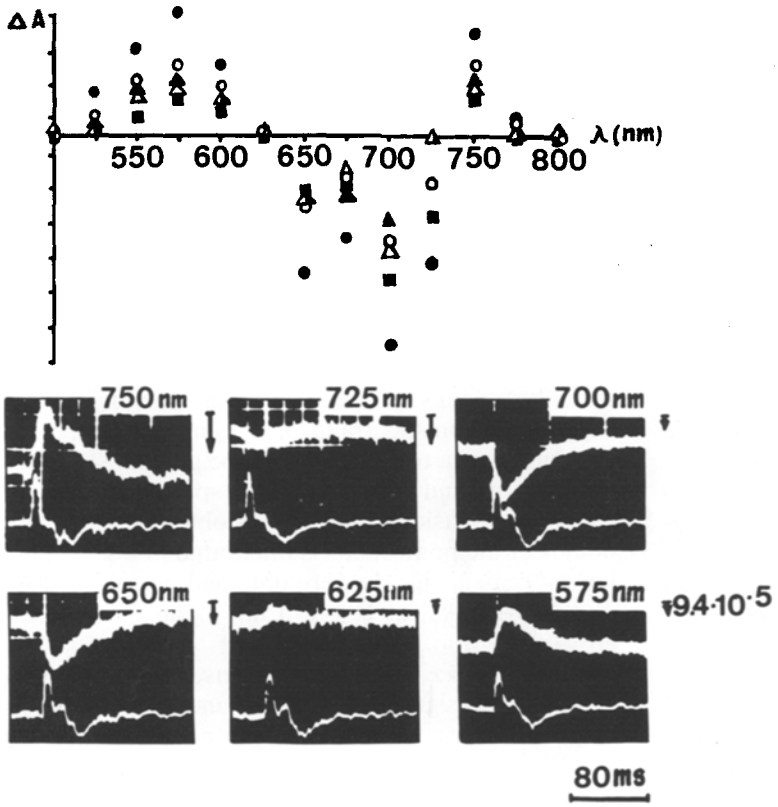

Fig. 6. Absorption spectra of the optical signals from *Planorbis* nerve trunks at pH 7.4 (●, ○) and pH 6.0 (■, △, ▲). The peak transmission of the interference filter is shown on the abscissa. Shown in the photographs are examples of absorption changes (upper traces) that accompanied action potentials (lower traces). Nerve regions of 50 μm were used for the optical recordings

These potentials were detected optically when the potentiometric probes penetrated the connective sheath and stained the neurons and axons. The optical signals we obtained in insects appear to arise from connective tissue for the following reasons: i) in all preparations, we obtained only decrementally spreading optical signals even though action potentials were recorded by suction electrodes; ii) these optical potentials appear at high voltage stimulation; and iii) in *Gryllus*, the nerve trunks and ganglia, after removal of the connective sheath, appeared unstained (visual observation).

In this paper, we show that the dye WW 375 had a triphasic action spectrum in invertebrate preparations. The main conclusion of this work is that the species-specific effects of the dye on different invertebrate preparations has a common feature: the existence of three peaks in the change of absorption (at 575, 675, and 750 nm) in both kinds of WW 375 action spectra (monophasic or triphasic). The amplitude of the optical signals is defined by many factors and fluctuated somewhat from preparation to preparation. But, the species-specific shape of the action spectrum is clear (*for example*, Fig. 6). In addition, these peaks may also be found in barnacle neurons (Ross & Reichardt, 1979). This main tendency was obtained in all of our preparations. It should be noted that the shape of the action spectrum is identical in nervous tissue (fibers and cells) and in the connective sheath.

In both kinds of action spectra, the wavelengths occur where the sign of the optical signal may be in different preparations of the same species, and where the general curve crossed the abscissa (for triphasic action spectrum, Fig. 4*A*) or where the curve was near it (monophasic action spectrum, Fig. 4*B*). In the case of triphasic action spectra, these wavelengths are 625 and 725 nm.

Our investigations indicate that the action spectra of the dye WW 375 is not related to the external environment of the probe, but to the complex of optical probe and membrane. In this respect, these data confirm Ross and Reichardt (1979). But in contrast to these authors, our data suggested the distinction in action spectra is not due to difference in invertebrate and vertebrate neuronal membranes.

The authors are indebted to S.V. Levin and L.B. Cohen for much helpful advice and the comments on the manuscript. We thank A.S. Waggoner for providing the voltage-sensitive dyes.

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Received 15 August 1988