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Evaluation of Optimal Voltage-sensitive Dyes for Optical Monitoring of Embryonic Neural Activity

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Abstract. To evaluate the suitability of a variety of fast voltage-sensitive dyes for optical recording of rapid transmembrane potential activity in the embryonic nervous system, we screened over twenty dyes, including several newly synthesized probes, in three different embryonic neural preparations: cervical vagus nerve bundle, nodose ganglion, and brainstem from 7-day old chick embryos. Measurements of voltage-related optical signals were made using a multiple-site optical recording system. Signal size, signal-to-noise ratio, photobleaching, and phototoxicity were examined. Several promising new merocyanine-rhodanine dyes for embryonic nervous systems were found.

Key words: Optical monitoring — Voltage-sensitive dyes — Merocyanine-rhodanines — Embryonic neural activity — Embryonic nervous tissues

Introduction

The ontogenetic approach to electrophysiological behavior could be a useful and viable strategy to analyze progressively the complicated functional organization/architecture of nervous systems in a manner reminiscent of the expansion of a complex function in a power series. However, conventional electrophysiological measurements are technically difficult or impossible in the early developing embryonic nervous system because of the small size and fragility of the cells.

As a result, extrinsic (requiring voltage-sensitive dye) optical signals have provided a powerful means for monitoring electrical activity (*for reviews see* Cohen & Salzberg, 1978; Salzberg, 1983; Grinvald, 1985; Loew, 1988; Kamino, 1991). Indeed, using optical techniques and voltage-sensitive dyes, we have been able to overcome several obstacles to the electrophysiological study of the early embryonic nervous system (Kamino et al., 1989*b;* Komuro et al., 1991; Momose-Sato et al., 1991; Komuro et al., 1993; Sato et al., 1993) and heart (Kamino, Hirota & Komuro, 1989*a;* Kamino, 1990; 1991).

Much progress has been achieved in the development of voltage-sensitive dyes for the measurement of rapid changes in membrane potential (Davila et al., 1973; Cohen et al., 1974; Ross et al., 1977; Gupta et al., 1981; Grinvald et al., 1982; Loew et al., 1985; 1992). On the other hand, with some dyes, Ross and Reichardt (1979) and Sensman and Salzberg (1980) reported species-specific effects on the spectrum of the action potential signal. In addition, for application to early developing embryonic preparations, certain physicochemical properties including osmotic behavior of the dyes make optical measurements difficult, because the early embryonic cells are primitive and they may be very sensitive to small changes in the environment. Thus, the choice of optimal dyes is a serious and important consideration (Kamino, 1991). We have used a merocyanine-rhodanine dye (NK2761: Kamino, Hirota & Fujii, 1981; Fujii, Hirota & Kamino, 1981) as an optimal voltage-sensitive dye.

This merocyanine-rhodanine dye (NK2761) is an analogue of Dye XVII (Ross et al., 1977). It was first introduced for experiments on early embryonic hearts (Kamino et al., 1981; Fujii et al., 1981), and subsequently used in experiments on embryonic neural preparations (Sakai et al., 1985; Kamino et al., 1989*b;* 1990) and intact nerve terminals (Salzberg et al., 1983; Obaid et al., 1985; Obaid, Flores & Salzberg, 1989). Furthermore, we have continued to look for additional new dyes to allow monitoring of embryonic neural activity. We

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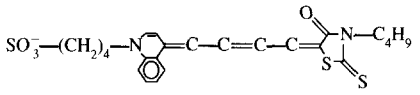
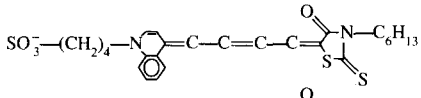
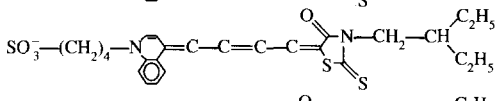
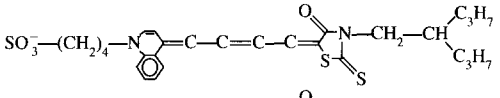
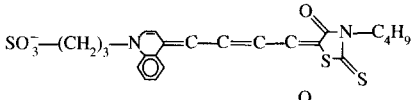
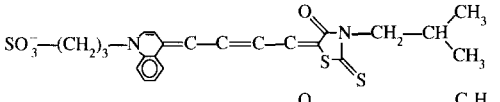
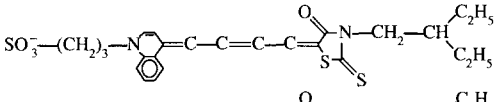
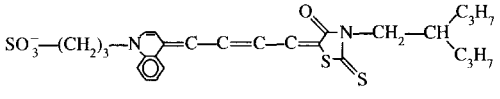
Table 1. The dyes tested in the present experiments

	Dyes	Sources	Tested preparations
Merocyanine-rhodanines			
NK2761	3-n-Butyl-5-{4-[1-(4-sulfobutyl)-4(1H-quinolylidene]-2-butenylidene} rhodanine, sodium salt	NK	BS, VN, NG
[a]NK2776	3-Hexyl-5-{4-[1-(4-sulfobutyl)-4(1H)-quinolylidene]-2-butenylidene} rhodanine, sodium salt	NK	BS, VN, NG
[a]NK3224	3-Isobutyl-5-{4-[1-(4-sulfopropyl)-4(1H)-quinolylidene]-2-butenylidene} rhodanine, compd. with triethylamine	NK	BS, VN
[a]NK3225	3-Butyl-5-{4-[1-(4-sulfopropyl)-4(1H)-quinolylidene]-2-butenylidene} rhodanine, compd. with triethylamine	NK	BS, VN
[a]NK3360	3-(2-Ethylbutyl)-5-{4-[1-(3-sulfopropyl)-4(1H)-quinolylidene]-2-butenylidene} rhodanine, compd. with triethylamine	NK	BS, VN, NG
[a]NK3361	3-(2-Ethylbutyl)-5-{4-[1-(4-sulfobutyl)-4(1H)-quinolylidene]-2-butenylidene} rhodanine, compd. with triethylamine	NK	BS, VN, NG
[a]NK3365	3-(2-Propyl-pentyl)-5-{4-[1-(4-sulfopropyl)-4(1H)-quinolylidene]-2-butenylidene} rhodanine, compd. with triethylamine	NK	BS, VN, NG
[a]NK3376	3-(2,2-Diphenylethyl)-5-{4-[1-(4-sulfobutyl)-4(1H)-quinolylidene]-2-butenylidene} rhodanine, compd. with trimethylamine	NK	BS, VN, NG
NK2495 (Dye XVII)	3-Ethyl-5-{4-[1-(3-sulfopropyl)-4(1H)-quinolylidene]-2-butenylidene} rhodanine, sodium salt	NK	BS, VN, NG
Dye XXIII (RGA84,NK2765)	3-Propyl-5-{4-[1-(4-sulfobutyl)-4(1H)-quinolylidene]-2-butenylidene} rhodanine, sodium salt	EO	BS, VN, NG
Merocyanine-oxazolones			
NK2367	3-Ethyl-5-{4-[1-(3-sulfopropyl)-4(1H)-quinolylidene]-2-butenylidene}-2-thio-2,4-oxazolidinedione	NK	NG
Other merocyanines			
Dye I (M-540, NK2272)	1,3-Di-n-butyl-5-{4-[3-(3-sulfopropyl)-2-benzoxazolinylidene]-2-butenylidene}-2-thiobarbituric acid, sodium salt	EO	NG
NK1311	3-Ethyl-5-{4-[1-ethyl-4(1H)-pyridylidene]-2-butenylidene} rhodanine	NK	NG
NK2385	3-Sulfoethyl-5-{4-[1-methyl-4(1H)-quinolylidene]-2-butenylidene} rhodanine, compd. with triethylamine	NK	NG
NK2389	3-Sulfoethyl-5-{4-[1-(4-sulfobutyl)-4(1H)-quinolylidene]-2-butenylidene} rhodanine, compd. with triethylamine and potassium salt	NK	VN
Oxonols			
NK2935 (WW781)	4-[5-(1,3-Di-n-butyl-2,4-dioxo-6-hydroxy-1,2,3,4-tetrahydro-5-pyrimidinyl)-2,4-pentadinylidene]-3-methyl-1-(4-sulfophenyl)-2-pyrazoline-5-on, disodium salt	NK	BS, VN, NG
NK3041 (RH155)	4-{5-[5-Hydroxy-3-methyl-1-(4-sulfophenyl)-4-pyrazolyl]-3-phenyl-2,4-pentadienylidene}-3-methyl-1-(4-sulfophenyl)-2-pyrazoline-5-on, trisodium salt	NK	BS, NG
NK3630 (RH482)	4-{5-[5-Hydroxy-3-propyl-1-(4-sulfophenyl)-4-pyrazolyl]-3-phenyl-2,4-pentadienylidene}-3-propyl-1-(4-sulfophenyl)-2-pyrazoline-5-on, trisodium salt	NK	BS
NK2936	1,3-Di-n-butyl-5-[5-(1,3-di-n-butyl-2,4-dioxo-6-hydroxy-1,2,3,4-tetrahydro-5-pyrimidinyl)-2,4-pentadienylidene] barbituric acid compd. with triethylamine	NK	VN
Oxazolidines (electrochromic dyes)			
ECD-1	2-(4-Dimethylaminostyryl)-3,3-dimethylindolino [2,1-b] oxazolidine	NK	NG
ECD-2	2-[4-(4-Dimethylaminophenyl)-1,3-butadienyl}-3,3-dimethylindolino [2,1-b] oxazolidine	NK	NG
ECD-3	2-(4-Dimethylaminostyryl)-3,3-dimethyl-5-methylsulfonylindolino [2,1-b] oxazolidine	NK	NG
ECD-6	3,3-Dimethyl-2-[2-(9-ethyl-3-carbazolyl) vinyl] indolino [2,1-b] oxazolidine	NK	VN

[a] Newly synthesized dyes; NK: Nippon Kankoh Shikiso Kenkyusho Co.; EO: Eastman Organic Chemicals; BS: Brainstem; VN: Vagus nerve bundle; NG: Nodose ganglion

Table 2. Chemical structures of merocyanine-rhodanine dyes with relatively large optical signals

	NK2761
	NK2776
	NK3361
	NK3376
	NK3225
	NK3224
	NK3360
	NK3365

report here the properties of twenty-three dyes, including seven newly synthesized merocyanine-rhodanine dyes, and evaluate the utility of the dyes for early embryonic neural preparations. Preliminary results have appeared previously (Hirota et al., 1992).

Materials and Methods

Preparations

Brainstems

In the present study, we have used 7-day old embryonic chick brainstems. Fertilized eggs of chicks (white Leghorn) were incubated in a forced draft incubator (Type P-03, Showa Incubator, Urawa, Japan) at a temperature of 37°C and 60% humidity, and were turned once each hour. The brainstems with vagus nerve fibers attached were dissected from the embryos, and the pia mater attached to the brainstem was carefully removed. The intact vagus-brainstem preparations were attached dorsally to the silicone (KE 106; Shin-etsu Chemical, Tokyo, Japan) bottom of a simple chamber by pinning with tungsten wires. Slice preparations about 1 mm thick were cut transversely from the isolated brainstems at the level of the vagus nerve root and were pinned to the chamber with the spinal cord side up. The preparations were kept in a physiological bathing solution (*see* below).

Cervical Vagus Nerve Bundles and Nodose Ganglia

The cervical vagus nerve bundles and the nodose ganglia were dissected from 7-day old chick embryos. The isolated cervical vagus nerve bundle and the nodose ganglion were attached to the silicone bottom of a simple chamber by pinning them with tungsten wires. The sheath of connective tissue attached to the cervical vagus nerve bundle and nodose ganglion was carefully removed in the bathing solution under a dissecting microscope.

Bathing Solution

The bathing solution had the following composition (in mM): NaCl, 138; KCl, 5.4; $CaCl_2$, 1.8; $MgCl_2$, 0.5; glucose, 10; and Tris-HCl buffer (pH 7.2), 10. The solution was equilibrated with oxygen.

Dye Staining

The isolated preparations were incubated for 20 min in the bathing solution to which 0.2 mg/ml of the dyes was added. After staining, the preparation was washed with several changes of normal bathing solution. The sources of the dyes used in the present experiments are given in Table 1; NK and ECD were from the catalogue of Nippon Kankoh-Shikiso Kenkyusho, Dye I from Cohen and Salzberg (1978), WW from Gupta et al. (1981), and RH from Grinvald et al. (1980).

Electrical Stimulation

For preparations in which the cervical vagus nerve was stimulated, the cut end of the nerve was drawn into a suction electrode fabricated from TERUMO-haematocrit tubing (VC-H075P; TERUMO, Tokyo, Japan), which had been hand-pulled to a fine tip (about 100 μm in caliber) over a low-temperature flame.

Optical Recording

The preparation chamber was mounted on the stage of an Olympus Vanox microscope (Type AHB-L-1). Bright-field illumination was provided by a 300 W tungsten-halogen lamp (Type JC-24V-300W, Kondo Sylvania, Tokyo, Japan) driven by a stable dc-power supply (Model PAD 35-20L, 0-35V 20A, Kikusui Electronic, Kawasaki, Japan). Incident light was made quasimonochromatic by a given interference filter (Type 1F-S, Vacuum Optics, Tokyo, Japan) placed between the light source and the preparation. A microscope objective (S plan Apo, 0.40 n.a.) and a photographic eyepiece formed a magnified (×25) real image of the preparation at the image plane. The transmitted light intensity at the image plane was detected using a 12 × 12 square array of silicon photodiodes (MD-144-4PV; Centronic, Croydon, U.K.). The output of each detector in the diode array was passed to an amplifier, in which simple RC low pass and high pass filters (time constants: about 500 μsec and 3 sec) were built, via a current to voltage converter. The amplified outputs from 127 elements of the detector were first recorded simultaneously on a 128-channel recording system (RP-890 series, NF Electronic Instruments, Yokohama, Japan), and then passed to a computer (LSI-11/73 system, Digital Equipment, Tewksbury, MA). The 128-channel data recording system was composed of a main processor (RP-891), eight I/O processors (RP-893), a 64K word wave-memory (RP-892) and a videotape recorder. The program for the computer was written in the assembly language (Macro-11) called by FORTRAN, under the RT-11 operating system (Version 5.1). All experiments were carried out at room temperature, 26–28°C.

Results

First we examined eight merocyanine-rhodanine dyes applied to preparations of cervical vagus nerve bundles,

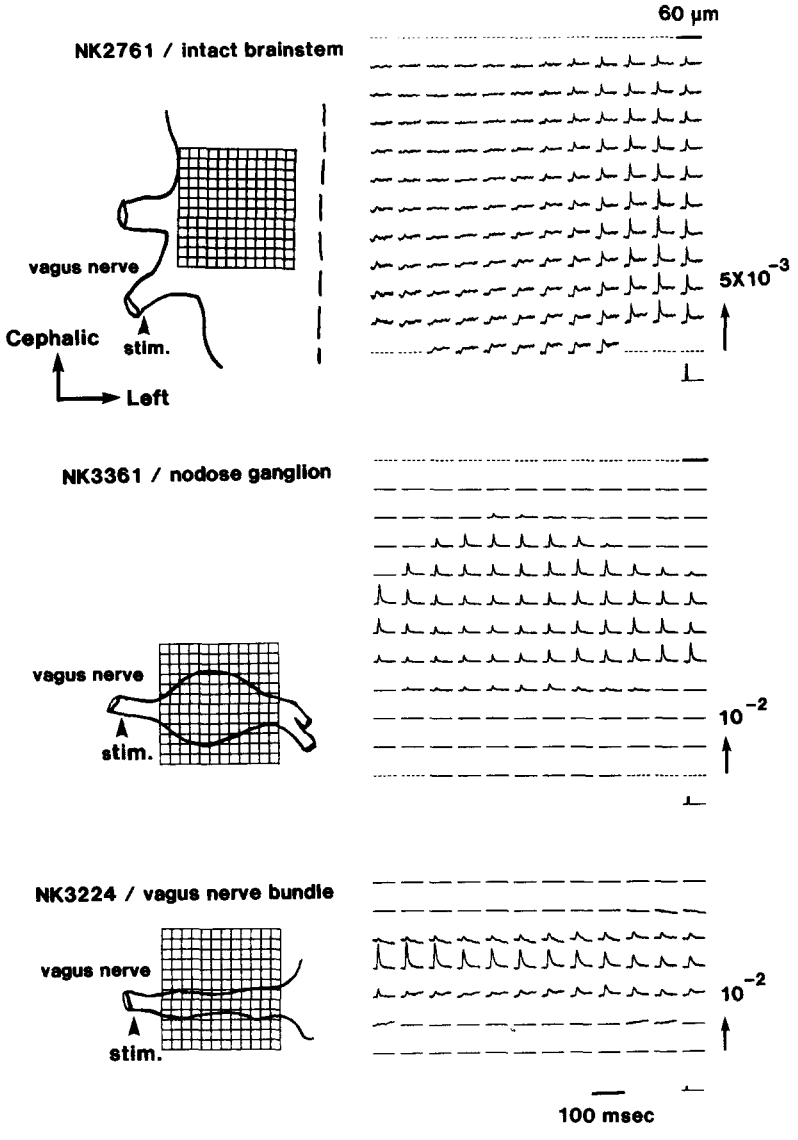


Fig. 1. Three examples of optical recordings obtained from three different preparations: the intact brainstem, a nodose ganglion and a cervical vagus nerve bundle dissected from 7-day old chick embryos, using three different voltage-sensitive dyes. The intact brainstem was stained with NK2761, the nodose ganglion with NK3361 and the vagus nerve bundle with NK3224. A square current pulse was applied to the right vagus nerve in the brainstem preparation, to the distal cut ends of the nodose ganglion and the vagus nerve bundle. The optical signals were maximum signals obtained in response to increasing stimulating currents, and were recorded in a single sweep. The direction of the arrows in the lower right corner of each recording indicates a decrease in transmission, and the length of the arrows represents the stated value of the fractional change. The recordings were made with a 702 ± 6.5 nm interference filter and in a single sweep.

nodose ganglia, and brainstems dissected from 7-day old chick embryos. The chemical structures of NK2761 and seven newly synthesized dyes are shown in Table 2. In these dyes, the alkyl chain attached to the rhodanine nucleus was varied. Of the dyes tested, NK2761, NK2776, NK3224 and NK3225 were highly soluble in Ringer's solution. Since molar extinction coefficients of these merocyanine-rhodanine dyes are relatively large, low concentrations were used for the staining of the preparations. (In the present experiments, the dye concentration was fixed at 0.2 mg/ml.) However, NK3361, NK3376, NK3360 and NK3365 were only slightly soluble in water, as a result of their more hydrophobic substituents. Accordingly, we first dissolved them in ethanol containing 20% pluronic F127 (BASF Wyandotte: Davila et al., 1973), and then diluted the original solution 100 times with Ringer's solution.

Figure 1 shows three examples of original recordings of optical signals made in various preparations stained with a merocyanine-rhodanine dye (NK2761, NK3361 or NK3224). The optical signals were evoked by a brief square current pulse applied to the end of the vagus nerve, and the recordings were made using a 12 × 12-element photodiode array without averaging. In the recording from the intact brainstem preparation stained with NK2761, fast spike-like signals (related to the action potential) and slow signals (related to postsynaptic potentials) (Komuro et al., 1991) were resolved. In the present experiments, we focused on the fast spike-like signal and compared the maximum sizes of the spike-like signals using the various dyes for various preparations, i.e., the cervical vagus nerve bundle, the nodose ganglion, and the brainstem.

These optical signals all depended upon the wave-

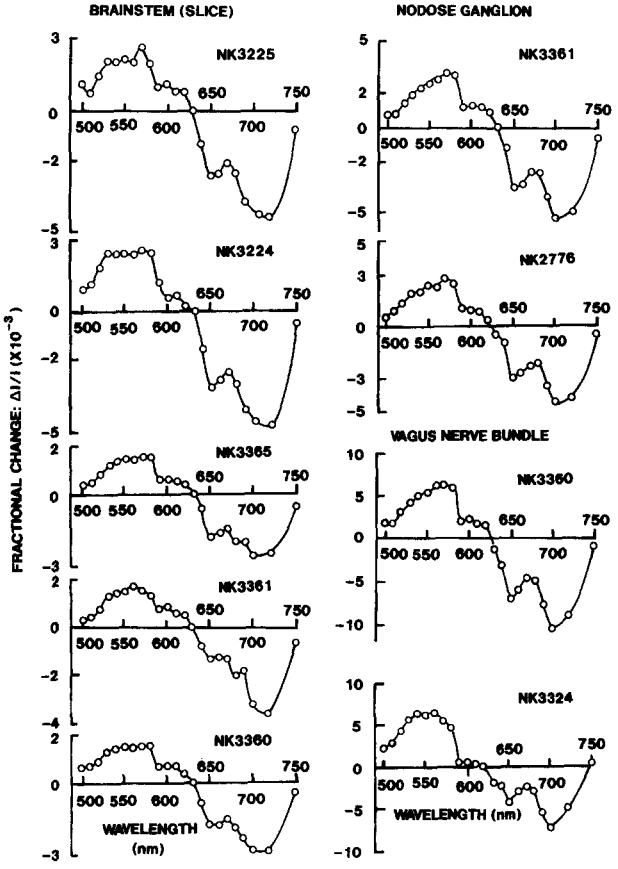


Fig. 2. The evoked optical action signals (fractional changes in the transmitted light) as a function of the wavelength of the incident light. In these plots, the largest signals were chosen for each preparation. The recordings were made with 499.5 ± 4.5 nm, 507 ± 4 nm, 520 ± 4.5 nm, 531.5 ± 4.3 nm, 543 ± 4.8 nm, 551.5 ± 4 nm, 561 ± 4.5 nm, 572.5 ± 4 nm, 577.5 ± 4.8 nm, 593.5 ± 4.5 nm, 603 ± 4 nm, 608.5 ± 4.8 nm, 619.5 ± 4.8 nm, 630 ± 4.8 nm, 637.5 ± 4.8 nm, 653 ± 5 nm, 633 ± 5.3 nm, 671 ± 7 nm, 679.5 ± 4.5 nm, 692.5 ± 4.8 nm, 702 ± 6.5 nm, 718 ± 5 nm and 752 ± 5 nm interference filters.

length of the incident light, and basically showed the same action spectra. Nine examples are shown in Fig. 2. The transmitted light intensity changed in the positive direction in the range of 500–620 nm, and in the negative direction in the range of 630–750 nm, with the cross-over occurring at around 630 nm. No optical signals were detected from unstained preparations. Thus, we concluded that the optical signals were due to absorption changes of the dyes related to transmembrane potential changes and were not related to any artifacts.

Similar experiments were carried out using two other merocyanine-rhodanines (NK2495/Dye XVII, Dye XXIII/RGA84/NK2765), a merocyanine-oxazolone (NK2367), four merocyanines (Dye I/NK2272, NK1331, NK2385, NK2389), four oxonols (NK2935/WW781, NK3041/RH155, NK3630/RH482, NK2936) and four oxazolidines (electrochromic dyes: ECD-1, ECD-2, ECD-3, ECD-6) (*original recordings not shown; see* Tables 1 and 3).

Signal Size

The sensitivity of the dyes to transmembrane voltage changes was assessed by measuring the fractional changes in transmitted light ($\Delta I/I$) in response to action potentials evoked by electrical stimulation.

When the merocyanine-rhodanine dyes were applied to the brainstem, the nodose ganglion, and the cervical vagus nerve bundle, NK2776, NK3224 and NK3225 often gave relatively large action signals as did NK2761. The maximum signal sizes recorded at 700 nm were different for the different preparations: $\Delta I/I$ was on the order of 10^{-2} for the cervical vagus nerve bundle, and 10^{-3} for the brainstem and the nodose ganglion. Although the other merocyanine-rhodanine dyes and an oxonol (NK3041/RH155) provided relatively good signals, their signal sizes were smaller than those of NK2761, NK2776, NK3224 and NK3225. These results are summarized in Table 3. In the present experiments, we compared the best signals for each dye obtained from several different preparations.

Signal-to-Noise Ratio

The signal-to-noise ratio (S/N) provides a good indication of which dyes are likely to be useful for monitoring transmembrane potential (Ross et al., 1977). Thus, we examined S/N for the three kinds of preparations stained with various dyes. In Fig. 3, the signal-to-noise ratios are plotted against the square root of transmitted background light intensities. We varied the light intensity by changing the output current of the stable power supply.

The relationships between S/N and transmitted background light intensity for these dyes exhibited similar shapes for all the preparations. They were composed of two components, a simple curve (at lower intensities) and a straight line (at higher intensities) corresponding to an asymptote of the curve. When the straight lines were extrapolated on the y-axis (S/N-axis), they all appeared to converge nearly on the same value. This extrapolated value was considered to reflect deviation from the relationship between the light intensity and signal-to-shot noise ratio (S/N_{shot}) expected theoretically (*see* Appendix). Similar graphical features were also obtained with preparations of the nodose ganglion and cervical vagus nerve bundle. This graphical characteristic strongly suggests that in a particular preparation, e.g., brainstem, nodose ganglion or cervical vagus nerve bundle, the extrapolated value was independent of the dye used, and

Table 3. Summary of the parameters of optical signals

Dyes	Materials								Wavelength (nm)
	Brainstem (slice)		Brainstem (intact)		Vagus nerve bundle		Nodose ganglion		
	Maximum signal size ($\times 10^{-4}$)	$d(S/N)/d(I^{1/2})$	Maximum signal size ($\times 10^{-4}$)	$d(S/N)/d(I^{1/2})$	Maximum signal size ($\times 10^{-4}$)	$d(S/N)/d(I^{1/2})$	Maximum signal size ($\times 10^{-4}$)	$d(S/N)/d(I^{1/2})$	
Merocyanine-rhodanines									
NK2761	70	4.9	23		135	9.0	113	9.0	700
[a]NK2776	47	5.7			100	4.8	46	2.6	700
[a]NK3224	51	4.5	8		78	4.5			700
[a]NK3225	60	4.6	11		46	2.4			700
[a]NK3360	32	3.1			106	7.2	>40	4.3	700
[a]NK3361	39	2.6	6		>40	5.0	53	3.0	700
[a]NK3365	34	4.0			44	3.1	29	2.7	700
[a]NK3376	30	2.0			22	1.4	19	1.5	700
NK2495 (Dye XVII)	20	1.4			59	3.7	17	2.5	700
Dye XXIII(NK2765, RGA84)	44	3.6			86	4.6	25	4.8	700
Merocyanine-oxazolones									
NK2367							27	1.4	680
Other merocyanines									
Dye 1 (NK2272, M-540)							25[b]	0.7	570
NK1311							NO		
NK2385							NO		
NK2389					NO				
Oxonols									
NK2935 (WW781)	13		8		21	0.8	6		630
NK3041 (RH155)	15	1.0	5				17	1.1	700
NK3630 (RH482)	24								720
NK2936					1				630
Oxazolidines									
(Electrochromic dyes)									
ECD-1							3[b]		600
ECD-2							11[b]		640
ECD-3							NO		
ECD-6					NO				

[a] Newly synthesized dyes; [b]with large phototoxic damage

was determined exclusively by the physical characteristics of the apparatus and the tissue used in the measurement.

There are many sources of noise that may contribute to optical recording of membrane potential changes (*cf.* Cohen & Lesher, 1986; Salzberg, 1983; Salzberg et al., 1977). Signal-to-shot noise ratio is proportional to the square root of the transmitted background light intensity. Thus, it is plausible that the dominant noise at higher intensities of the transmitted light is "shot noise," and that the noise at lower intensities is that from physical characteristics of the apparatus (e.g., dark noise).

The slope of the straight line varied with the dyes, providing a parameter indicating an effective value of S/N for each dye. Thus, the slopes show that NK2761, NK2776, NK3224, NK3225 and NK3365 were good dyes for embryonic neural preparations (*also see* Table 2).

Photobleaching

We examined the photobleaching time course of eleven dyes. In brainstem slice preparations stained with each dye, the time course of the changes in transmitted background light intensity (I) was measured by repeating the experiment without electrical stimulation under continuous illumination. In Fig. 4, the abscissa is the illumination time through a 702 ± 6.5 nm interference filter, and the ordinate is the normalized background intensity of transmitted light from the stained preparation. (However, we did not systematically examine the relationship between photobleaching and the amount of dyes bound to membranes. We used the concentrations used in our usual experiments.) In these experiments, the same intensity of illumination was used. The rate of increase in the transmitted background light intensity (decreased absorption) using NK2761, NK3365 and Dye XVII

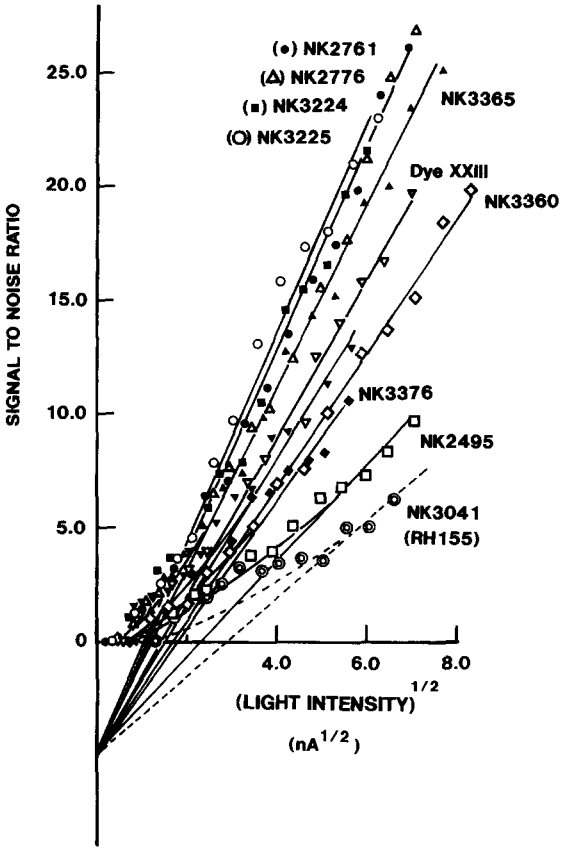


Fig. 3. Signal-to-noise ratio as a function of the square root of transmitted light intensity. The noise was measured in peak-to-peak. Data were obtained from brainstem slice preparations stained with NK2761, NK2776, NK3224, NK3225, Dye XXIII, NK3360, NK3365, NK3376, NK2495 or RH155 (NK3041). The recordings were made with a 702 ± 6.5 nm interference filter.

(NK2495) was smallest and the background absorption was reduced by about 5% after illumination for 200 sec. On the other hand, the bleaching of NK3361 and RH155, was relatively rapid. (However, RH155 could be used, at reduced illumination intensity, to record continuously for up to 3 hr from cultured *Aplysia* neurons (Parsons et al., 1991).)

Photodynamic Damage

The ten merocyanine-rhodanine dyes and four oxonol dyes used here permitted optical recording from embryonic neural preparations with no discernible photodynamic damage or pharmacological effects over illumination periods of about 30 min. On the other hand, when a merocyanine (Dye I: NK2272/M-540: Davila et al., 1973) and two oxazolidine dyes (electrochromic dyes: ECD-1 and ECD-2) were applied to the nodose ganglion, strong photodynamic damage was observed. In Fig. 5, an example of photodynamic damage induced by Dye I is shown (*cf.* Salzberg, Davila & Cohen, 1973). Under continuous illumination, the size of action signals was reduced by 50% after 54 sec. The photodynamic damage induced by the two oxazolidine dyes (ECD-1 and ECD-2) was much more severe.

Discussion

The ideal voltage-sensitive dye for an individual preparation is very sensitive to changes in transmembrane potential and has little or no tendency to harm the preparation as a result of pharmacological or phototoxic actions. In addition, it is required that photobleaching of the dye is small. For reasons discussed in the introduction, in early embryonic excitable tissues, it is necessary to select suitable dyes.

The present results indicate that relatively sensitive absorption probes of membrane potential are available from among the merocyanine-rhodanine class of dyes: large optical signals were obtained using NK2761, NK2776, NK3224, NK3225, NK3360, NK3361, NK3365 and NK3376 with no serious pharmacological or phototoxic action; their bleaching times were relatively long. In addition, because a lower concentration (0.1–0.2 mg/ml: 200–400 μM) can be used for staining, osmotic effects on cells can be minimized. These characteristics of the dyes are advantageous, especially for monitoring neural activity from early embryonic preparations. Of the merocyanine-rhodanine dyes, NK2761 has often proven to be best. On the other hand, although an oxonol dye (RH155/NK3041) is useful for juvenile *Aplysia* neurons in culture (Parsons et al., 1989; 1991), the adult *Aplysia* abdominal ganglion (Falk et al., 1993), neocortical slices from adult guinea pigs (Albowitz, Kuhnt & Ehrenreich, 1990), and cerebellar slices from skates (Konnerth, Obaid & Salzberg, 1987), this dye was inferior to the merocyanine-rhodanine dyes for early embryonic preparations.

In the present experiments, we examined the dyes with an emphasis on early embryonic preparations. We were also persuaded that NK2761 is well suited for heart preparations from the adult frog (Sawanobori et al., 1981; Komuro et al., 1986) and the adult rat (Sakai et al., 1993). Furthermore, this dye is also suitable for the neurohypophysis of adult *Xenopus* (Salzberg et al., 1983; Obaid et al., 1989), and for hippocampal slices from the adult rat (K. Sato, Y. Momose-Sato and T. Sakai, *unpublished results*). However, this dye is not suitable for the abdominal ganglion of adult *Aplysia* (L.B. Cohen and J.-Y. Wu, *personal communication*), or for the hypothalamus of adult rats (H. Komuro, *personal communication*). From these results, it is suggested that the relationship between voltage-sensitivity and physicochemical properties of dyes is a complex function in a variety of preparations.

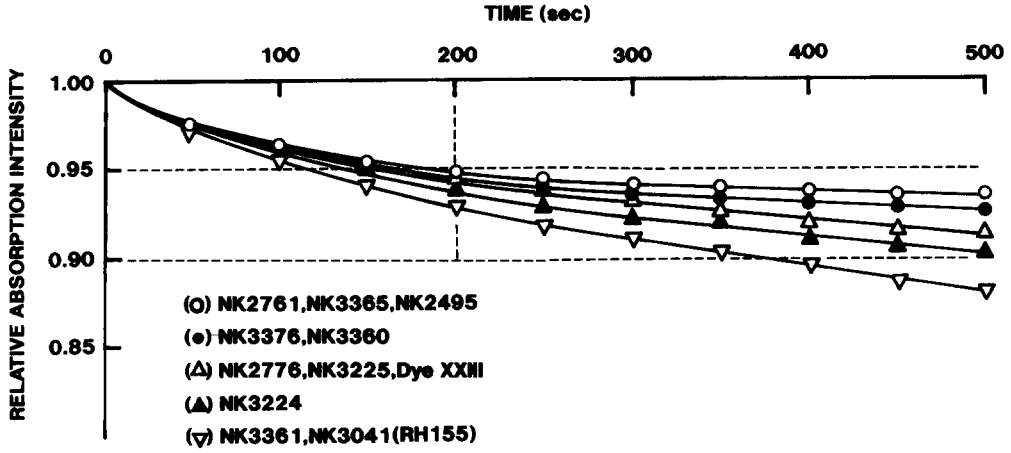


Fig. 4. The time course of photobleaching of eleven different dyes. The ordinate represents the relative absorption intensity, the abscissa is the time after the beginning of illumination of the stained preparations.

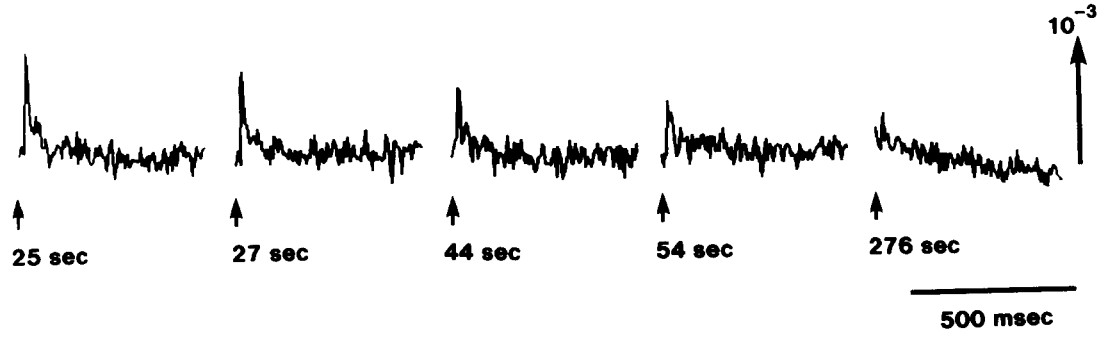


Fig. 5. A typical example of photodynamic effects on optical action signals observed in a 7-day old embryonic nodose ganglion stained with Dye I. Arrows under the signals indicate the stated time after onset of illumination.

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Appendix

Formulation of the Empirical Relationship Between S/N and $I^{1/2}$

1. Mathematically, the signal-to-noise ratio in transmitted light measurements (shot noise limited) is given by

$$S/N_{\text{shot}} = k \cdot I^{1/2} \tag{A1}$$

where S is the signal, N_{shot} the shot noise, I the background light intensity, and k, a constant (e.g., Kingston, 1978).

In the present experiment, the curves of the signal-to-noise ratio (S/N) *vs.* the square root of the background intensity ($I^{1/2}$) plots deviated from the linear relationship between the S/N_{shot} and $I^{1/2}$ (Fig. 3).

As illustrated in Fig. A1, the S/N–$I^{1/2}$ curves are considered to have an asymptotic line which is parallel with Eq. *A1*, so that the

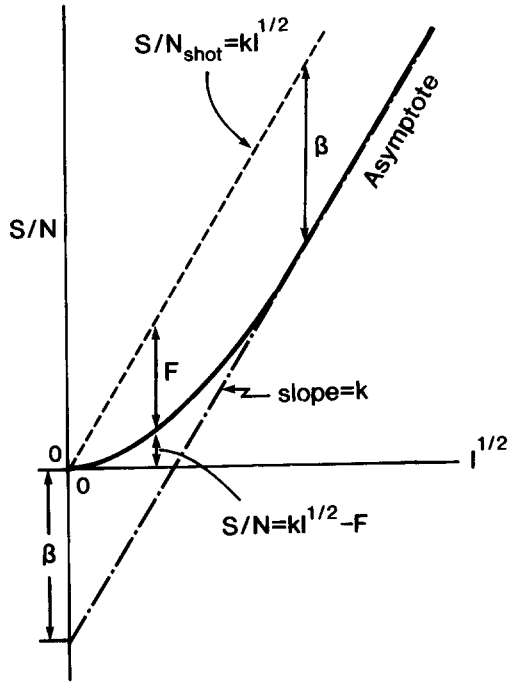

Fig. A1. Schematic illustration of theoretical (shot noise limited) and experimental relationship between signal-to-noise ratio (*S/N*) and background light intensity (*I*), and definition of *F*, and β for a certain set of a given dye. The long broken line expresses the theoretical $S/N_{shot} - I^{1/2}$ relation, the heavy line the experimental curve for a given dye, and the chainline the expected asymptotic line for the experimental curve.

present experimental relationship between the *S/N* and $I^{1/2}$ can be expressed in the form

$$S/N \quad k \cdot I^{1/2} - F, \tag{A2}$$

where *F* is a "deviation term." *F* should be a function of $I^{1/2}$ which has an asymptotic line (F β) as shown in Fig. A2.

Here, it can be assumed that *F* is a hyperbolic or exponential function of $I^{1/2}$, namely

$$\text{hyperbolic: } F = \beta - \frac{\alpha\beta}{I^{1/2} + \alpha} \tag{A3}$$

$$\text{exponential: } F = \beta - \beta \exp(-\alpha \cdot I^{1/2}), \tag{A4}$$

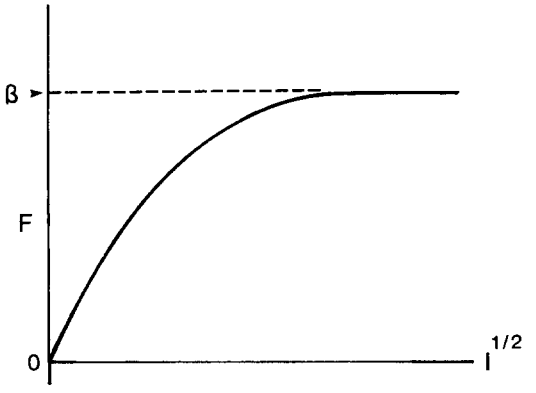

Fig. A2. Graphical characteristics of the relationship between *F* and $I^{1/2}$, expected mathematically.

where, α and β are constants.

Thus, we obtain the following two possible equations for the experimental relationship between the *S/N* and $I^{1/2}$.

$$S/N = k \cdot I^{1/2} - \left(\beta - \frac{\alpha\beta}{I^{1/2} + \alpha}\right) \tag{A5}$$

$$S/N = k \cdot I^{1/2} - [\beta - \beta \exp(-\alpha \cdot I^{1/2})]. \tag{A6}$$

Now, in Eqs. A3 and A4, *F* approaches β as *I* increases. Consequently, the asymptotic line of Eqs. A5 and A6 are uniquely given by

$$S/N \quad k \cdot I^{1/2} - \beta. \tag{A7}$$

Accordingly, the extrapolated value on the y-axis of the linear part of the experimental curve in Fig. 3 corresponds to the "−β" in Eq. A7.

2. On the other hand, assuming that the noise consists of an intensity-independent part and a part due to the quantum nature of light, i.e., shot noise, the total noise is $N \quad a + b \cdot I^{1/2}$. Since the signal is proportional to the light intensity then:

$$S/N \quad c \cdot I/(a + b \cdot I^{1/2}) \tag{A8}$$

where *a*, *b*, and *c* are constant. Equation A8 can be rewritten as follows:

$$S/N \quad (c/b)I^{1/2} - ac/b^2 + (a^2c/b^3)/(I^{1/2} + a/b) \tag{A9}$$

Here, putting $k \quad c/b$, $\alpha = a/b$, and $\beta = ac/b^2$, Eq. A8 is equivalent to Eq. A5. Thus, Eq. A5 probably fits the data well and is better justified than Eq. A6.