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The Optical Spike [and Discussion]

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The optical spike

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Among the signs of activity in excitable membranes, the action current (electrical spike) has been extensively studied. Recently, a new approach with optical methods has been rewarding. In nerves, a transient, rapid change of light scattering, birefringence and induced fluorescence can be observed during the passage of the action current. These optical effects are synchronous with the electrical spike and are therefore called the optical spikes. Birefringence decreases during excitation in the giant axon of the squid, the walking nerves of *Maia*, the vagus nerve of the rabbit, but it increases in the olfactory nerve of the pike, which contains 4 million non-medullated nerve fibres. Light scattering increases or decreases depending on the angle of observation. Vitally stained nerves with fluorescent probes show an increase and a shift in the wavelength distribution of the fluorescent spike.

Introduction

In 1968 a new approach in our understanding of the basic 'machinery' of excitation in nerves was opened up by the pioneering work of Cohen & Keynes (1968, 1969*a*, *b*) who presented at the meeting of the Physiological Society evidence of a rapid, transient change of light scattering and of birefringence in an excited nerve, appearing synchronously with the electrical spike (action potential). This discovery led immediately to further studies in Cambridge and Plymouth (England), in Bethesda and Berkeley (U.S.A.), in Russia and in Bern (Switzerland). Three similar, but not identical transient optical effects, intimately connected with the electrical spike in an excited nerve were studied: light scattering at various angles, birefringence and fluorescence, induced by vital-staining of the nerve with fluorescent probes. I propose to call the transient optical signs of excitation the 'optical spike' and to subdivide them into 'light scattering spike', 'birefringence spike' and 'fluorescence spike'. For the analysis of these phenomena, the technique of applying hyperpolarizing and depolarizing pulses under clamp conditions has furnished very useful information and for these effects the term 'optical pulse response' will be chosen.

Materials and techniques

The best suited object for the study of the optical spike is the giant axon of *Loligo*, the atlantic squid. Second best is the olfactory nerve of *Esox*, the freshwater pike, which has 4 million un-medullated nerve fibres of a modal diameter of 0.2 μm and a very narrow size distribution. Other nerves studied are the nerves of the walking leg of *Maia*, lobster nerves, the olfactory nerve of the garfish *Belone* and the vagal nerve of the rabbit. The use of the giant axon provides advantages, which none of the other nerves offer: it can be impaled with an internal electrode, micro-injections can be made and the axon can be extruded and replaced by a perfusion. The optical signals are, however, very small and the signal to noise ratio is such that averaging methods have to be used in order to improve the ratio, a process in which

* In memory of my ancestor, the anatomist Joh. von Muralt from Zürich, who presented a paper to the Royal Society 305 years ago (*Phil. Trans.* **49**, 1669).

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small but important timing coincidences may get lost. The olfactory nerve of the pike produces optical spikes which are almost 10^3 times larger and can be recorded in one single sweep on the oscilloscope. This nerve, however, has the disadvantage that it cannot be clamped for optical pulse responses.

The extreme faintness of the optical spikes calls for averaging techniques, light sources, photocells and electronic equipment with low noise and careful anti-vibration measures. (See for light scattering: Cohen, Keynes & Landowne (1972*a*, *b*), for birefringence: Cohen *et al.* (1971 *a*, *b*), for fluorescence: Cohen *et al.* (1974).) Special aspects of the methods will be discussed under the following separate headings.

Light scattering

There are two different aspects of light scattering: if the scattering 'particles' are large compared to the wavelength of the incident light: 'white' scattering, if they are small compared to the wavelength: 'blue' scattering is observed. In biological objects 'white' scattering is usually so strong that the 'blue' component is completely covered up. The giant axon of the squid scatters much more light in the forward direction than at right angles. At an angle of 10° about 10^3 times more than at 90°, which is a typical sign of 'white' scattering. Illumination of the axon with natural light produces a partial polarization of the scattered light at 90° with a polarization parallel to the axis of the axon. In these studies one has to keep in mind that the squid axon is built up of three structurally different tissues, each taking its share of the light scattering in the resting nerve: (*a*) the axon, a protein-structure with an alinement parallel to the fibre axis; (*b*) the axon membrane, containing the excitable elements, with a dominant radial structure of lipoproteins, which are amphipathic, with a hydrophobic and a hydrophilic portion and lipids; (*c*) the Schwann cell, with intra- and extra-cellular spaces.

The expectation that the optical spike during excitation is a measurable sign of conformational changes within the excitable membrane, has led to many even somewhat contradictory aspects.

The following report is based on the work of Cohen *et al.* (1972*a*, *b*). The transient change of light scattering during excitation is always measured by $\Delta I/I$, where ΔI is the size of the optical spike and I the intensity of the resting light scattering. This quotient is of the order of 10^{-6}, with an unfavourable signal to noise ratio. So 10^3–10^4 sweeps have to be averaged in order to get a signal, such as is shown in figure 1, which represents the transient right-angle light scattering change in a squid axon, 8000 sweeps averaged. The heavy line is the optical record, the thin line is the simultaneously recorded action potential.

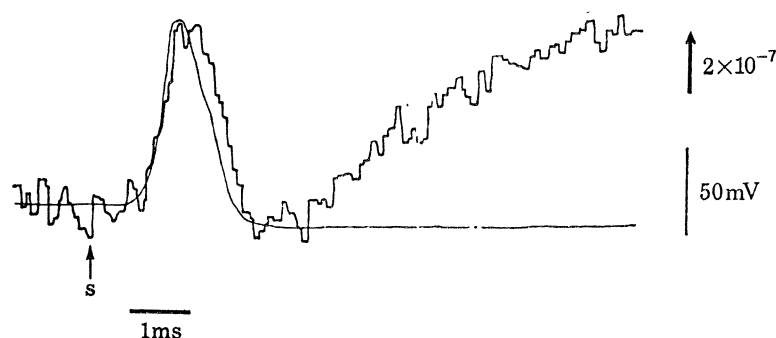

Figure 1

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There are obviously two separate optical responses: a rapid, transient change *synchronous* with the action potential, for which we shall use the term 'optical spike' and a *delayed*, long lasting change after excitation, reaching a peak 20 ms after excitation and tapering back to resting level with a half-time of 50–100 ms (not shown in figure 1).

Light scattering recorded at an angle of 70, 90 and 120° shows a *positive* optical spike (increase of light scattering), at 10 and 27° a *negative* optical spike and at 45° no scattering change was recorded. The optical spikes are not only different in sign, but also in shape, suggesting different structural origins. Curiously, their size depends not only on the action potential but also on the state in which the axon is with respect to fatigue. In a fresh axon, the size of the forward-angle, negative optical spike *decreases* linearly with a decrease of the size of the action potential, in an axon which is subjected to fatigue, the right-angle positive optical spike *increases* as the action potential becomes smaller, showing that the optical spike is not only potential-dependent, but also influenced by other factors.

Three interdependent elements in the excitation mechanism must be considered separately: (*a*) the changes in membrane potential; (*b*) the changes in membrane conductance; (*c*) the ionic currents. One may hope that it will be possible to relate the various recorded optical spikes to these changes, in order to get insight into their molecular basis. One approach in order to get such insight is the study of the optical pulse responses during voltage-clamp steps.

The light scattering studies of *current-dependent responses* revealed three distinct time courses. At an angle of 90° there is a marked positive optical pulse response to a 50 mV depolarizing step of 2–5 ms duration. This pulse produces a large *inward* current through the membrane, but the main peak of the optical response was reached later than the current-flow, so it did not appear to be related to the instantaneous current density but rather to the time integral of the ionic current. If the sodium in the outside was replaced by choline, the 50 mV depolarizing step resulted in an equal increase of conductance, but there was only a very small current flow. Under these conditions no optical pulse response appeared (the experiment was carried out with forward scattering at an angle of 25°). Blocking the delayed *outward* current with a micro-injection of tetraethylammonium and applying a depolarizing step so near the equilibrium potential that the current was very small, gave no optical pulse response. Under these conditions the conductance increase is large and yet there is no optical response. So this is rather disappointing because an optical change directly linked with changes of membrane conductance might have given valuable information about molecular events in and around the ionic channels. The study of the *potential-dependent responses* revealed two different changes, one at forward angles and one at right angles with different time constants: at 90° $\tau = 24$ μs and at forward angles two exponentials, with $\tau = 23$ μs and $\tau = 1$ ms. A rise in temperature had no effect on 90° scattering, but reduced the forward scattering. In the presence of butanol and octanol, which penetrate into nerve lipid monolayers, the 90° scattering was increased and the forward scattering remained unaffected, suggesting an involvement of 90° scattering with the lipid portion of the membrane. On the whole the size of the potential-dependent responses was proportional to the square of the potential. Keynes (1972) in summarizing his own experiments on light scattering, came to the conclusion that they 'do not seem to offer an especially promising approach'.

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Birefringence

Materials and techniques

Birefringence is an optical property which is shared by many biological structures, such as nerve, muscle, hair, bone, cartilage etc. Its basis is the orientation of submicroscopical structural elements in these fibrous tissues either parallel to the fibre axis, orthogonal to it or in a helix. The classical method of measuring birefringence makes use of a calibrated compensator (Babinet, Berek, Brace, etc.) which introduces into the beam of polarized light a variable retardation R_c. The biological object produces by birefringence a retardation R_x and the compensator is set in such a way that $R_c = R_x$. This method, however, cannot be used for recording rapid birefringence spikes. Cohen, Hille & Keynes (1970) were the pioneers who introduced an entirely new method. They recorded the extremely small intensity change ΔI of the outcoming polarized light component, produced by a change ΔR of the retardation in the nerve during excitation. Their theory of this new approach is based on equations Fresnel developed in 1821! The formula derived for the purpose of measuring the optical spike is:

$$\Delta R = \tfrac{1}{2}R - \Delta I/I, \tag{1}$$

where ΔR is the change of retardation, ΔI the recorded change of intensity, I the resting intensity of the light and R the retardation due to the resting birefringence of the nerve.

There are three causes of birefringence which must be considered:

(*a*) intrinsic birefringence: this type of birefringence occurs if anisotropic molecules or macromolecules are oriented in one direction;

(*b*) form birefringence: it occurs if isotropic structural elements are oriented in one direction and if the spaces between the elements are filled with an isotropic substance having another refractive index;

(*c*) accidental birefringence: it occurs if the degree of freedom of valence electrons is reduced by stress or pressure, electric or magnetic fields.

Birefringence is measured by the difference between the refractive index n_a parallel to the axis of a biological structure and n_o orthogonal to it. The relation between retardation R, the phase difference $\frac{1}{2}\Theta$, the wavelength of light λ, the length d of the light path in the birefringent object and the birefringence $n_a - n_o$ is:

$$n_a - n_o = \frac{R}{d} = \Theta \frac{\lambda}{2\pi} \frac{1}{d}. \tag{2}$$

Optical birefringence spikes were obtained by Cohen, Hille & Keynes in the nerves of the walking leg of *Maia*, in the squid axon (normal and perfused) and in the vagal nerve of the rabbit. The number of sweeps which had to be averaged was several thousands. The observed optical birefringence spike during excitation was shown, by a number of very careful control experiments, to be entirely a change in retardation under the experimental conditions. In the squid $\Delta I/I$ is of the order of 10^{-5}, but in most cases the recording was not restricted to the extreme edge of the axon image, where the polarized light passes through the cylindrical 'shell', the excitable membrane, with birefringent, radially oriented lipoproteins and lipids. That this region is the origin of the optical spike was shown in a fine experiment, where the recording was restricted to narrow strips of the axon image. In the centre of the axon $\Delta I/I$ is zero and rises to the 'edges' on both sides according to the predicted retardation for a thin birefringent shell, surrounding the axon.

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The resting retardation R of the axon is of the order of 56 nm and $\Delta I/I$ about 2×10^{-5}. This gives a value of $\Delta R = 5.6 \times 10^{-4}$ nm, which corresponds to a phase difference of the two components of the elliptically polarized light of one millionth of a wavelength! The biologists have surpassed the physicists, who with their best methods had been able to measure only phase-differences of $10^{-4}\,\lambda$ (Szivessy 1935).

The author had the rare pleasure of assisting at these remarkable experiments in Plymouth and stimulated by this great experience remembered that there is another very interesting nerve – well known to physiologists at the beginning of this century – the olfactory nerve of the pike *Esox*. Experiments with this nerve in Bern gave a new and fascinating result: the resting birefringence of the whole nerve is negative with respect to the fibre axis and the resting retardation R is 24.8 nm. $\Delta I/I$ is 3.3×10^{-3} and $\Delta R = 4.1 \times 10^{-2}$ nm, 100 times larger than in the squid. So *one* sweep was sufficient to record a good optical spike.

There is a possibility of increasing the signal to noise ratio quite considerably (Cohen, Keynes, von Muralt & Rojas 1970; von Muralt 1974).

For small values of ΔI one can write

$$\Delta I = 2I_0 \sin \tfrac{1}{2}\Theta \cos \tfrac{1}{2}\Theta \, \Delta\tfrac{1}{2}\Theta, \tag{3}$$

where I_0 is the intensity of the incoming linear polarized light and $\frac{1}{2}\Theta$ the phase difference, $\Delta\frac{1}{2}\Theta$ the phase difference producing the optical spike. By increasing $\frac{1}{2}\Theta$ artificially by inserting into the light path an additional phase difference with a compensator or a quarter wavelength plate, one can increase the signal ΔI considerably up to the value $\frac{1}{2}\Theta = 45°$, where the sin–cos function has its maximum (0.5). But in doing this, one increases also I, according to

$$I = I_0 \sin^2 \tfrac{1}{2}\Theta \tag{4}$$

and with it the electronic noise in the photocells, which increases with $\sqrt{I}$. Experimentally one can easily determine the optimal signal to noise ratio by varying the added retardation, f.i. with a $\frac{1}{4}\lambda$ plate in the light path. This improvement in technique permits the recording of the optical birefringence spike in one single sweep!

Results

Birefringence changes in nerves have been described since 1968 by many authors (Cohen, Keynes & Hille 1968; Tasaki, Watanabe, Sandlin & Carnay 1968; Entine 1969; Berestovsky, Lunevsky, Razhin & Musienko 1969; Berestovsky, Liberman, Lunevsky & Frank 1970*b*; Berestovsky *et al.* 1970*a*) using crab nerve, lobster nerve, crayfish nerve, rabbit vagus nerve and mainly squid giant axons. Cohen, Hille & Keynes 1970; Cohen *et al.* 1971, found in the squid axon that the optical spike follows almost closely the time course of the intracellularly recorded action potential. The same birefringence spike is obtained from fresh axons and axon membranes, where the axoplasm has been removed and replaced by a potassium fluoride solution. With changes of temperature the time courses of the birefringence and the electrical spike remain synchronous. A very small delay between the birefringence spike and the action potential was observed consistently at lower temperatures, which is important! The birefrigence spike has its origin in the excitable membrane, possibly with a small contribution from the Schwann cells and their extracellular spaces. The study of optical pulse responses revealed three separate voltage-dependent components. A hyperpolarizing pulse gives a rapid

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optical response, termed the *fast phase* proportional to the potential squared, followed by a slow return towards the base line, termed the *rebound*. Treatment of the axon with tetrodotoxin, terbium, high calcium, etc., slows down the fast phase, increases its size and a new *slow component* with a time constant of 2 ms is added to it. The rebound disappears, an effect which can also be produced by high calcium. An indication of a small current- or conductance-dependent component was also observed. So there is more than one structural source for the retardation change! The recent evidence that in 1 μm^2 of nerve membrane, there are only some fifty sodium-sites (Keynes, Ritchie & Rojas 1971), compared to several million phospholipid and other molecules, lowers the prospect of obtaining much *direct* information of conformational changes involved in the activation and inactivation of the sodium and potassium channels. We do not know, however, whether this activation and inactivation is a process strictly localized to the channel site, or whether the environment of a large membrane with voltage-dependent structural responses is not a necessary triggering device for such conductance changes.

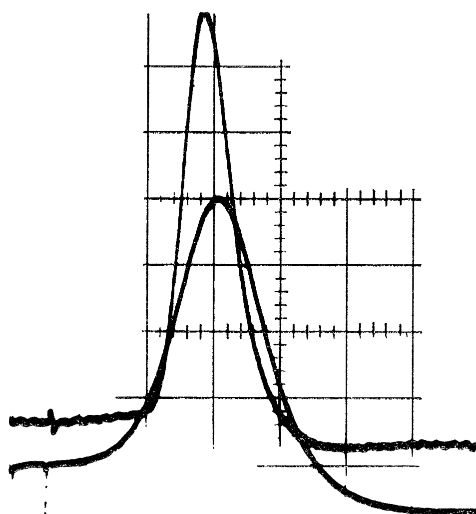

FIGURE 2. Upper tracing: optical spike, 200 μV/division; lower tracing: action-potential, 1 mV/division; sweep: 100 ms/division; temperature: 0 °C.
Duration of spikes corrected for zero conduction, optical: 240 ms; electrical: 400 ms.

The olfactory nerve of the pike with its 4 million unmedullated nerve fibres is unique. It has a negative birefringence with regard to the fibre axis and the optical birefringence spike of this nerve is due to an increase of its negativity. Figure 2 shows this remarkable optical spike, recorded at a temperature of 0 °C. The birefringence spike seems synchronous with the action potential, but on closed inspection it is evident that it reaches its maximum before the action potential and drops to the base line earlier than the action potential (von Muralt 1971, 1974). For the time being it was not possible to locate the recording electrode exactly at the spot, where the optical spike was recorded, but calculations show that the spreading of the excitation wave from the 'optical spot' to the 'electrical spot' is not sufficient to account for the rather pronounced difference in time-course between the birefringence spike and the action potential. Until the experimental conditions for an exact coincidence of the two recordings are not reached (a technique which is under way), it is safer not to attempt an interpretation of these differences in time relations.

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Discussion

The results show clearly that during excitation there is an appreciable, transient variation of the molecular structure in the excitable membrane or close to it. The basis of the optical spike of the olfactory nerve must be an increase of retardation of the radially oriented elements in the excitable membrane, the lipoproteins. This explains also the negative optical spike, found in all other nerves, where the positive birefringence of the axon dominates and the negative, radial component is small. A retardation increase in the radial elements must result in a decrease of the compound birefringence. Cohen *et al.* (1971) have discussed in detail the possible sources of this retardation change, but with the new evidence from the olfactory nerve in certain points, the interpretation of the results has to be revised. The interesting fact that all nerves have a membrane in which potential-dependent and to some extent also current-dependent structural changes occur during excitation and also in response to electrical pulses, is important enough! The increase of this response by an addition of butanol or octanol to the bathing solution points to the dominant rôle of the membrane lipids.

Fluorescence

Introduction

The attempt to use fluorescent probes in order to produce an extrinsic fluorescence of nerve and to study the changes of this fluorescence during excitation was successfully tried by Tasaki, Watanabe, Sandlin and Carnay in 1968. The quantum yield and the spectral distribution of the fluorescent light are both sensitive to changes in viscosity, pH, solvent polarity and macromolecular and molecular conformational changes in the nerve membrane. Tasaki and his collaborators started with the dye 1,8 ANS (1-anilino-8-naphthalene sulphonate) and then shifted to 1,8 TNS (1-toluidinylnaphthalene-8 sulphonate). They were able to record transient changes of fluorescence during excitation, with other words *fluorescence spikes*. Soon they were aware that many other dyes give the same optical effects, which were studied in detail in the following papers: Tasaki, Carnay & Watanabe (1969); Conti & Tasaki (1970); Watanabe, Tasaki & Hallett (1970); Conti & Wanke (1971); Conti, Tasaki & Wanke (1971); Tasaki, Watanabe & Hallett (1971); Hallett, Tasaki, Schneider & Carbone (1972); Tasaki, Watanabe & Hallett (1972); Tasaki, Carbone, Sisco & Singer (1973); Tasaki, Hallett & Carbone (1973).

When Cohen returned from Plymouth and Cambridge to Yale University he started similar studies on 300 fluorescent probes, which he described with his collaborators in the following papers: Cohen, Landowne, Shrivastav & Ritchie (1970); Cohen, Davila & Waggoner (1971); Cohen, Landowne & Shrivastav (1971); Davila, Salzberg, Cohen & Waggoner (1972, 1973); Salzberg, Davila & Cohen (1973); Davila, Cohen, Salzberg & Shrivastar (1974); Cohen *et al.* (1974).

The most spectacular of the substances which they studied is merocyanine, which gives such a strong fluorescence that the optical change can be registered as a response to one excitation in a single sweep of the oscillograph, a real optical spike! (Davila *et al.* 1973).

An important and disturbing factor in the study of fluorescence is the photodynamic damage of the exciting u.v. light on the excitable membrane. (Booth, von Muralt & Stämpfli 1950; Arvanitaki & Chalazonitis 1961; Pooler 1972; Fox & Stämpfli 1973). The photodynamic damage, however, is negligible if green light of 546 nm can be used as exciting light. This is

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the case for the excitation of fluorescence with merocyanine, which makes this new fluorescent probe even more attractive for biological studies!

Materials and techniques

In the experiments, so far described, the giant axon of the squid has been used mostly. Since the fluorescence of ANS and TNS are different if the probe is applied from outside or from within, the giant axon is the only nerve where such studies are feasible, in addition to the possibility of measuring optical pulse responses for which an internal electrode is necessary. A few experiments were carried out with walking leg nerves of lobster, the olfactory nerve of garfish, the claw nerve of the spider crab and the rabbit vagal nerve.

The dyes are used at concentrations of 0.01–05 mg/ml, but for each dye the optimal concentration has to be determined experimentally, since at lower and higher concentrations, the optical spike decreases. Unsoluble dyes were applied in 1 % ethanol with the addition of 0.01–02 % Pluronic F-127 or in a mixture of seawater and acetone. To dampen the photodynamic action of the exciting light nitrogen gas was bubbled through the solutions. (For all details see Watanabe, Tasaki & Hallett (1970) and Cohen *et al.* (1974).)

In some experiments the exciting u.v. light was polarized and the fluorescent light was analysed with respect to its plane of polarization. Disturbance of the recorded fluorescence response by scattered light was avoided by using filters, which were only transparent for the long wavelengths of the fluorescent light. In the cyanine and merocyanine dyes the absorption- and emission-wavelengths are close. Filters which pass only the short wavelength shoulder of the exciting light were used. The fluorescent probes excitable with u.v. light (365 nm) were 1,8-ANS, 2,6-ANS, 1,8-TNS, 2,6-TNS, the group of aminonaphthalene sulphonates 1,8-AmNS, 2,6-AmNS. The whole group of merocyanine and cyanine dyes can be excited with light between 480–660 nm.

The chemical purity of the fluorescence probes is very important, because most commercially available dyes contain fluorescent impurities, which may lead to an incorrect attribution of optical effects to the major component, when it actually is due to the impurity. Caution is recommended!

Results

It is best to begin with the latest achievement: Davila *et al.* (1973) succeeded in obtaining an optical fluorescence spike in one sweep on the oscillograph, represented in figure 3. The upper tracing is the optical spike, produced by merocyanine fluorescence. $\Delta I/I$ is 10^{-3} and this spike is many times larger than *any* fluorescence change obtained previously. They used this dye, following a suggestion of Dr G. Strichartz. The lower tracing is the change in membrane potential due to excitation. It is obvious that the optical spike follows the change in membrane potential, even to the small hump in the rising phase. (Time constant of light system 0.56 ms, axon diameter 400 μm, temperature 14 °C.) The axon fluorescence produced by staining with merocyanine is linearly dependent upon the membrane potential. This offers a new and very promising aspect, since this dye and its green exciting light have no deleterious effects on the axon. Merocyanine allows non-destructive monitoring of membrane potential in membranes even under conditions, where electrodes cannot be used!

There have been some indications that ANS and TNS treated axons gave not only potential dependent fluorescence pulse responses, but also responses which could be related to conductance

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changes, because these fluorescence responses to a depolarizing pulse were larger than to a hyperpolarizing pulse and the observed changes were more rapid in the hyperpolarizing direction than in the depolarizing direction (Conti *et al.* 1971; Conti & Wanke 1971). It is known how difficult it is to obtain a satisfactory voltage and space clamp and until more convincing evidence for a conductance-dependent fluorescence pulse response is furnished, this problem remains open.

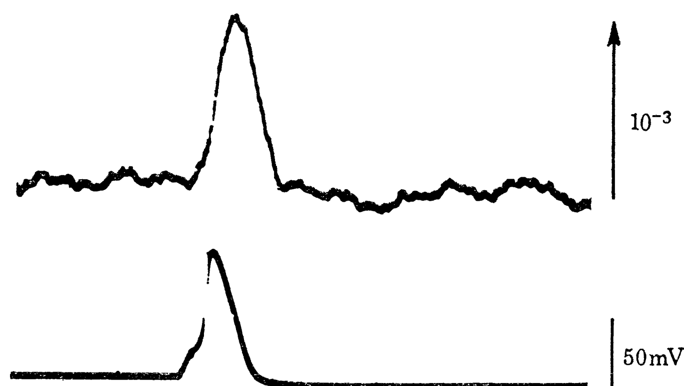

FIGURE 3.

Optical pulse responses have been measured on 38 other dyes in squid axon, and all the fluorescence responses were potential-dependent (Cohen *et al.* 1970; Cohen *et al.* 1971*a*; Cohen *et al.* 1971*b*; Davila, Cohen & Waggoner 1972). Only two dyes, tetracycline and coriphosphine O, seem to have a dependence on membrane potential *squared*.

The sign of the fluorescence spike with ANS depends upon the method of application. Injection into the inside of the axon gave a negative, but the application from the outside a positive spike. This behaviour is unique for ANS and not shown by other dyes which were tried internally and externally, such as acridine orange, chlorotetracycline, neutral red, neutral violet, pyronin B, rhodamine B and tetracycline, all giving changes of the same sign on both sides. The pulse reponses for depolarization correspond to the sign of the spike for all acridines, quinolines, azins, oxazins and thiazins. In pyronin B, rhodamine B and rhodamine S, curiously enough, the response was opposite.

A very interesting approach is the use of polarized exciting light and the analysis of the plane of polarization of the fluorescent light, introduced by Tasaki *et al.* in 1971, using 2′6′-TNS as fluorescent dye. If the plane of polarization of the exciting light (365 nm) is parallel to the axis of the illuminated nerve and the analyser in such a position that light parallel to this axis passes through, the negative optical spike is recorded (symbol $\|$, $\|$). If the position is $\perp$, $\|$ or $\|$, $\perp$ no signal is observed! However, in the position $\perp$, $\perp$ a small positive optical spike of about 30 % is seen. ANS does not show such highly polarized fluorescence and the signals with the various four combinations of polarizer and analyser showed only such small differences that they almost fall within the limits of error. Pyronin B was found to give results similar to TNS but the optical spike is *diphasic*, with an initial negative component followed by a slow positive component. In the position $\|$, $\|$ the negative component was present and absent for $\|$, $\perp$. The positive component of pyronin B is not polarized and always present. The optical pulse response in TNS stained nerves corresponded exactly to the results with the optical spikes in sign. (Tasaki *et al.* 1972; Tasaki *et al.* 1973).

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The major portion of the fluorescent dye in an axon is undoubtedly bound to non-excitable sites. But the polarization experiments show clearly that a long molecule like 2′6′-TNS is not only incorporated but also oriented in the excitable membrane. Since the internal structure of the excitable membrane (the lipoproteins and lipids) have a radial orientation, it is difficult to understand, why a polarization of the light parallel to the fibre axis produces a strong negative and a polarization perpendicular to it a weak positive fluorescent spike (Fig. 3 in the paper of Tasaki, Hallett & Carbone 1973). The authors assume a rigid paracrystalline structure, which orients the long axis of the probe molecules parallel to the long axis of the axon. The negative fluorescence spike is ascribed to a transient fall in the quantum yield during excitation and the small positive fluorescence spike is interpreted by assuming that a small fraction of the molecules is arranged perpendicularly to the membrane. These conceptions are difficult to conciliate with the birefringence studies, where the radial orientation of the birefringent elements in the axon membrane is the dominant structure.

Another approach is the spectral analysis of the emitted fluorescent light. A squid giant axon stained with 2′6′-TNS shows a rather broad spectrum of fluorescent light from 400–500 nm, with a maximum at 440 nm, stained with 2′6′-ANS the spectrum is similar, but somewhat sharper. During the optical spike there is a transient shift towards shorter wave lengths and a sharpening of the spectrum, especially with 2′6′-ANS and the maximum is at 410–320 nm (Tasaki, Carbone, Sisco & Singer 1973).

The interpretation and evaluation of all these findings with fluorescent probes is difficult. There are at least four almost trivial possibilities to explain the fluorescent optical spike: (*a*) the membrane potential has a direct effect on fluorescent emission; (*b*) the dyes have dipole moments and tend to aline in a changing potential gradient; (*c*) concentration change of the dye during excitation; (*d*) electrophoresis of the dye into another environment. But there remains also the possibility that some of the changes are really related to conformational changes in the membrane, *triggering* the permeability changes in the sodium- and potassium-channels. Further experiments in fluorescence and the use of new probes seem necessary in this field!

Looking forward

The discovery and the start of the studies on the optical spikes (light scattering, birefringence and fluorescence) was full of hopes. It was felt that this new approach might bring us new insight into the secret nature of the remarkable sequential conductivity changes for Na^+ and K^+ ions, producing the electrical spike. The study of the optical pulse responses revealed the almost complete potential-dependence of the observed optical spikes and cooled the early enthusiasm considerably. The extreme smallness of the optical spikes made it necessary to feed hundreds and thousands of sweeps into averaging computers, with the risk that details of the time relations get lost. At this moment new hope arises: it is now possible to record one spike in birefringence and in fluorescence and to vary rapidly the external conditions under which the nerve is working. This opens up a new approach to the fascinating problem of the structural mechanism of excitation. Research always develops in waves – let us hope that a new wave opens up new horizons.

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Discussion

R. D. KEYNES

As Professor von Muralt has explained, the change in light intensity seen when birefringence is being measured is negative during the conducted action potential in crab and squid nerve, but in his elegant records from pike nerve it is positive. This does not arise from any difference in the fundamental nature of the molecular reorientations in the membrane, which in every case cause an increase of negative retardation, but is simply a consequence of the fact that the net resting retardation is positive with reference to the longitudinal fibre axis in crab and squid nerve, and negative in pike nerve. I should like to take this opportunity of presenting an explanation for an unexpected observation that I made with Professor J. M. Ritchie and Professor L. B. Cohen a year or two ago, to the effect that in gar nerve the sign of the light intensity change reverses with temperature. Figure 4 shows that this is due to a reversal of the net resting retardation, which is negative as in pike nerve at low temperatures, but positive as in crab nerve at room temperature. In pike the net retardation remained negative at all temperatures, while in crab and squid it is always positive. The origin of these differences in resting retardation has not been investigated, but they could be explained by a species variation

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in the relative contributions of negative intrinsic birefringence from the membrane and positive intrinsic or form birefringence from other unidentified sources, the amount of membrane being greatest in gar and pike nerve.

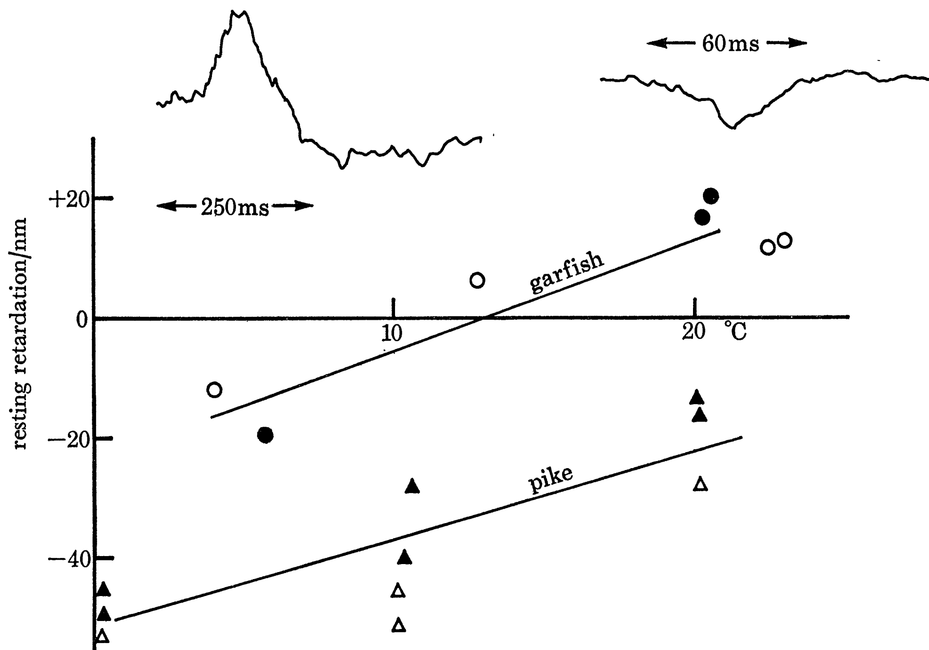

FIGURE 4. Some measurements of the effect of ambient temperature on the net optical retardation of olfactory nerves from gar and pike. The tracings above show the optical spike recorded in one of the gar nerves at 5 and 21 °C.

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