Fluorescence Changes in Nerve
Induced by Stimulation

Their relation to protein configuration

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ABSTRACT It was previously assumed, on the basis of changes in the ultraviolet absorption spectrum and of increase in ionizable sulfhydryl groups, that during excitation the proteins of excitable structures undergo some structural rearrangements, and these rearrangements may be similar to those designated by the term transconformation. In the present experiments, it was observed that electrical stimulation of peripheral nerves from rat, guinea pig, frog, and crab causes a decrease in their fluorescence. The peaks of the emission and activation spectra correspond to those attributed to proteins. Denaturing agents, such as urea, were also found to decrease the fluorescence of nerve extracts. It is, therefore, probable that the decrease in fluorescence, associated with the excited state, is due to a change in the configuration of the nerve proteins. The fluorescent method is applicable not only to tissue extracts but allows the observation of surviving nerve fibers before, during, and after stimulation. It showed that fluorescence of the fibers decreases invariably during stimulation and tends to return to the control level during restoration. The reduction in fluorescence is quantitatively related to the number of stimuli received by the nerve.

It was reported earlier that electrical stimulation of nerve tissue gives rise to structural rearrangements in certain protein constituents. This conclusion was based on a shift of the ultraviolet spectrum (1) and subsequently supported by the results of amperometric titration of sulfhydryl groups (2). Our observations were confirmed in other laboratories by means of the same (3, 4) or different methods (5, 6).

We felt, however, that the physiological significance of these observations, made in extracts of nerve tissue frozen in the resting or active state, would remain doubtful unless they are confirmed in living structures. Such a confirmation became possible, thanks to recent observations on the fluorescence
properties of proteins. The present paper deals with fluorescence changes observed in stimulated nerves which support our earlier conclusion.

**METHODS**

Fluorescence studies in proteins are of recent date (7–10) and their conclusions are still tentative. It is known, however, that protein fluorescence is due to the presence of tryptophan and tyrosine. Proteins which contain both amino acids emit fluorescence at the wavelength of tryptophan. It seems also that either amino acid can quench the fluorescence of the other, suggesting intramolecular transfer of energy between side groups. For a discussion of the problem of protein fluorescence, see Udenfriend (11).

The influence of denaturation on the fluorescence of proteins is not well known. When treated with 6 to 8 M urea, some proteins (trypsin, chymotrypsin) show an increase while others (albumins, lactic dehydrogenase) a decrease in fluorescence (10).

In the present experiments, fluorescence was measured by means of an Aminco-Bowman spectrophotofluorometer. In the studies with extracts, the nerve was frozen in the resting or excited state, as described previously (1). The frozen tissue was weighed, homogenized in Ringer's solution, and centrifuged at 12,000 × G for 30 minutes. These operations were done between 0 and 4°C. The supernatant was then diluted with Ringer's solution so that each milliliter was the equivalent of 5 mg of wet tissue. The portion of the nerve which had been in contact with the electrodes was not included in the extract.

Fluorescence readings were done at slit arrangement 4. The exciting wavelength of 280 mμ was used for determining the fluorescence spectrum and the emission wavelength of 345 mμ served for obtaining the activation spectrum. Since the protein content of individual nerves showed slight variations, the fluorescence values were corrected accordingly. When urea was used, the extract was divided into two samples: one was diluted with Ringer's solution, the other with 6 M urea dissolved in Ringer's.

Absolute values of fluorescence intensity have admittedly little significance because they are influenced by technical factors inherent in the instrument used and by other conditions such as temperature, absorption, scattering, quenching, etc. The spectra, therefore, are given on a relative scale in which the emission or activation peak is taken as 1.0. In experiments with stimulated nerves, the relative fluorescence is given in comparison with that of the resting nerve whose fluorescence is taken as 1.0. In a few cases, the actual instrumental readings will be given to show the intensity of the fluorescence measured in these experiments.

When fluorescence was measured in living nerve fibers, the nerves (sciatic of *Rana pipiens*, guinea pig, or rat, leg nerve of *Carcinus maenas*) were dissected out and placed in the appropriate Ringer's solution. At one end of the nerve the fibers were teased apart with a fine needle in order to make the greatest possible number of fibers accessible to the activating light. The nerve was placed into a quartz micro-cuvette of the fluorometer, the other end being attached to a pair of platinum electrodes as shown in Fig. 1. The preparation was allowed to rest for 30 to 60 minutes before the first fluorescence measurement. Subsequent measurements were made at
30 minute intervals for a total duration of 5 hours. Readings were made rapidly, so as to reduce exposure to ultraviolet light to a minimum. In the intervals between readings, the preparation was removed from the instrument and kept at room temperature.

Fluorescence was measured at the activation and emission peaks indicated above. None of the recommended slit arrangements proved entirely satisfactory and the optimum combination was found by trial and error. A certain amount of scattering was inevitable but this has no significant influence on the results since each nerve was its own control. Variation in readings due to the instability of the instrument was accounted for by means of a standard solution fluorescing at the same wavelength and read together with the experimental samples. Absolute fluorescence intensity has even less meaning in these experiments than in those involving extracts. The amount of axoplasm exposed to the activating light varies with the extent to which the fibers have been teased apart and this is obviously an uncontrolled variable. The results are expressed, therefore, in terms of relative fluorescence; the mean value obtained during the first hour being taken as 1.0.

Nerves were stimulated by a Grass stimulator supplying 10 volt square waves of 1 msec. duration at the specified frequencies. The efficacy of the stimuli was verified on the nerve in situ with the innervated muscles attached.

RESULTS

Nerve Extracts Spectra obtained with rat, guinea pig, frog, and crab nerve extracts showed maximum fluorescence between 340 and 345 mμ and maximum activation between 275 and 280 mμ. When the extract was treated with 6 M urea for 2 hours, the peaks decreased significantly. Fig. 2 shows the spectra of frog nerve extracts. Substantially the same results were observed in the other species.

Decreased fluorescence was also observed in extracts of nerves stimulated for 2 hours at 180 pulses per sec., as shown in Fig. 3. The decrease, as com-
pared with that of resting nerves, was even more marked than after urea treatment.

Extracts were also prepared from nerves submitted to stimulation for varying times and at varying frequencies. Fig. 4 shows that the reduction in fluorescence is quantitatively but not linearly related to the number of impulses received by the nerve. The mean of the actual instrumental readings of the six control values was $48 \pm 9$ (SD).

![Fluorescence and Activation Spectra](image)

**Figure 2.** Emission and activation spectra of frog sciatic nerve extract. Abscissa, wavelength; ordinate, relative fluorescence (peak of control sample = 1.0). Upper lines, normal, resting nerve extract in Ringer's solution; lower lines, the same extract 2 hours after addition of urea at the total concentration of 6 M.

**Living Nerve Fibers** Fluorescence measurements were made in living nerve fibers by the technique described above. The results obtained in frog sciatic nerve are summarized in Fig. 5. All measurements were done at the fluorescent wavelength of 345 m\(\mu\) with excitation at 280 m\(\mu\) which in preliminary experiments had been found to yield the peak values.

It is seen that without electrical stimulation (curve C) there are slight fluctuations during the 5 hours of observation but no definite downward or upward trend. In the other series of experiments, the first hour of rest was followed by a 2 hour period of stimulation and a further 2 hours were allowed for recovery.

Each point of the figure represents the mean value of six experiments. Stimulation was always followed by a reduction in fluorescence, the rate and the extent of which were roughly related to the frequency, at least up to 180
pulses per sec. It is also clear that the fluorescence tends to return to the initial level when stimulation is stopped.

It should be emphasized that, for reasons mentioned above, these results

![Graph of fluorescence changes in nerve](image)

**Figure 3.** Emission and activation spectra of resting and stimulated frog sciatic nerve extracts. Abscissa, wave length; ordinate, relative fluorescence (peak of resting sample = 1.0). Upper lines, extract from resting nerve; lower lines, extract from nerves stimulated at 10 v, 180 pulses per sec. for 2 hours.

![Graph of reduction in fluorescence](image)

**Figure 4.** Reduction in fluorescence as a function of the duration and frequency of stimulation. Abscissa, at left, duration of stimulus at constant frequency of 60 pulses per sec.; at right, frequency of stimulus at constant duration of 1 hour. Ordinate, relative fluorescence (emission of resting nerve = 1.0). In all measurements, the activating wavelength was 280 m\(\mu\) and the fluorescent wavelength was 345 m\(\mu\). Guinea pig sciatic nerves.
FIGURE 5. Effect of stimulation on the fluorescence of living frog nerve fibers. Abscissa, time in minutes (the dark block indicates stimulation at the stated frequency); ordinate, relative fluorescence (mean fluorescence during the 60 minute rest period = 1.0). At left, non-stimulated control nerves (C), 30 and 60 pulses; at right, 100, 180, and 240 pulses per sec. Each point represents the mean value of six experiments.

FIGURE 6. Effect of stimulation on the fluorescence of living crab nerve fibers. Abscissa, time in minutes (the dark block indicates stimulation at the stated frequency); ordinate, relative fluorescence.
are valid only if each nerve is considered as its own control. The mean value of the first instrumental reading in all thirty-six experiments is $55 \pm 19$ (SD) showing the variability between individual nerves. In terms of relative fluorescence, however (if the mean of the first three measurements is taken at 1.0), the results are much more consistent. In the six experiments in which the nerves were observed for 5 hours without stimulation, the mean of sixty-six measurements was $0.997 \pm 0.072$ (SD). The lowest value obtained after 2 hours' stimulation with 180 pulses per sec., was $0.66 \pm 0.09$ (SD). The most significant fact is that, under the influence of stimulation, fluorescence was reduced in each of the thirty experiments without exception.

Similar results were obtained with crab nerve, as shown in Fig. 6; stimulation at 30 pulses per sec. was more effective than at 60. In mammalian fibers, the results were less consistent than those obtained with extracts. This may be due to the stricter oxygen and temperature requirements which cannot be fulfilled under the present experimental conditions.

Fig. 7 shows the fluorescence decrease as a function of the total number of impulses sent through the nerve. It supplies a comparison between the results obtained in extracts and intact fibers.

**DISCUSSION**

According to our present knowledge of protein fluorescence, the changes just described can be interpreted as indicating a rearrangement of protein molecules in the direction of a less orderly structure. This interpretation is sup-
ported by the position of the activation and emission peaks which suggests
that the fluorescence of nerves is due primarily to their proteins. With
the exception of free tryptophan, no other material known to be present in nerve
has exactly the same characteristics. Purine bases whose activation peaks are
in the 270 to 280 m\(\mu\) range, emit at higher wavelengths than do the proteins.
To ascertain that the fluorescence of nerve extracts was not due to free trypto-
phan or other small molecules, the extracts were dialyzed for 18 hours against
Ringer's solution. The fluorescent properties of the solution, when corrected
for the volume changes, were not significantly different from those of the
original, non-dialyzed extract.

The similarity between the effect of the denaturing agent, urea, and that
elicited by stimulation is a good indication of the direction of the changes
occurring in the proteins. By comparing Fig. 2 with Fig. 3, it is obvious, how-
ever, that the two effects, although similar, are not identical. The crossing of
the spectrum at 365 m\(\mu\), seen with urea-treated extracts, was observed con-
sistently in all experiments and in all species. It was absent in all stimulation
experiments. The term denaturation, therefore, is inadequate to characterize
the protein changes associated with the excited state. The word transcon-
formation proposed by Lumry and Eyring (12) is probably more appropriate
to designate those reversible alterations in the secondary and tertiary structure
of the proteins which are associated with enzyme action, transport phenomena,
and the reception and storage of information.

The fluorescence method has allowed us to show that transconformation,
previously shown in extracts, does probably occur in living systems. Further-
more, the remarkable agreement between the results obtained in extracts and
in living fibers, as shown in Fig. 7, justifies the use of extracts in the biochemi-
cal study of excitation.

It should be noted that in the surviving preparation, the fluorescent ma-
terial remained inside the fibers. When, at the end of the experiment, the
nerve was removed and the bathing solution examined, its fluorescence was
not significantly different from that of a Ringer's solution.

Compared with the other methods used previously in extracts, the fluores-
cent technique is relatively insensitive. Ultraviolet spectrophotometry (1) has
allowed detection of significant changes after 10^8 impulses and titration of
sulfhydryl groups after 2 to 3 \(\times\) 10^4 impulses. Decrease in fluorescence became
significant only when the number of stimuli approached 10^8. Another dis-
advantage of the method, as used in whole fibers, is the possibility of inflicting
damage on the excitable elements by prolonged or repeated exposure to
ultraviolet radiation. This consideration probably limits the application of
the method.

It is possible, however, that fluorescence measurements, especially if they
are linked with polarization studies, will give us some insight into the energy
transfer processes which take place during excitation and the role played in them by proteins.

Received for publication, May 15, 1962.

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