ELECTRIC-FIELD-INDUCED SHIFTS IN THE INFRARED SPECTRUM OF CONDUCTING NERVE AXONS

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ABSTRACT Difference spectra between resting and excited nerve in the infrared region between 2000 and 1000 cm^{-1} have been examined with a resolution of 0.5 cm^{-1}. Spectra were obtained with a modified Perkin-Elmer model 521 grating infrared spectrophotometer (Perkin-Elmer Corp., Instrument Div., Norwalk, Conn.), and the signal-to-noise (S/N) ratio was improved by time averaging and digital smoothing. Peaks occurring in the regions around 1030 and 1066 cm^{-1} are identified as P–O–C stretch, at 1410–1414 cm^{-1} as C–H deformation, and at 1750 cm^{-1} as carbonyl stretch. The difference peaks appear to be due to a shift of about 1 cm^{-1} in the absorption band to lower frequencies for the three lower frequency bands and to a higher frequency for 1750 cm^{-1} band. Since the difference peaks appear when the nerve is modulated by a propagated action potential it is concluded that the changing electrical field across the nerve membrane is perturbing the absorption spectrum. From evidence presented it appears likely that these difference peaks are due to phospholipids in the nerve membrane and that they may be related to conformational changes associated with membrane permeability.

INTRODUCTION

The mechanism of the permeability changes that occur with excitation in nerve and muscle is presently being studied by a variety of biophysical techniques including optical scattering and birefringence (Cohen et al., 1968, 1969, 1970, 1971), fluorescent probes (Tasaki et al., 1969), nuclear magnetic resonance (Fritz and Swift, 1967), ultraviolet spectrophotometry (Makarov and Krasovitskaya, 1970), and spin label EPR (Hubbell and McConnell, 1968; Calvin et al., 1969).

A method of studying molecular conformation during excitation using infrared spectrophotometry is described here. This method is based on the difference spectra between excited and resting nerve axons and yields information about changes in molecular vibrations of molecules that occur while an action potential is being propagated. Since the infrared absorption bands can provide information on the conformation of specific molecular groups, this technique is a powerful method of

studying the molecular basis of nerve excitation. Theoretical studies by Goldman (1964) and Wei (1969) have suggested that molecular dipoles play an important role in controlling K^+ and Na^+ ion permeabilities.

Spectroscopy in the infrared is a sensitive method of investigating the influence of the resting and action potentials (which correspond to electrical field changes of 10^5 v/cm) on membrane structure and function. Perturbation of the dipole moment in membrane molecules by the electrical field can produce a shift or shape change in the infrared absorption lines. Infrared emission from nerve between 5000 and 500 cm^{-1} has been reported by Fraser and Frey (1968) and discussed theoretically by Moisescu and Mărgineanu (1970).

METHODS

Biological Preparations

The sciatic nerve of frog *Rana pipiens*, the nerves in the walking legs of lobsters *Homarus*, and the olfactory and trigeminal nerves of garfish *Lepisosteus osseus* have been used. When the pattern of stimulation was adjusted to account for the difference in conduction velocity, comparable results were observed in all preparations.

Most experiments have been performed on the sciatic nerve of frog because they were readily available. The wide distribution of fiber diameters and conduction velocities and the mixture of myelinated and nommyelinated axons have, however, introduced additional problems in interpreting the results.

The lobsters were obtained locally from a seafood distributor and were kept for as long as a week in a seawater aquarium. The garfish were caught in Lake St. Clair by the University of Guelph and the Ontario Department of Lands and Forests. This lake has been closed to commerical fishing because of the high levels of mercury found in some species of fish but the levels in garfish are not known. The possible influence of mercury on the nerves of garfish was not considered in these studies since the garfish appeared healthy and the propagated action potentials appeared normal. They were maintained for up to 2 wk in an aquarium. The isolated nerves would conduct impulses after several days when stored in refrigerated Ringer's solution as described by Easton (1971).

The nerves were removed from the animal and the connective tissue and sheath dissected away under buffered Ringer's solution appropriate for that species. They were carefully transferred to a plate on the holder which served as a window for infrared transmission. The axons were spread as evenly as possible across the area of the window which was 0.7×2.0 cm. A chamber was formed between the two plates of window material with a washer of Mylar film 16 μ thick (Dow "Saran Wrap," Dow Chemical Co., Midland, Mich.). A pair of platinum electrodes is placed at one end for stimulating and a second pair at the opposite end for monitoring the propagated action potentials. The nerve holder has been continuously developed and refined. The present model is shown in Fig. 1. The window material used between 4000 and 1100 or 1000 cm^{-1} is CaF_2 while AgCl is used between 1100 and 650 cm^{-1}. Short lifetimes of the nerve when using AgCl windows made us suspect that Ag^+ from the window was poisoning the nerve even though its solubility is low. The lifetime of the nerves between AgCl plates was extended to as long as with CaF_2 by coating the AgCl plates with a thin layer of petroleum jelly.

Most of the experiments were performed at a temperature of about 30°C as measured by a thermocouple probe. A stream of air was directed at the holder to prevent the temperature

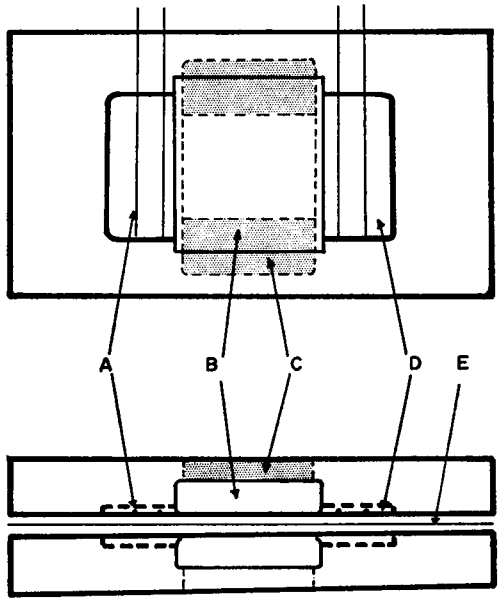

FIGURE 1 Nerve holder. The nerve holder has been designed to provide a chamber *D* at each end of the axons where the stimulating electrodes *A* are placed to avoid reducing the excitability with pressure. *B* is the window material, CaF_2, while *C* is a copper block that serves as a heat transfer plate between the constant temperature chamber (Fig. 2) and the CaF_2 plate. *E* is a Mylar washer 16 μ thick placed between the two window assemblies to provide a 16 μ chamber for the nerve in the central region. The window assemblies are made of acrylic plastic.

of the nerve in the infrared beam from rising much above this temperature. To lower the temperature of the nerve and lengthen the life of the preparation, the cooling chamber shown in Fig. 2 was constructed. The spectrometer was purged with dry nitrogen to prevent condensation on the windows.

The basic stimulus pattern was determined by the chopping period of the instrument and the response and refractory period of the axons. To obtain the maximum signal it is desirable to depolarize the axons for as much of the 38 msec chopping cycle as possible and to have the axons polarized (at rest) during the following 38 msec period. The stimulus patterns used varied from eight to one per 38 msec active period of the chopping cycle depending on temperature and type of nerve. The final choice was based on achieving the maximum signal in the spectrum. The stimulating and propagated action potentials were displayed on a double-beam oscilloscope (Tektronix 555 with types E and C plug-in vertical amplifiers, Tektronix, Inc., Beaverton, Ore.). The stimulating voltage was increased or the experiment was terminated when the amplitude of the propagated action potential fell to a predetermined level, usually 50% of the initial amplitude. The useful lifetime of the preparation varied from 30 min to 4 hr depending on the temperature, species, and the individual animal.

Description of the Spectrophotometer

The spectrophotometer (Perkin-Elmer model 521), a grating instrument covering 4000–250 cm^{-1}, was modified in order to obtain difference spectra from a single sample. The sample is

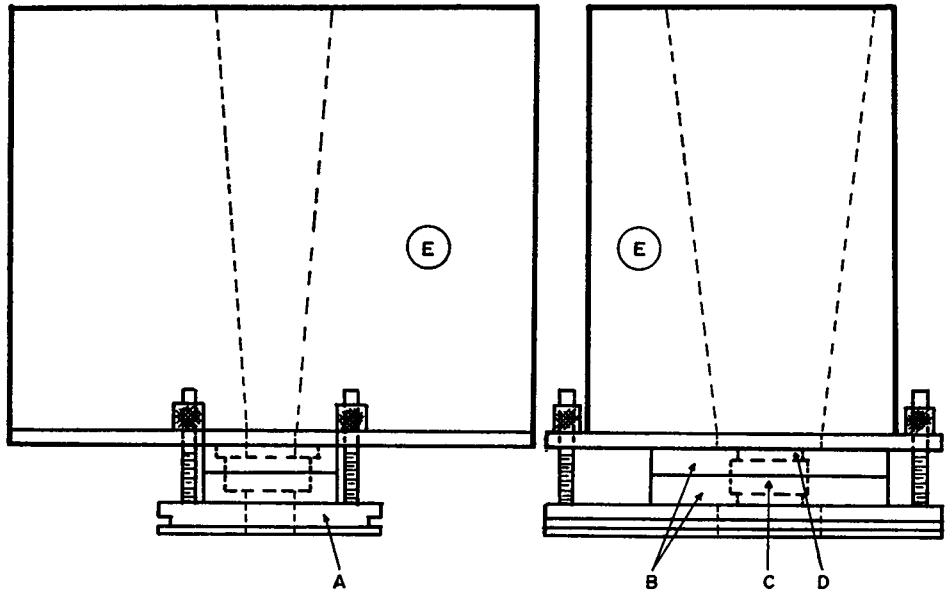

FIGURE 2 Holder and cooling chamber. The nerve holder is shown attached to the cooling chamber. *A* fits into the sample holder on the spectrometer and is made of acrylic plastic. *B* points to the window holders shown in Fig. 1. *C* is the CaF_2 window, and *D* is the copper block in contact with the cooling chamber *E*. It is insulated with foamed styrene and can be filled with ice or connected to a constant temperature circulating bath.

a bundle of nerve axons that alternately are stimulated during a period of 38 msec and rest during the following 38 msec. The difference in transmission of infrared energy at a specific wavelength was recorded between the two halves of the 76 msec chopping cycle. By adjustment of the stimulation pattern the nerves were propagating impulses across the window during one-half of the chopping cycle and were resting during the second half of the cycle. Variation of the stimulation pattern was used to maximize the response from nerves having a range of conduction velocities. A simplified block diagram of the apparatus is shown in Fig. 3.

The instrument was modified by disabling the optical wedge with the attenuator speed control and stopping the motor that drives the sector (chopping) mirror. An external 13 Hz sine wave is used to drive the synchronous demodulator and to gate the pulse generator (Argonaut LRG 051, Argonaut Associates, Inc., Beaverton, Ore.). The output signal is taken from the demodulator and is proportional to the difference in transmission between the two halves of the chopping cycle. After passing through a low-pass filter to remove high frequency noise the signal is recorded on a potentiometric recorder (Leeds and Northrup Co., North Wales, Pa., Speedomax G). For digital processing the electrical output is converted to perforated paper tape by means of an analogue to teleprinter coupler (Beckman 3108, Beckman Instruments, Inc., Fullerton, Calif.), and teleprinter (Teletype ASR-33, Teletype Corporation, Skokie, Ill.). Signal processing is done off-line on a digital computer (Digital, Equipment PDP-10/50, Digital Equipment Corp., Maynard, Mass., or Datagen Nova 1200, Data General Corp., Southboro, Mass.). The command to digitize the output at 0.2 cm^{-1} increments is controlled by a pulse derived from the wave number counter using a modifica-

tion of the method described by Peterson et al. (1966). Details of the instrumentation are described by Sherebrin and MacClement (1972).

The spectral range, line width, and amplitude in the difference spectra were not known when we started this research. For this reason a wide range of scan rates and slit widths were tried in order to achieve the best response. Because of the variability of the preparations and the noisy signal, optimization was difficult.

A study of the shift in an infrared absorption band due to an applied electric field by Handler and Aspnes (1967) showed that the change in absorption was a few parts in 10^6 and the resulting spectral shift was a few hundredths of a wave number. Using this information we correctly concluded that narrow slit widths and very slow scanning rates would be required to observe any difference spectra for nerve. Table I gives the instrument parameters which were used for most experiments.

Signal Processing

The S/N ratio of an individual scan was so low (≤ 1) that no useful information could be gained from a single scan. Standard methods of signal averaging were used with many scans to improve the S/N ratio. Elementary statistical theory shows that averaging n spectra together improves this S/N ratio by the $\sqrt{n}$. This improvement applies to the ideal case where

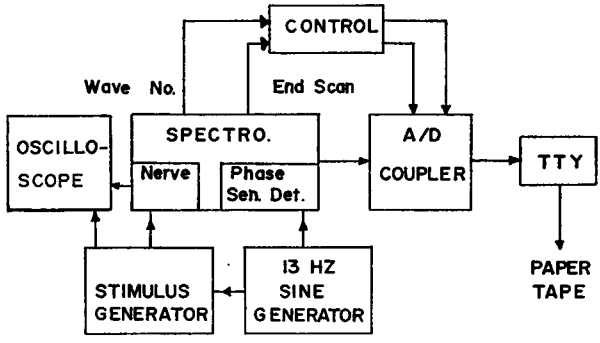

FIGURE 3. A simplified block diagram of the apparatus.

TABLE I

INSTRUMENT PARAMETERS USED IN OBTAINING SPECTRA

Frequency	Scanning rate	Mechanical slit width	Spectral slit width*
cm^{-1}	cm^{-1}/sec	μ	cm^{-1}
2000	0.17	200	3.1
1600	0.17	200	2.0
1250	0.17	300	1.7
1000	0.17	350	1.3

Time constants of recording system: 4 and 8 sec. Source current: 0.8 amp. Wave number increment for digitized spectra: 0.2 cm^{-1}.

* From data provided by Perkin-Elmer.

the signal is identical in all scans even though it is obscured by noise. The signal we describe may change slightly from scan to scan as the nerve degenerates during the course of an experiment. The preparation is not identical in thickness or area from sample to sample and therefore the absolute magnitude of the difference in transmission will vary among results that are being averaged together. Under these conditions of variability the process of time averaging can degrade as well as enhance the S/N ratio so that the spectrum must be examined at each step of the averaging process.

Digital filtering of the time-averaged spectra has also been used to improve the S/N ratio. A smoothing method using a seven-term polynomial (Savitzky and Golay, 1964) has yielded good results. Various methods of signal enhancement are discussed by Ernst (1966), and he concludes that a combination of time averaging and curve smoothing yields optimal results. Our work confirms his conclusion.

In order to perform digital calculations such as time averaging and smoothing, the signal was sampled every 0.2 cm^{-1}. Since the scanning rate was sometimes varied, the time between sampling was from 1 to 3 sec and the time constant of the low-pass filter was selected to be either 4 or 8 sec, accordingly. Because the signal was sampled at a rate that was greater than twice per time constant, some of the measurements are not completely independent. The statistical analysis takes this into account.

RESULTS

In order to test that the spectral peaks were real and not the result of chance, statistical methods were used as a guide. These methods are illustrated for the region around 1030 cm^{-1} shown in Fig. 4. Each of the two curves is the average of eight consecutive scans using frog sciatic nerve. The two sets of data were taken on separate days. Both curves show a maximum ΔT at 1030.6–1030.4 cm^{-1}. There are

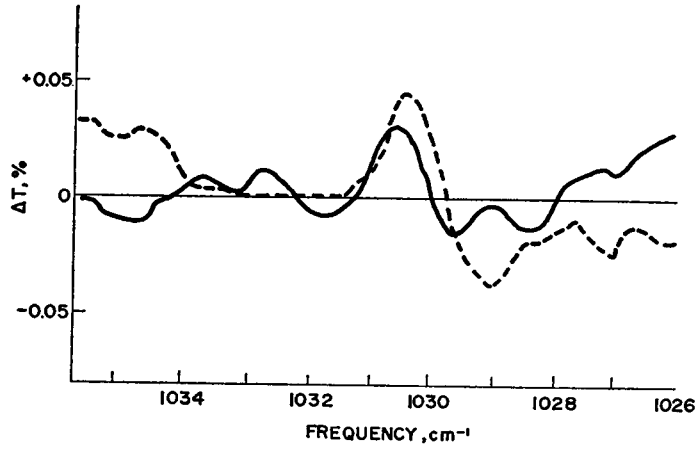

FIGURE 4 Difference spectrum, P–O–C stretch. Solid line is for eight scans of the transmission difference spectrum of frog sciatic nerve at 35°C (experiment 150471, 3–18) while the dashed line is also for eight scans done 2 days earlier (experiment 130471, 1–16). Stimulus pattern was eight pulses, each of 0.3 msec duration, separated by 4 msec, triggered once every 76 msec by the 13 Hz generator. The agreement between these independent experiments is a measure of the reproducibility.

minima at 1029.6–1029.0 cm^{-1}. The error in reproducibility of wave number is 0.25 cm^{-1} on the frequency dial of the spectrophotometer and 0.20 cm^{-1} on the digitized controller. The expected error in reproducibility of measurement is therefore about 0.5 cm^{-1} not including absolute errors in calibration.

The individual scans for the dashed curve in Fig. 4 (experiment 130471) were examined and the value of ΔT was larger in each scan at 1030.4 cm^{-1} than at 1029.0 cm^{-1}. A t test applied to the difference between the maximum and minimum shows that this difference is significant with a $P < 0.005$. Since the points of maximum and minimum value were chosen as a result of the experiment, the analysis is not entirely valid. It must be noted, however, that the shape of the spectrum contains more information than just the maximum and minimum which the t test utilizes.

Another statistical method is the F test which is used to compare the variance of the mean values of ΔT at each of the 80 points of the sampled spectrum with the variance of all the individual readings. The number of degrees of freedom was reduced since consecutive readings are not entirely independent but are related by the time constant of the measuring circuit. The results show that the spectrum for experiment 130471 differs significantly from a flat base line with $P < 0.05$. A disadvantage of the F test is that it becomes less sensitive if large regions with no signal are included in the spectrum being examined. Another weakness is its inability to take into account the shape of the spectrum.

The results quoted for experiment 130471 are better than for most other experiments. The P values are normally about 0.05 for the t test and 0.1 for the F test. Considering that the S/N ratio is estimated to be 0.3 for a single scan and that it is increased by the $\sqrt{n}$ we cannot reasonably expect values of P to be lower than 0.3 for eight scans. Using some specific assumptions about the measurement of signal and noise, Bureš et al. (1967) calculate the probability that the output belongs to a signal. Their values are given in Table II. They conclude that it is not possible to judge on the presence of a signal unless the S/N ratio is >4 but note that S/N ratio is the real ratio of powers at the time of measurement. For this reason the measured value or estimate of the S/N ratio may be in considerable error.

TABLE II

THE PROBABILITY P THAT A SIGNAL IS PRESENT WHEN THE SIGNAL-TO-NOISE RATIO IS MEASURED OR ESTIMATED

S/N	P
0	1.00
1	0.32
4	0.05
9	0.003
11	0.001

* From calculations by Bureš et al. (1967).

In order to ensure that the spectral peaks are not the results of instrumental artifacts, control experiments were done with dead nerve and with an empty holder. The S/N ratio decreased almost exactly as $1/\sqrt{n}$ when n scans were averaged (as predicted by theory for pure noise.)

A summary of the preliminary results for the spectral region between 4000 and 700 cm^{-1} has been reported (Sherebrin, 1972). Several of these bands have been investigated in further detail and are described below.

P–O–C Vibration

Peaks in the difference spectra appear in the region around 1030 and 1066 cm^{-1} and are shown in Figs. 4 and 5. We have observed these peaks in nerve from frog and garfish under a range of stimulus patterns and temperatures from 15 to 35°C.

C–H Symmetric Bend in $N–CH_3$ or $C–CH_3$

The region around 1414 cm^{-1} has been studied most intensively because of the relatively large peaks at 1410 and 1414 cm^{-1} shown in Fig. 6. Peaks from 1420 to 1405 cm^{-1} have been observed on several hundred scans using various preparations of frog, lobster, and garfish at different temperatures and rates of stimulus.

C = O Carbonyl Stretch

Fig. 7 shows the difference spectra for the carbonyl stretching frequency of the C = O group on the glyceride residue of the phosphatides.

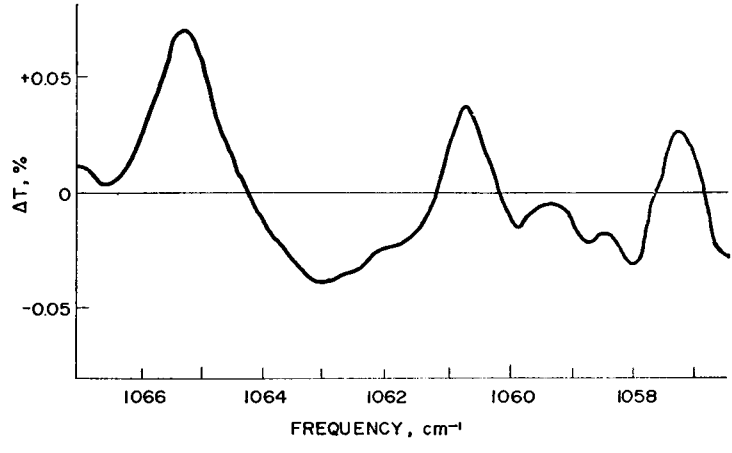

FIGURE 5 Difference spectrum, P–O–C stretch. Eight scans, unmyelinated garfish olfactory nerve (experiment 160871, 20–27). Stimulus pattern, one 40-msec pulse every 76 msec. Temperature 35°C.

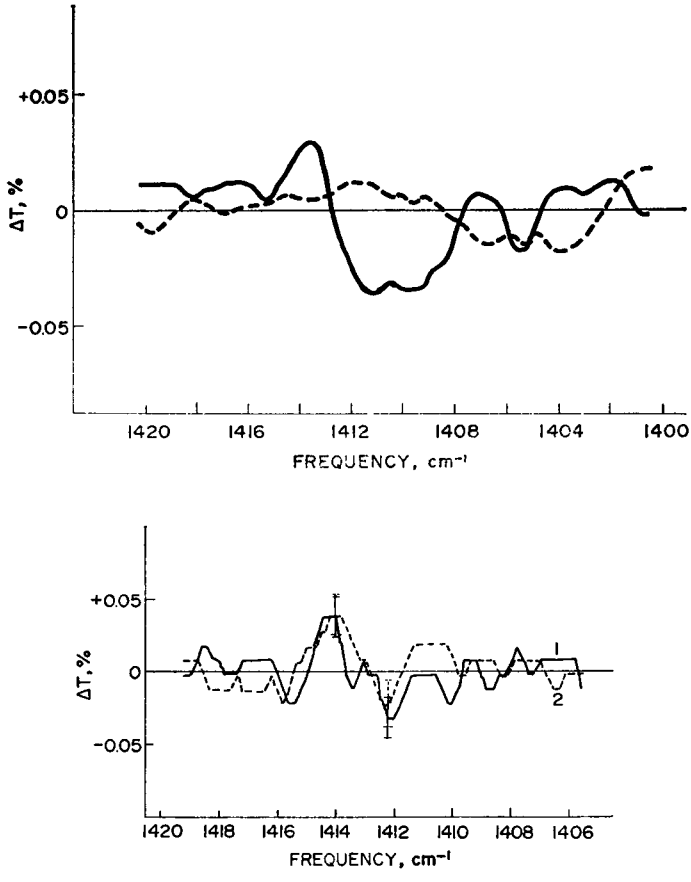

FIGURE 6 Difference spectra, C–H deformation. Curves are produced by averaging and then smoothing. (*a*) Frog sciatic nerve at 35°C (experiment 091270, 1–16). Solid line is for eight scans while stimulating with the pattern described for Fig. 4. Dashed line is for eight scans when the nerve was not stimulated. (*b*) Frog sciatic nerve at 12°C. Stimulus: one 10-msec pulse every 76 msec. Bars represent standard error of the mean. Curve 1: 36 scans done on five nerves (experiment 23-, 241071). Curve 2: 24 scans done on eight nerves experiment 09-, 10-, 11-, 140971.

DISCUSSION

The appearance of a difference spectrum depends on several factors. It can arise because of the shift of an absorption line with excitation as illustrated in Fig. 8. A narrowing of the width of a line or a change in the magnitude of the absorption can also cause a difference peak. Changes in polarization may also be implicated because the grating monochromator favors light polarized perpendicular to the slit over parallel by about 2:1. Precisely because it is a difference spectrum we see only

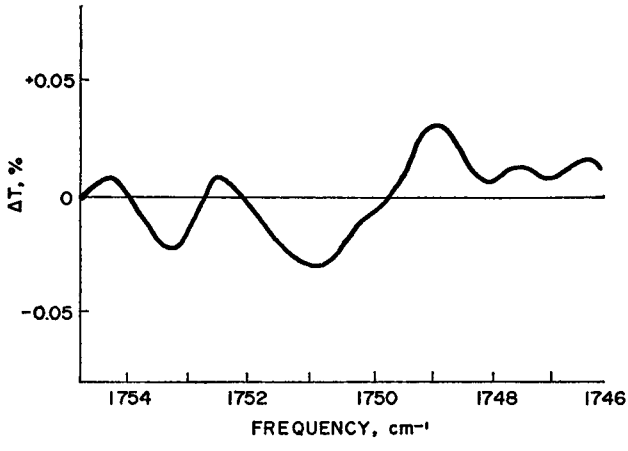

FIGURE 7

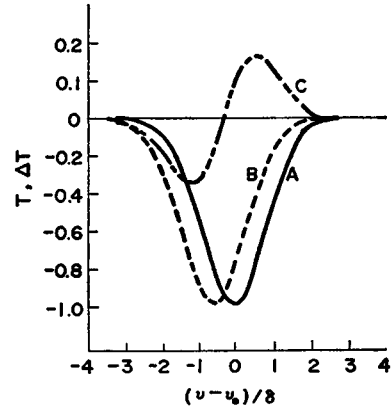

FIGURE 8

FIGURE 7 Difference spectrum, C=O stretch. Frog sciatic nerve at 35°C (experiments 130571, 140571, and 170571). Stimulus pattern is as described for Fig. 4. Transmission difference spectrum is for 16 scans averaged.

FIGURE 8 Difference peak due to a shift in absorption. Curve *A* is a gaussian absorption line, *B* is the same line shifted to lower frequencies, while *C* is the difference between *A* and *B*. The ordinate is a normalized scale for transmission *T* and change in transmission ΔT. The abcissa is a normalized deviation from the center of the absorption line. $\nu - \nu_0$ is the frequency deviation and δ is the half-line width at half-maximum intensity. Based on figures from Petrakis (1971).

those molecular bonds that are perturbed by the changing electrical field during excitation.

With this technique it is not possible to differentiate between passive effects due to the changing electrical field and active events that control the changes in membrane permeability to sodium and potassium. The 20 Hz cut-off frequency of the infrared detector precludes measuring kinetics of excitation. Several approaches are being considered to enable kinetics to be studied.

The spectral peaks in Figs. 4–7 appear to be due to a shift of the absorption line (as in Fig. 8) in various bonds of phospholipids when the membrane is depolarized. For the absorption lines at 1030, 1066, and 1414 cm^{-1}, there is an increase in transmission at higher frequencies and a decrease in transmission at lower frequencies. The opposite is true at 1750 cm^{-1}. Since higher frequencies of vibration are of higher energy, the three populations of bands at 1030, 1066, and 1414 cm^{-1} are shifting to lower energy configuration when the membrane is depolarized and the electric field is near zero. The line at 1750 cm^{-1} behaves in the opposite manner and it shifts to a higher energy configuration.

In order to determine the location of the molecular bonds in terms of specific compounds in the membrane it is necessary to examine the ordinary infrared absorption of materials found in axon membranes and also in model compounds that have similar bonds. Phosphatidylcholine and -serine, whose structures are shown in

Fig. 9, have a dipole between the phosphorus and nitrogen groups because of charge separation, and it is likely that this part of the molecule will be affected by the changing electrical field during excitation. The infrared absorption spectra of anhydrous 1,2-distearoyl-L-phosphatidylcholine has been given by Chapman et al. (1967). Several of our difference peaks as reported (Sherebrin, 1972) are in reasonable agreement with his lines but other lines are significantly different. Our calibration was done in the 1410 cm^{-1} region using the lines of water vapor and are expected to be correct to less than 1.0 cm^{-1}. The major difference is then probably due to the variation in the type of phospholipids and their interaction with other membrane components.

Let us now examine why we believe the difference curves to be due to phospholipids rather than proteins. The top diagram in Fig. 10, *N*-methylacetamide, has a peptide bond similar to that linking the amino acid residues together in a protein. The middle diagram in Fig. 10 represents a general protein where R's are amino acid chains. The C = O absorption line for peptide linkages occurs between 1690 and 1660 cm^{-1} for solutions and 1675 and 1630 cm^{-1} for solids. On the other hand, the bottom diagram of Fig. 10 represents the C = O group in a glycerol residue of

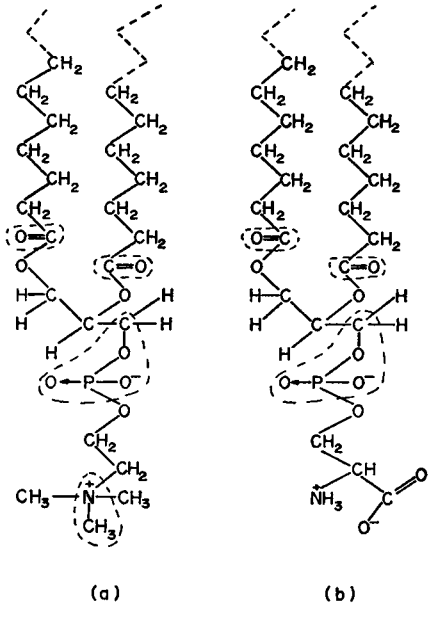

FIGURE 9

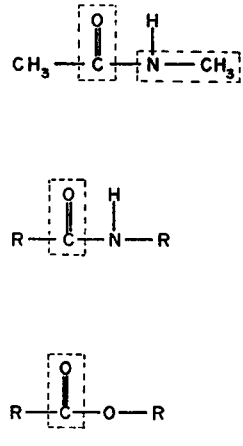

FIGURE 10

FIGURE 9 Molecular structure of (*a*) phosphatidylcholine and (*b*) phosphatidylserine. Note the charge separation between the phosphorus and nitrogen atoms which gives these molecules a dipole moment.

FIGURE 10 Structures of model compounds. Top, model compound containing a peptide bond; middle, a protein where R groups are amino acid chains; bottom, a carbonyl group in a glyceride residue.

a phosphatide. The range of the C = O absorption for this group is 1735–1750 cm^{-1}. From this evidence it appears that the C = O difference peak at 1751 cm^{-1} is due to a bond in the phospholipid rather than in protein. An absorption line near this frequency has also been reported by Davies and Thomas (1955) for brain tissue.

The line at 1414 cm^{-1} is almost certainly due to a symmetric bend deformation mode of C–H in a $\overset{+}{N}$–CH_3 group. Referring again to the top diagram in Fig. 10 and the lower part of Fig. 9 *a*, we see that this group occurs in both phosphatidylcholine and protein peptide bonds. Since, however, the positive charge may not necessarily be separated from the negative in the protein, it is reasonable to assign the difference peak to the phospholipid and leave the question of the protein open at this time.

The P–O–C stretching modes at 1030 and 1066 cm^{-1} are probably due to phospholipids, because the phosphorus atom appears at one end of the dipole. In basic studies of these modes of vibration in the compound *o,o*-dimethylphosphorochloridiothioate, Nyquist and Muelder (1968) discuss band assignments in the 1020–1080 cm^{-1} region. They assign the line near 1050 cm^{-1} to the symmetrical stretch of $P(-O-C)_2$ and the line near 1035 to antisymmetrical stretch of the same group. The phospholipids in nerve have the skeleton $(-CH_2-O-P) = O$ which may account for the differences in frequency. Until more is known about the molecules in nerve and their absorption spectra it is difficult to discuss how perturbation by a change in electrical field would alter the modes of vibration.

The investigation in pure chemistry and physics of electrical-field–induced shifts in optical, ultraviolet, and infrared absorption is relatively recent (Handler and Aspnes, 1967; Hochstrasser and Noe, 1968; Cardona, 1969; May et al., 1970; Bücher et al., 1969). The classical result is the line splitting observed in gases known as the Stark effect. The use of electrical fields in spectroscopy for probing biological structures has considerable promise. A great deal of work remains to be done on the four difference peaks discussed here to extract all the available information from them.

CONCLUSIONS

(*a*) Infrared difference spectra have been observed between nerve in the active and resting stages. The shape of each difference peak appears to be due to a shift in absorption band on the order of 1 cm^{-1}.

(*b*) The difference spectra are related to changes in nerve that occur with the propagation of an action potential. The voltage clamp studies of the last 20 years have shown that the permeability of the membrane is a function of transmembrane potential (electrical field). It is also known that the membrane contains phospholipids with a net dipole moment. From this information it is concluded that the difference peaks observed are related to the electrical field across the nerve membrane.

(*c*) The frequencies at which we have observed difference peaks correspond to

absorption lines for molecular groups found in molecules that form the nerve membrane.

(*d*) The molecules that share these group frequencies are phospholipids which are found in high concentration in the nerve membrane. The contribution of protein to the difference spectra must await more detailed investigation.

(*e*) Difference peaks have been assigned to carbonyl stretching (1750 cm^{-1}), CH_3 symmetric deformation (1414 cm^{-1}), and P–O–C stretching (1030, 1066 cm^{-1}) in phospholipid molecules.

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