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Peter Gwan Pa Ang
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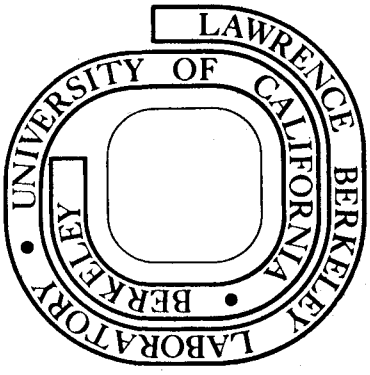

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ELECTRO-OPTIC SPECTROSCOPY AND CHEMICAL MODIFICATION OF NERVE AXON MEMBRANES

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This work was done with support from the U.S. Energy Research and Development Administration.

ELECTRO-OPTIC SPECTROSCOPY AND CHEMICAL MODIFICATION OF NERVE AXON MEMBRANES

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ABSTRACT

The optical retardation or birefringence of the nerve responds to perturbation of the membrane potential as detected from the changes in the intensity of polarized light transmitted through the nerve. The technique developed for separating the total transmitted light intensity changes into a pure retardation component and a pure forward direction scattering component involves introducing a quarter-wave plate into the optical system. The absolute magnitude of the retardation change is calculated from the geometric average of the relative light intensity changes at two different orientations of the phase plate. The signal to noise ratio is improved by the use of a computer averaging system. Transient changes in the phase angle of the optical retardation of the order of 10^{-5} radian can be detected.

By applying positive (depolarizing) and negative (hyperpolarizing) voltage pulses (50 mV) through two pairs of external electrodes on a small bundle of lobster walking leg nerves, the membrane potential is perturbed transiently. The resting potential is normally about -70 mV, which corresponds to an electric field of about 10^5 V/cm across the membrane. An attempt is made to investigate the molecular structures that may be responsible for the voltage dependent retardation change, $\Delta\Theta$. The magnitudes of $\Delta\Theta$ as well as the static retardation Θ_o, before and after a chemical modification, are compared.

Increasing the concentration of potassium and/or calcium results in a decrease of $\Delta\Theta$ and Θ_o. In calcium-free medium lowering the pH results in an increase of $\Delta\Theta$ with little effect on Θ_o. However, if calcium is present in the medium this pH induced increase of $\Delta\Theta$ is only small. This is then followed in time by a rapid decrease of both $\Delta\Theta$ and Θ_o. Treatment with high pH (above pH 10.5), phenylglyoxal, and protein denaturants ($HgCl_2$, N-Ethylmaleimide, para chloro mercuribenzoate) reduce both $\Delta\Theta$ and Θ_o. Glutaraldehyde also reduces $\Delta\Theta$ significantly but increases Θ_o slightly. Uranyl ion, which binds strongly to the phosphate head group of phospholipids, does not have any effect on $\Delta\Theta$ and Θ_o. Other phospholipid modifiers, including phospholipases and detergents, reduce $\Delta\Theta$ with relatively little or no effect on Θ_o. The decrease of $\Delta\Theta$ in this case could be due to a general disruption of the membrane structure.

Among the cholinergic compounds, acetylcholine has an interesting effect. In the regular saline solution it strongly reduces $\Delta\Theta$ and Θ_o. However, in calcium-free saline it increases $\Delta\Theta$ with no effect on Θ_o. thus resembling the effect of low pH. Other cholinergic compounds bearing quarternary amines have no significant effect on the optical retardation. Eserine and nicotine decrease $\Delta\Theta$ quite strongly in the regular saline. However, in calcium-free solution they increase $\Delta\Theta$ with relatively little effect on Θ_o.

Local anesthetics and barbital sodium are able to induce a large increase in $\Delta\Theta$ and Θ_o, especially in the calcium-free medium. The increase in Θ_o indicates a loss of the negative retardation component of the nerve, which is likely caused by the disordering effect of the drugs on the lipid structures. The increase of $\Delta\Theta$ is believed to be

due to an increase in the fluidity of the membrane. In the regular saline solution calcium usually interferes and causes a rapid decrease of both $\Delta\Theta$ and Θ_o.

Ouabain, an ATPase inhibitor, gradually increases $\Delta\Theta$ with little effect on Θ_o. ATP itself has relatively little effect.

The increase of $\Delta\Theta$ induced by eserine and nicotine suggests the involvement of a cholinergic binding macromolecule, located at the internal surface of the membrane. Some other drugs, e.g. ouabain and local anesthetics may also interact with it. It is suggested that $\Delta\Theta$ may be associated with an electric field induced displacement of a positively charged guanidinium group that is a part of the macromolecule.

Melvin Calvin

DEDICATION

To my parents.

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ELECTRO-OPTIC SPECTROSCOPY AND CHEMICAL MODIFICATION OF NERVE AXON MEMBRANES

CONTENTS

CONTENTS Continued)

I. INTRODUCTION

The electrophysiological characteristics of ion-permeability control in excitable membranes are becoming increasingly clear. The macroscopic phenomena accompanying the action potential in neural membranes have been described in detail for some time now. Membrane currents of voltage-clamped squid giant axons have been analyzed by many investigators (Hodgkin and Huxley, 1952; Cole, 1968; Armstrong and Bezanilla, 1974). However, the molecular mechanisms responsible for these macroscopic events are still vague. The identification of the biochemical constituents involved remains relatively unexplored. The discovery of pharmacological agents, most notably tetrodotoxin and tetraethylammonium ion, that block either the inward or the outward current with a very high degree of selectivity, have greatly aided the electrophysiological studies. There are many other drugs, e.g. local anesthetics, that are able to affect both the inward and outward currents. The chemical interaction of all these compounds with excitable membranes is, however, not well defined. To this aspect, spectroscopical experiments may complement the electrophysiological studies of nerves.

In this thesis the electro-optical properties of nerves are investigated. The birefringence or optical retardation changes of nerves have been reported by several groups (Tasaki et al., 1968; Cohen et al., 1968-1973; Berestovsky et al., 1969; Sato et al., 1973; Kaplan, 1972; Kaplan and Klein, 1974; Watanabe et al., 1973; Ang and Klein, 1974 ; Von Muralt, 1975). The source of the birefringence change is still obscure. The axon has many different components and it is therefore of interest to identify the molecular species that are involved in the

retardation changes. The research described in this thesis involves chemical modifications of the lobster nerves. The resulting changes in their optical retardation characteristics are examined.

This thesis is divided into several parts. The first part contains a brief description of excitable membranes. Nerve and artificial membrane models are discussed in some detail. A review of the electrophysiological and spectroscopical experiments on nerve is also presented. The technique of electro-optic spectroscopy is presented in detail. The use of a quarter-wave plate in order to improve the signal to noise ratio and to calculate the static retardation as well as the magnitude of the field induced retardation changes is explained.

Data are presented, showing the effects of treating the axon with different cation compositions (variation of potassium, calcium and pH), protein reagents, phospholipid modifiers, cholinergic compounds, local anesthetics, barbiturates and ATPase modifiers. The results are discussed with respect to the various mechanisms that can be postulated to explain the changes in the optical retardation properties of the nerves.

The results of a few voltage-clamp experiments performed at the Marine Biological Laboratory in Woods Hole, Massachusetts are included in the appendix.

II. EXCITABLE MEMBRANES

A. Electrophysiology

A neuron, or a nerve cell may be generally described as having these basic functional units: the soma, the dendrites, the axon and the synaptic endings. The soma, or perikaryon, in many respects resembles the cell bodies of less specialized cells. The unique function of the neuron, that of transmitting information from one part of the organism to another, is made possible by the unique nature of the branches that extend from the soma. These are of two types, the dendrites and the axon. Dendrites are relatively short structures found on all neuronal cell bodies. They serve as sites for incoming information and they have many synaptic connections with other neurons. The axon serves as a self amplifying cable for long distance intracellular communication. A voltage spike or an action potential travels the length of the axon until it reaches the terminal, where the axon makes synaptic contacts with another nerve or a muscle.

All living cells possess a membrane. Membranes are generally composed of proteins, phospholipids, cholesterols, carbohydrates, and also combinations of these as glycoproteins and glycolipids. The nerve axon can be simply regarded as a long cylinder of plasma membrane with electrolyte solutions of differing ionic compositions on the inside and outside. The ionic concentration gradient across the membrane is maintained by a chemical energy-dependent selective active transport of Na^+ ions outward and K^+ ions inward. This Na^+-K^+ pumping is not related directly to excitability and will not be discussed in further detail.

The interior of an axon is an aqueous electrolytic gel having approximately the following ionic concentration: (Na^+)= 60 mM, (K^+)=400 mM, (Cl^-)= 40-100 mM, and other anions such as isothionate and aspartate in concentrations yielding an electroneutral balance. The axoplasm contains an extensive network of axially oriented proteinaceous neurotubules and neurofilaments (Metuzals and Izzard, 1969). The bulk of the axoplasm is apparently not directly involved in action potential propagation, since most of it may be squeezed out of a squid giant axon and replaced with electrolyte (such as potassium fluoride) without detrimental effect (see review by Gilbert, 1971). The exterior saline solution concentrations are approximately: (Na^+)= 460 mM, (K^+)= 10 mM, (Ca^{++})= 50 mM, (Mg^{++})= 10 mM and (Cl^-)= 540 mM. Between the inside and the outside of the axon one can measure a potential difference of about -70 mV, the resting membrane potential. This potential can be approximated by the Goldman-Hodgkin-Katz equation (Goldman, 1943; Hodgkin and Katz, 1949):

$$\Delta\psi = \psi_i - \psi_o = \frac{RT}{F} \ln \frac{(K^+)_o + (Na^+)_o P_{Na}/P_K + (Cl^-)_i P_{Cl}/P_K}{(K^+)_i + (Na^+)_i P_{Na}/P_K + (Cl^-)_o P_{Cl}/P_K}$$

This equation takes into account the permeability of other ions relative to the permeability of potassium. Because the resting membrane is more permeable to K^+ ions than to others, the resting membrane potential stays close to the potassium equilibrium potential. The origin of the membrane potential can be very complicated, particularly if one considers the effects of fixed charges on the membrane surface. The potential profile in the membrane and the contributions of the surface potentials to the membrane potential have not been well

established, but quite a number of investigators have applied the fixed charge theory to biological membranes (Gilbert, 1971; Ohki, 1973).

By changing the ionic composition of the media, or by passing currents across the membrane, one can depolarize or hyperpolarize the membrane potential, i.e. making it more positive or more negative respectively. A threshold potential is the membrane potential above which an all-or-none action potential is produced and below which none is produced. The action potential of the axon is a pulse of about 100 mV in amplitude and about 1 msec in duration. This self-propagating information travels along the nerve in the form of a moving region of locally depolarized membrane.

B. Models

Numerous models for the excitable membrane have been proposed. These models can be useful since they may suggest new electrophysiological, spectroscopical and biochemical tests whose results may narrow the field in model competition. The test of a mathematical or physical model for membrane excitation is not only whether it satisfies electrophysiological characteristics, but whether it makes chemical sense, i.e. it must be consistent with the biophysical and biochemical properties of the excitable membrane.

1) Hodgkin-Huxley Model (1952).

Their model was based upon the "sodium hypothesis" which states that the action potential overshoot is due to an influx of Na^+ ions during the initial phase of the action potential. Experimental support was given by their work on the giant axon of the squid using the "voltage clamp" technique. It was found that upon placing axial

current and voltage electrodes into the interior of the axon, it was possible to fix the voltage across the membrane using a network of feedback amplifiers, and then measure the resulting transmembrane currents (see also Cole, 1968). When the membrane potential was clamped to a depolarized level, an initial transient inward current, followed by a steady-state outward current was detected. The transient inward current was identified as the sodium current, since its magnitude was dependent upon the external Na^+ concentration. Experiments using radioactive tracers have shown that the steady-state outward current is due to a K^+ flux through the membrane in a low-resistance state.

The Hodgkin-Huxley model stated that the permeabilities of the membrane to Na^+ and K^+ ions are specific and independent of each other, and that these permeabilities are explicit, smooth functions of voltage and time. Ions pass through the membrane by way of "channels" or "pores" and these pores are controlled by membrane molecules which serve as "gates". These gating molecules are postulated to be charged or have dipole moments, which makes their conformation sensitive to the electric field in the membrane. The charge movement associated with changes in the electric field, or "gating current" should be measurable. Hodgkin and Huxley were unable to detect it in their experiments. However, by combining the techniques of internal perfusion, voltage clamp and signal averaging, Armstrong and Bezanilla (1974) and Keynes and Rojas (1974) were able to measure the gating current associated with the sodium channel. The gating current showed up as an outward current rising rapidly to a peak on depolarization of the membrane and then declining

exponentially to zero, followed at the end of the depolarizing pulse by an inward surge of current with a similar time course. The chemical nature of the gating molecules have not been well established.

In general, the Hodgkin-Huxley model provided a fairly complete description and analysis of the voltage clamp currents of the axon membrane along with a demonstration that the action potential could be calculated from the model formulation using the cable equation. The formulation was partly phenomenological and in a number of places detailed equations were given which were purely empirical. This model is by far the most quoted of all, and experimental results are usually phrased in terms of the model's mathematical parameters. An objection to the sodium hypothesis of the Hodgkin-Huxley theory was raised when Tasaki (1968) found that apparently normal action potentials can be generated with a divalent cation external solution and a sodium phosphate internal solution.

2) Tasaki Model (1968)

The excitable membrane of a nerve cell is considered to be composed of macromolecular complexes of proteins and phospholipids. In the physiological pH range the amphoteric nature of such macromolecules results in an excess of negative charge within the external membrane layer and also on the inner surface of the axon membrane. The charge layer makes the membrane act like an anion-impermeable cation exchanger, able to exchange equivalent amounts of cations with the electrolyte media contiguous with the membrane surfaces. Tasaki has emphasized the indispensability of divalent cations in the external medium. In the resting state anionic sites in the membrane are primarily occupied by divalent cations such as Ca^{++}.

Excitation is initiated by an outward flowing stimulating current of monovalent cations, which replace the divalent cations on the anionic sites. When a critical univalent/divalent ratio is reached, a cooperative phase transition in membrane macromolecules occurs. The membrane macromolecules are believed to have two stable states. Excitation is visualized as a phase transition of the macromolecular complex from a stable "resting" divalent cation-rich state to the depolarized, univalent cation-rich "excited" state. The phase transition results in drastic changes in membrane selectivities, increases in the densities of charged sites participating in cation exchange and increase in the water content. As a consequence the membrane conductance for all cations increases drastically and the potential change observed during the action potential ensues due to the interdiffusion of cations. Adjacent membrane patches will be similarly excited by outward flowing counter-currents to the net inward current during the excited state. Termination of the excited state occurs as a result of the inward diffusion of divalent cations and their recombination with the anionic membrane sites, returning the membrane to its low conductance resting state.

3) Changeux Model

Changeux et al. (1967) have discussed certain general aspects of the cooperative interaction between micellar units, possibly consisting of lipoprotein, arranged in a two-dimensional lattice. The treatment is general and largely phenomenological. One can show the possibility of "flip-flop" transformations between conformational states. The theory became the basis of a model by Blumenthal et. al. (1970), in which the two states are characterized by a discontinuous change

in the affinity and permeability of some ionic or molecular species.

4) Adam Model

The use of cooperative phenomena as a basis for excitability has also been developed by Adam (1968) based on a lattice of cation exchanger units which bind or release calcium competitively with sodium, thereby controlling the current flow. This also leads to a "flip-flop" system and limited attempts have been made to calculate some aspects of the voltage clamped current curves.

5) Wobschall Model

Wobschall (1968) has involved the idea of electrets of phospholipid dipole domains. This is another example of an approach based on a study of cooperative interactions. He has expressed his ideas in analytical form and has carried out some computer simulations of changes in Na^+ and K^+ conductances as functions of voltage that closely resemble those found experimentally in both action potential propagation and in voltage clamp situations.

6) Tobias Phospholipid Model

Tobias (1964) has proposed a model similar to Tasaki's. It is hypothesized that outward flowing cationic currents drive potassium from the axoplasm into the membrane where it displaces calcium from the polar carboxyl and/or phosphate groups of phosphatidylserine. The phospholipids then undergo an orientational change, inducing a conformational change in the lipoprotein membrane structure which is expressed in the electrophysiological properties of the excited state.

7) Wei Transistor Model

Another model which also places heavy emphasis on the role of oriented phospholipids is that of Wei (1969). He has suggested the use of transistor mechanisms as an analogue of membrane excitation, due to the fact that the membrane has many properties similar to those of semiconductors. Excitation is initiated by a voltage induced "flip-flop" of the oriented phospholipid head group's electrical dipoles, which lowers the potential energy barrier at the membrane surface, thus allowing the Na^+ ions to flow along their electrochemical potential gradient. A description of the K^+ fluxes is not given, nor is the role of Ca^{++} treated.

8) Goldman Model

Goldman (1964) has postulated that the ion flow control mechanism incorporates an effective dipole whose rotation under the influence of the electric field opens or closes the appropriate ports. The conformation of a macromolecule critically involved in excitation depends upon the electrostatic attraction between the singly-charged positive and negative ends of the dipole, assumed to be connected by a flexible molecular chain. These charges attract each other when no counter-ions are present. They do not interact when the negative charge is electrostatically bound to a univalent cation, and they repel when the negative charge is bound to a divalent cation. Goldman assumes that the resting potential across the membrane tends to keep the positive ends of the dipole buried in the membrane in a mode that gives preference to the binding of Ca^{++} at the anionic sites. Depolarization allows the dipole to emerge from the membrane, release Ca^{++} and bind Na^+ and then K^+ in successive conformational states.

By assigning a specific affinity for Na^+, K^+ or Ca^{++} to each of these conformations, as well as appropriate electric field-dependent rate constants for transitions between them, a mathematical model which predicts a lot of physiological data can be worked out.

9) Weiss Spring Model

Another model which assumes that lipoproteins can act as exchange sites for monovalent and divalent cations has been proposed by Weiss (1969). The model envisions the peptide portion in a spring-like helical conformation which is contracted in the resting state, with Ca^{++} bound to outer sites and K^+ bound to inner sites. An outward stimulus current displaces the bound Ca^{++}, which allows the spring to expand to a Na^+ selective conformation thereby increases its permeability. Ca^{++} diffuses back into the membrane with Na^+, causing the springs to recontract into a conformation favorable to binding and passage of K^+ ions. The K^+ current displaces Ca^{++} from the inner sites to the outer sites, and the membrane thereby reverts to the resting state.

10) Nachmansohn Acetylcholine Receptor Model

A complex, multi-reaction model involving acetylcholine as the essential trigger for excitation has been proposed by Nachmansohn (1959). His chemical hypothesis of nerve excitability has emerged from biochemical and electrophysiological studies. It is postulated that acetylcholine is present within the excitable membrane bound to a storage protein. On excitation, acetylcholine is released within the membrane and initiates a series of reactions leading to a large amplification of the normal subthreshold activity. When released

from the storage site, acetylcholine is translocated to a receptor protein, inducing a conformational change. This conformational change is assumed to release Ca^{++} ions, which are bound to the receptor in the resting condition. The released Ca^{++} ions act on the elements in the gateway, producing the changes that permit the flow of ions. The conformational change of the receptor protein is assumed to translocate acetylcholine to an acetylcholinesterase enzyme. The acetylcholine is hydrolyzed, and the receptor returns to its original conformation and again binds Ca^{++} ions; thus the barrier for ion movement is reestablished. It is assumed that the three proteins, the storage protein, the receptor protein and the enzyme protein, are interlocked in a complex which is located close to the gateway through which the ions are supposed to move during activity.

An attempt at an integral model of excitability was made recently by Neumann et al. (1973, 1974) on the basis of the classical chemical model elaborated by Nachmansohn. The integral model is an attempt to integrate much of basic data of biochemical and pharmaco-electrophysiological studies on excitable membrane. Some fundamental concepts are introduced: 1) the notion of a basic excitation unit, 2) the assumption of an acetylcholine storage site particularly sensitive to the electric field of the excitable membrane, 3) the idea of a continuous sequential translocation of acetylcholine through the cholinergic protein (acetylcholine cycle). The key processes of the model associated with excitation are formulated in terms of a series of chemical reactions. Supporting their model is evidence that acetylcholinesterase is localized in excitable membranes, and that acetylcholinesterase inhibitors block excitation in several types of nerve fibers.

In this thesis, the effects of some cholinergic compounds on the electro-optical properties of the nerves will be studied.

11) Artificial Membranes

Excitability is a membrane phenomenon, thus it is not limited only to nerves and muscles, but it can also be found in many other systems. Spike electrogenesis by chloride activation occurs normally in the fresh water algae Chara and Nitella (Gaffey and Mullins, 1958; Mullins, 1962). A protoplasmic drop isolated from an internodal cell of Nitella can become electrically excitable in a solution of appropriate electrolyte composition (Inoue et al., 1973).

Several compounds of fungal or bacterial origin, e.g. EIM (Excitability Inducing Material), alamethicin, monazomycin, can readily incorporate into preformed artificial membranes made of planar lipid bilayers, and generate voltage-dependent ion conductances (Mueller and Rudin, 1968; Mueller, 1975). Alamethicin has a complex behavior in the presence of protamine, where action potentials can be evoked. Since the kinetics for the gating mechanism in all excitable cell membranes and also in the artificial bilayers are identical, Mueller (1975) believes that the mechanism by which the membrane opens and closes for the flow of ions is essentially the same in all cases. He has proposed a model in which the gating involves the voltage-induced insertion of all or part of the translocator molecules from the membrane surface into the hydrocarbon region and their subsequent aggregation into open channels by lateral diffusion. The long axis of the translocators is assumed to span the hydrocarbon region forming the walls of the channel like the staves of a barrel. This model is attractive, since the proposed mechanism should be able to cause rather drastic changes in the optical properties of the membrane.

C. Spectroscopical Studies

To complement the classical electrophysiological studies, many investigators have attempted physical studies of excitable membranes. The goal is to detect and understand functionally related changes in the structure of axonal membranes. The physical studies in general fall into two categories, optical and magnetic resonance studies. The optical studies include the studies of changes in ultra - violet absorption (Kayushin et al., 1960; Makarov and Krasovitskaya, 1970), changes in infrared absorption (Sherebrin et al., 1972) and heat emission (Abbott, 1960; Howarth et al., 1968), changes in intrinsic fluorescence (Ungar and Romano, 1962; Landowne and Ritchie, 1971), changes in extrinsic dye fluorescence and in fluorescence polarization (Cohen et al., 1973, 1974; Tasaki et al., 1975), changes in dye absorption (Ross et al, 1974; Waggoner, 1976), changes in Rayleigh linewidths of inelastically scattered laser light (Fritz, 1975), changes in light scattering (Cohen et al., 1972a, 1972b, 1973; Tasaki et al., 1968, 1975) and changes in optical retardation (see introduction). An excellent review and discussion of many of the proposed mechanism for the optical changes is given by Cohen (1973) and also by Tasaki (1975).

Magnetic resonance spectroscopy on nerves have been attempted using the electron spin resonance (ESR) and the nuclear magnetic resonance (NMR) spectrometers. Hubbell and McConnell (1969) have done an exploratory study of the structure of a number of biological membranes, using the spin label 2,2,6,6,-tetramethylpiperidine-1-oxyl (TEMPO). The ESR spectra showed rapid tumbling of the label in nerves. They concluded that the excitable membranes contain liquid-like hydrophobic regions of low viscosity. Calvin et al. (1969) have explored the behavior of pair interaction in biradical spin labels incorporated

in nerves. The spectrum of one biradical indicated that it was dissolved in a liquid-like hydrophobic region of the nerve. The ESR spectrum did not change during action potentials, probably because the label was bound at sites where no structural changes occur during nerve excitation. Different types of spin labels have been incorporated in the nerve as local anesthetics (Giotta et al., 1973). They concluded that durations of anesthesia and ability of the drug to partition into the hydrocarbon region of the membrane are related. Another label has been attached to sulfhydryl groups of the nerve to detect chemical-induced protein conformational shift (Giotta and Wang, 1973). Further research is needed to detect potential-induced changes in the ESR spectrum. A strange ESR signal in nerves have been observed as a result of mechanical injury of the nerve (Commoner et al., 1969). The g-value depends on temperature and orientation suggesting ferromagnetism in the sample. Uninjured nerve lacks the ESR signal.

In recent years, nuclear magnetic resonance (NMR) spectroscopy has been applied to the study of nerve tissues (Fritz and Swift, 1967; Chapman and McLauchlan, 1967; Klein and Phelps, 1969; Dea, Chan and Dea, 1972). Most of these studies have been concerned with the state of water in the nerve. In addition to the strong water signals, Dea et al., (1972) have observed several relatively sharp, although weaker, resonances that could be contributed by the protons of phospholipids and cholesterol. A change in NMR spectrum of proton or other nuclei associated with the action potential of the nerve has never been reported. If this can be detected, it may reveal a lot of specific information about changes in the local environment of the membrane during excitation.

Unlike some other spectroscopical signals (extrinsic dye fluorescence, ESR) that require the incorporation of labels into the membrane, the changes in the optical retardation originate from the nerve itself. An exact interpretation of signals originating from extrinsic labels is difficult, because the binding sites of many of these labels in the nerve have not been well characterized (Cohen, 1973). In this thesis an attempt is made to investigate the molecular origin of the voltage dependent changes in the optical retardation. The changes in the electro-optical properties of the nerve following a chemical modification will be studied. The details of the technique is described in the next chapter.

III. ELECTRO-OPTIC SPECTROSCOPY

A. Optical Retardation and Birefringence

The index of refraction of a birefringent material depends upon the orientation of the plane of polarization of the traversing radiation. This type of optical anisotropy also means that the velocity of light in the medium will depend upon its polarization. In general, linearly polarized light incident on such material will have its state of polarization preserved only if the plane of oscillation of the electric field vector is oriented in either of two mutually perpendicular directions, called the principal axes. Each of these two directions is characterized by a separate index of refraction and dielectric constant. Therefore, two mutually perpendicular polarized beams which are aligned along the principal axes, and which are initially in phase upon entering the material, will emerge out of phase. The phase angle difference produced is given by

$$\Theta = \frac{2\pi d(n_x - n_y)}{\lambda}$$

The difference between the refractive indices, $n_x - n_y$, is called the birefringence of the material, and its product with the thickness, d, is the optical retardation given by

$$R = d(n_x - n_y)$$

The optical retardation can be measured using the compensation method. This is done by putting the material between two crossed polarizers and inserting a phase plate, e.g. a quarter-wave plate between one polarizer and the object. The phase plate can be used to add, subtract or compensate the optical retardation. The intensity of light transmitted through the system depends on the total optical retardation.

When the principal axes of the retarding materials are oriented at a 45° angle with respect to the polarizer axis (see Fig. 1), then the light intensity is given by (Szivessy, 1928)

$$I = I_o\, k(\sin \Theta/2)^2$$

The actual output of the photodiode that measures the total light emerging from the optical system is given by

$$I_{total} = I_o\, k(\sin\Theta/2)^2 + I_{leak} + I_{dark}$$

I_o is the intensity of the light incident upon the polarizer, k is an isotropic transmission coeffient representing losses due to absorption, light scattering, reflection etc., Θ is the phase retardation angle, I_{leak} is the voltage that is recorded due to imperfection of the crossed polarizers and I_{dark} is the voltage recorded with light off, thus representing the DC offset due to the battery in the photodiode circuit.

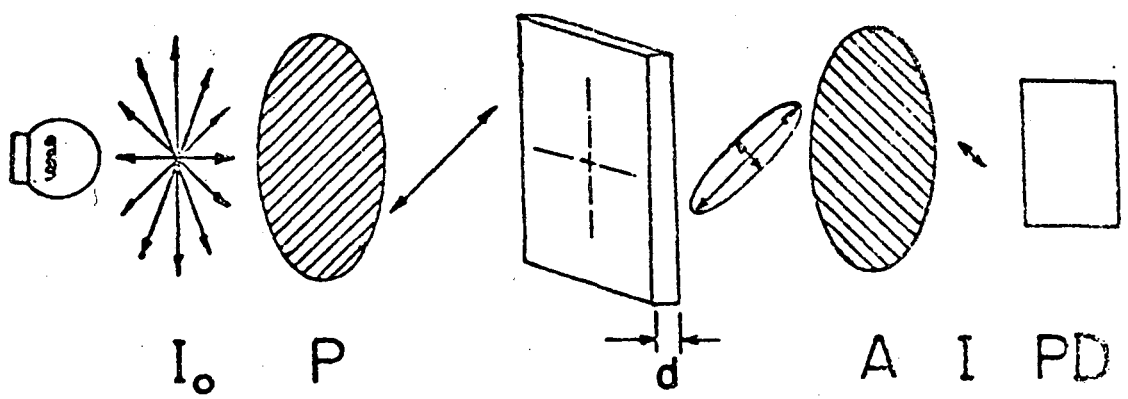


Fig. 1 Basic optical system:
I_o = intensity of light source, P = polarizer; a quarter-wave plate (not shown) can be inserted behind P, d = thickness of the retarding material, A = Analyzer. The emerging light intensity I is detected by the photodiode PD (from Kaplan, 1972, reproduced by permission).

$I_{leak} + I_{dark}$ can be measured directly when the static retardation of the object is fully compensated by the quarter-wave plate, i.e. when total $\Theta = 0$. This condition is met when the phase plate has been rotated to give minimum light transmission. At this orientation, the angle ψ between the slow axis of the quarter-wave plate and the polarizer axis, determines the static retardation Θ_o of the material, i.e.

$$\Theta_o = \left[\frac{2\pi}{\lambda} \quad \frac{\lambda \sin 2\psi}{4}\right]$$

It is possible to calculate $\Delta\Theta$, the change in the phase angle of the optical retardation, from the corresponding changes in light intensity. Kaplan and Klein (1974) have shown that differentiating the I_{total} equation yields

$$dI_{total} = dI = I_o (\sin \Theta/2)^2 \quad dk + I_o k (\sin \Theta/2) (\cos \Theta/2) \, d\Theta .$$

or, in abbreviated form

$$dI = dI_s + dI_r$$

where dI_s is the intensity change due to changes in forward direction light scattering, reflectance etc., and dI_r is the magnitude of the intensity changes due to changes in retardation. When the quarter-wave plate is oriented such that the total Θ is maximally positive at $\Theta = \Theta_o + \pi/2$ (see Fig. 2), depolarizing pulses will yield

$$dI_1(\Theta = \Theta_o + \pi/2) = dI_s + dI_r$$

and when the total Θ is made negative by rotating the quarter-wave plate, the intensity change is

$$dI_2(\Theta = \Theta_o - \pi/2) = dI_s - dI_r$$

It is easy to show that

$$dI_r(\Theta = \Theta_o + \pi/2) = - dI_r(\Theta = \Theta_o - \pi/2)$$

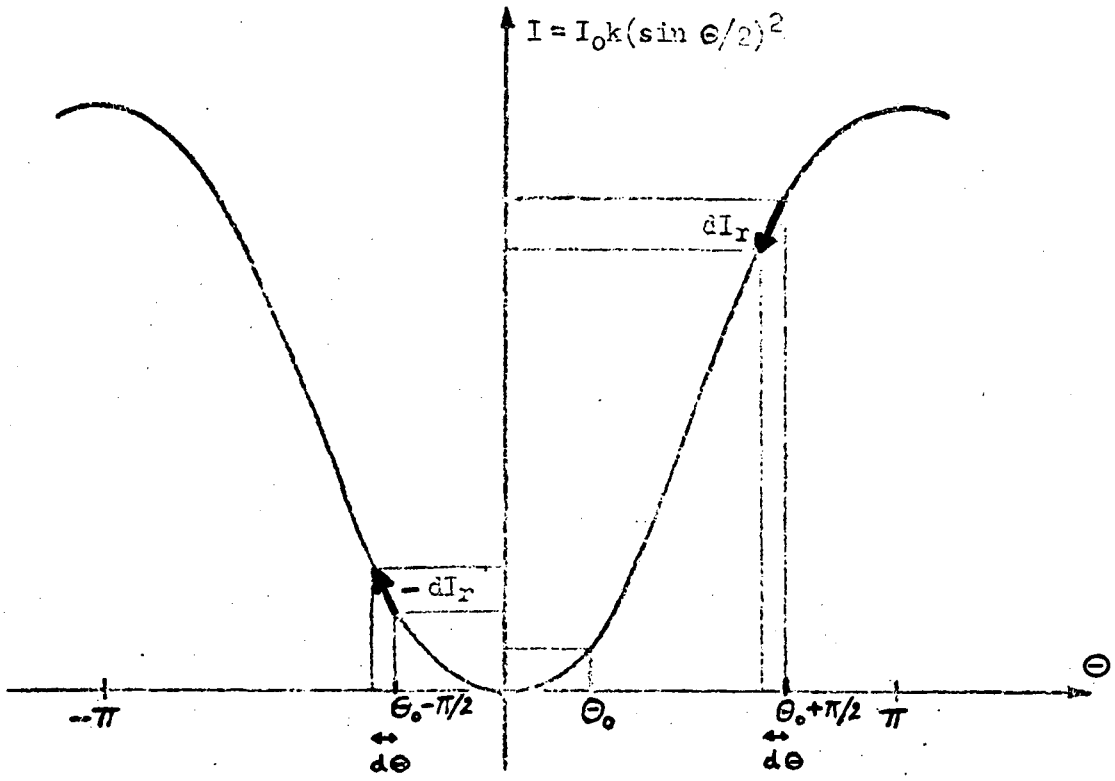


Fig. 2 Light intensity I as function of the phase angle Θ of the optical retardation. Arrows show the direction of light intensity changes due to a decrease of Θ. (Modified from Kaplan, 1972)

The pure retardation component is thus

$$dI_r = \left[dI_1(\Theta=\Theta_0+\pi/2) - dI_2(\Theta=\Theta_0-\pi/2)\right]/2$$

In this thesis the forward direction light scattering changes will not be analyzed in detail. This component is usually much smaller than dI_r and it is also not very reproducible.

We have defined previously $dI_r(\Theta) = I_0k(\sin\Theta/2)(\cos\Theta/2)d\Theta$ or subsituted as $dI_r(\Theta) = I(\Theta)\cot\Theta/2\, d\Theta$.

At $\Theta=\Theta_0+\pi/2$ and at $\Theta=\Theta_0-\pi/2$ we can have

$$\left[\frac{dI_r(\Theta)}{I(\Theta)}\right]_{\Theta=\Theta_0+\pi/2} = \cot\frac{\Theta_0+\pi/2}{2}\, d\Theta \quad \text{and}$$

$$\left[\frac{dI_r(\Theta)}{I(\Theta)}\right]_{\Theta=\Theta_0-\pi/2} = \cot\frac{\Theta_0-\pi/2}{2}\, d\Theta$$

respectively.

It can be easily shown that the product

$$\left[\cot\frac{\Theta_0+\pi/2}{2}\right]\left[\cot\frac{\Theta_0-\pi/2}{2}\right] = -1$$

Thus the change in phase angle of the optical retardation can be calculated as

$$d\Theta = \sqrt{-\left[\frac{dI_r(\Theta)}{I(\Theta)}\right]_{\Theta=\Theta_0+\pi/2} * \left[\frac{dI_r(\Theta)}{I(\Theta)}\right]_{\Theta=\Theta_0-\pi/2}}$$

The compensator can be used for measurements in white light. In this case the calibration value for $\lambda = 550$ nm, the centre of gravity of the visible spectrum, can be applied. For higher accuracy monochromatic light is necessary. However, as it can be seen in the expression of dI, it is advantagous to use a light source of high intensity.

Biological materials may exhibit two types of birefringence, i.e. intrinsic birefringence and form birefringence. Intrinsic or crystalline birefringence occurs in subtances having homogenous ordered arrays of molecules which are anisotropic in the polarizabilities of their chemical bonds. The form or textural birefringence occurs when particles or layers of a substance having one dielectric constant are arrayed in a substance having a different dielectric constant. In many cases, intrinsic and form birefringence occur simultaneously in the same material. The relative contribution of each type can often be determined by varying the refractive index of the surrounding medium until it matches that of the particles causing the form birefringence. The residual birefringence when the total birefringence reaches an extrenum is the pure intrinsic component.

Bear et al. (1937a, 1937b, 1937c) analyzed the birefringence of various regions of both myelinated and non-myelinated nerve fibers.

They found that the axially aligned proteins of the axoplasm of invertebrate giant axons (from squid and lobster) generate a positive uniaxial birefringence with respect to the long axis of the fiber. At the center of the axon, most of the resting retardation arises from the presence of longitudinally oriented fibrils and tubules in the axoplasm; removal of the axoplasm reduces the retardation of the center of the squid axon by 90% (Cohen et al., 1970). At the edges of the axon, the positive retardation is contributed mainly by the form and intrinsic birefringence of the Schwann cell and connective tissue. The plasma membrane itself appears to exhibit a negative intrinsic birefringence and a positive form birefringence with respect to the longitudinal axis. The negative birefringence is an indication of the radially oriented lipids in the membrane system (Bear et al., 1937a, 1937b; Schmitt and Bear, 1939).

It is known that during electrical activity the optical retardation, defined as $R = d(n_{//} - n_{\perp})$, where d is the thickness and $n_{//}$ and $n_{\perp}$ are the refractive indices of light with electric vector parallel or perpendicular to the nerve's long axis respectively, decreases with a time course resembling the action potential of the nerve. Treatment with proteases reduces the optical signal derived from the birefringence change (Sato et al., 1973; Kaplan and Klein, 1974), which indirectly indicates the involvement of proteins. However, the proteases are not very specific since they attack many different proteins. Cohen et al. (1971) have concluded that the retardation change does not occur in the lipid portion of the membrane, since it is not affected by the presence of octanol, butanol or procaine in the bathing medium. They have considered that electrostriction or molecular reorientation (Kerr effect),

due to a change of the electric field in the membrane, could give rise to changes in birefringence. They have also shown that the signal originates from a thin annular layer of uniaxial retarding material near the surface of the axon having a radial optic axis, and is mostly potential dependent. From the slow time constant of the signals it is assumed that the movement must take place in a region with high viscosity.

On the other hand, Sato et al. (1973) have suggested that the birefringence change is related to changes in longitudinally oriented macromolecular structures near the membrane, and is related to the mechanism controlling the membrane conductance. The involvement of a longitudinally oriented, crystal-like structure at or near the inner surface of the axon membrane in the process of nerve excitation was also suggested. Watanabe et al. (1973) presented evidence which supports the view that birefringence signal in the crab nerve is at least partly originated from the axoplasm. They believed that the birefringence signal is probably produced by an increased calcium influx during excitation, which disturbs the ordered structure of the axoplasm and decreases its anisotropy.

In this thesis an attempt is made to investigate the molecular species that may be responsible for the voltage dependent retardation changes.

B. Materials and Methods

The nerve bundles used for this study were obtained from the walking legs (pereiopods) of the American lobster , Homarus Americanus. The nerves were isolated simply by cutting the ligament around the joint between the meropodite and carpopodite of an isolated walking leg (1st, 2nd, or 3rd) and pulling them apart (Furusawa, 1929). Only a small fraction, about $\frac{1}{2}$ mm thick of the nerve bundle was used and the length was typically 15 mm.

The experimental chamber has three compartments for the electrolytes (Fig. 3). In the central chamber, about a 2 mm length of the fibre is bathed with the experimental solution. Both open (cut) ends of the nerve are dipped in saline (with 100 mM K^+) which fills the distal compartments situated on the left and right sides of the central chamber. The nerve portions (about 2 mm long) between the central and distal chambers are enclosed with 2% agar gel, which virtually isolates the chambers from each other.

We perturbed the membrane potential transiently by applying square voltage pulses (about 50 mV) through two pairs of external electrodes made of black platinized platinum. One pair of electrodes was positioned adjacent to the fibers in the central chamber. The other pair was positioned at either of the open ends of the nerve, which topologically have a close contact with the inside of the nerve. This symmetrical electrode and chamber assembly closely resembles the arrangement in the conventional double sucrose gap technique, except in our chamber agar is used instead of sucrose to separate the liquid pools.

In the early stages of this study we used electrodes made of smooth platinum instead of the black platinized platinum.

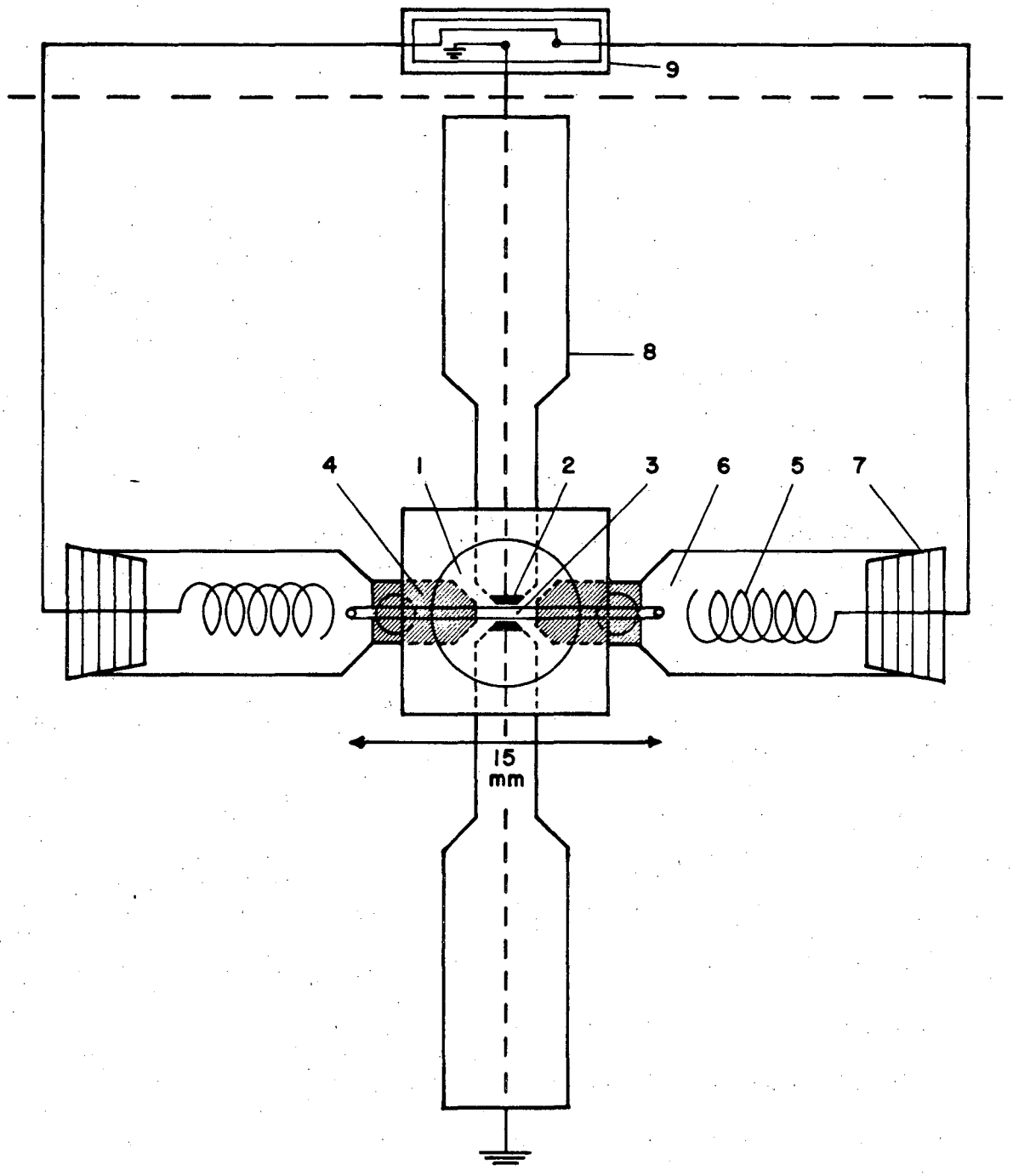


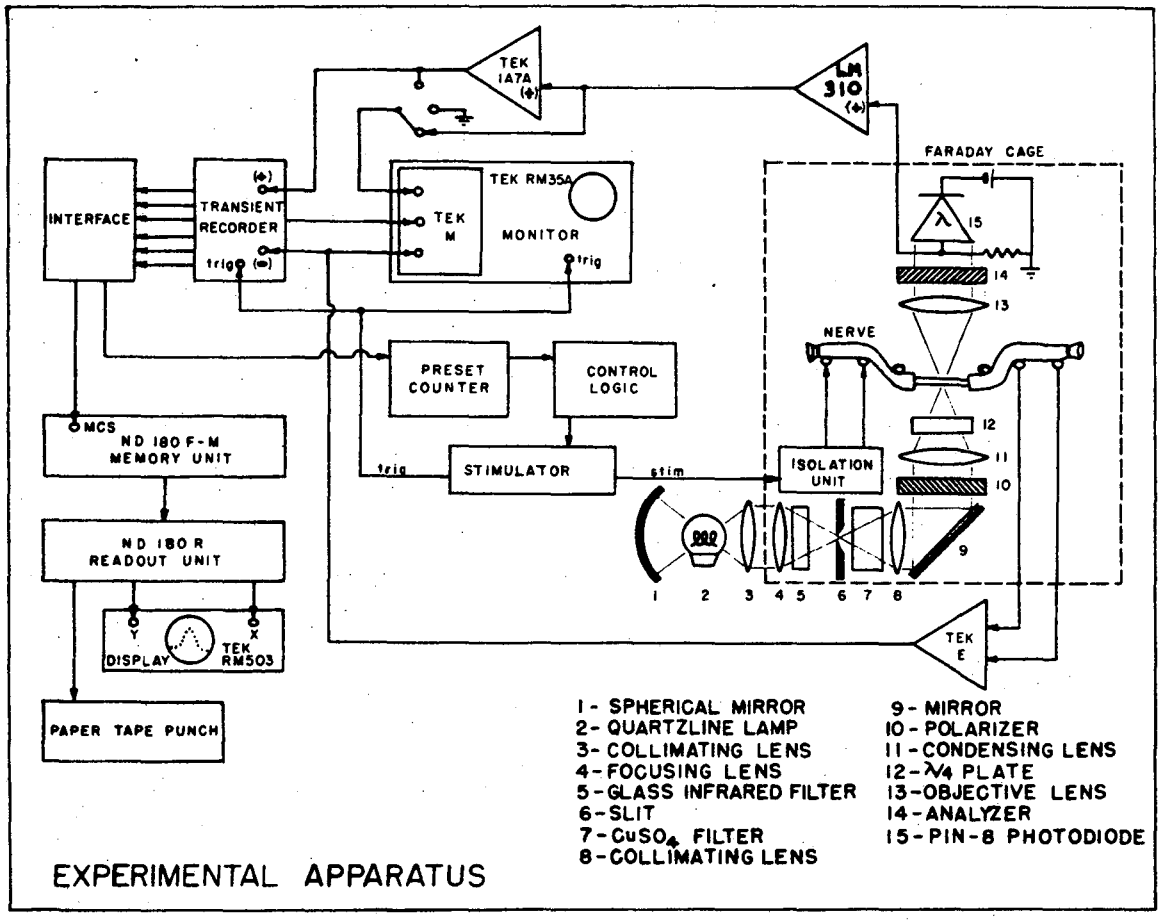
Fig. 3 : XBL 762-5659
Schematic diagram of the experimental chamber (top view):
1) central chamber filled with external fluid, 2) external electrodes, 3) nerve axons, 4) agar plug, 5) "internal" electrodes, 6) chamber with "internal" fluid, 7) stopper, 8) electrode holder, 9) pulse generator

The changes in optical retardation obtained with the smooth electrodes were much smaller and suffered a differentiation effect. These effects disappeared after the electrodes have been platinized in a standard platinizing medium. A train of hyper-and depolarizing pulses were applied to the nerve. These pulses originated from 2 Tektronix pulse generators (type 161, driven by type 162). They were attenuated by a potentiometer and passed through an amplifier (Harrison 6823A-Hewlett Packard) with an output impedance of less than 3 ohms. These voltage pulses had a rise and decay time of about 20 μsec.

Light from a DC operated quartzline lamp (GE EJL 24V, 200 Watts) traverses the central compartment through a pair of glass windows. The lamp is powered by a voltage regulated power supply (Lambda, model LE 102-FM). The glass reflector surrounding the bulb envelope was cut away with a diamond saw in order to produce an image of the filament. The lamp is positioned at the center of curvature of a spherical mirror. Light from the filament and its reflected image is first collimated and then focused upon a variable slit after passing through an infrared absorbing glass filter. After recollimation, the light is reflected from a 45° first surface mirror and passed through a film polarizer (Zeiss, # 47-36-00) mounted below a microscope condensor. The plane of polarization is set at a 45° angle with respect to the axes of the microscope stage. The light is condensed using an inverted Zeiss 10x Ultrafluor strain-free quartz objective lens. The beam is focused on an area of about 0.1 mm^2 at the center of the nerve fibers. The axons in the experimental chamber are mounted on the stage of the polarizing microscope, with their long axis oriented at a 45° angle from the polarizer axis. A quarter-wave

plate is inserted between the polarizer and the nerve, in a manner that allows the plate to be easily rotated. Light emerging from the nerve is collected by a second strain-free quartz 10x objective lens having a numerical aperture of 0.20 and passed through an eyepiece (Tiyoda Ki-eye 16 bi) and an analyzer (a second film polarizer with its axis rotated 90° from that of the polarizer). The eyepiece focuses the beam at a photodiode (PIN-8, United Detector Technology) which measures the light intensity. The photodiode has a very good quantum efficiency at high light intensities and has much less demanding power supply requirements than a photomultiplier. A 15 V mercury cell battery provides a stable bias potential. The photodiode current, which is proportional to the light intensity, is measured as the voltage across a 100 kΩ load resistor.

The experimental apparatus is shown in Fig. 4. That portion used for measuring and signal averaging the light intensity changes is basically similar to that used by Kaplan and Klein (1972, 1974). Modifications were made to permit the measurements of static light intensity and static retardation. The voltage across the 100 kΩ resistor is measured using an LM 310 voltage follower (National Semiconductor Corporation), which is internally connected as a unity-gain non-inverting amplifier. Typically this operational amplifier has an input resistance of $10^{12}\Omega$ and an output resistance of 1Ω. The DC output is monitored on a digital voltmeter (Dana, Model 5400). The signal is then amplified by a Tektronix type 1A7A amplifier powered by a Tektronix Type 127 power supply. The bandpass filters of the amplifier are set at a high frequency -3 dB point of 3 KHz and a low frequency -3 dB point



XBL 722-193

Fig. 4.

(from Kaplan, 1972, reproduced by permission and modified).

of 0.1 Hz. This blocks the slow drift sometimes seen at the DC coupled input stage if the light intensity emerging from the nerve drifts slightly, because of a slow deterioration of the tissue with time. The output of the amplifier is displayed on a Tektronix RM35A oscilloscope using one of the four channels of the type M preamplifier. Amplifier noise, shot noise of the light source and vibration noise dominate the signal. Care is taken also to eliminate 60 cycle noise by appropriate grounding and by mounting the microscope and photodiode system in a fine copper mesh Faraday cage.

The small component of the signal due to changes in the optical properties of the axon is resolved by employing the signal averaging technique. The signal to noise ratio increases proportional to $n^{\frac{1}{2}}$, where n is the number of passes made, usually 400. The output of the 1A7A amplifier, biased close to 0 V, is fed into a transient recorder (Biomation Model 610) which can store and digitize 128 words (addresses) of 6 bit data at a rate up to 100 nanosec/point. For the experiments with the nerve bundle, a digitization rate of 1 msec/point is normally used, which gives a sweep time of 128 msec. The signal averaging is done by a Nuclear Data 180 M memory unit operating in the multi-channel scaler (MCS) mode. It has four separate quadrants of memory that may be used separately. The interface built for this system utilizes the digital output of the transient recorder directly. Once a signal is stored and digitized by the transient recorder, it sends a signal to the interface to begin advancing the addresses of the transient recorder output and the Nuclear Data memory unit simultaneously. At each of the 128 addresses, six bits are transferred in parallel from the transient recorder to an interface buffer. A clock circuit

then begins to produce a pulse, subtracts one from the buffer, produces another pulse, etc. until the buffer is empty. The analog signal is therefore converted to a number of pulses between 0 and 63 for each of the 128 digitized points. After appropriate amplification and shaping, the pulses are fed from the interface into the Nuclear Data where they are added to the number already in the memory for that particular address. The averaged signal is displayed on a Tektronix RM503 oscilloscope and is recorded photographically using a Du Mont oscilloscope camera with a Polaroid film back. The optical recording time constant is about 60 μsec.

All experiments were performed at room temperature, about 20° C. The medium bathing the nerves was made according to the formulation given by Dalton (1958) and was usually buffered with 10 mM H E P E S (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, pK = 7.55) adjusted to pH 7.4-7.5 with NaOH or HCl. Its composition per liter is : 465 mM Na^+, 10 mM K^+, 25 mM Ca^{++}, 8 mM Mg^{++}, 533 mM Cl^-, 4 mM SO_4^{--} . All chemicals were reagent grade and used without further purification. The companies from which the chemicals were obtained are listed (with their abbreviated forms) as follows:

Aldrich Chemical Company, Inc. (ALH)

Allied Chemical Corp., B & A Laboratory (ACN)

J. T. Baker Chemical Co. (BKC)

Bio-Rad Laboratories (BRL)

Calbiochem (CBI)

I.C.N. - K & K Laboratories, Inc. (KNK)

Mallinckrodt Chemical Works (MAL)

Mann Research Laboratory, Becton-Dickinson Co. (MAN)

Merck & Co., Inc. (MRK)

Polysciences Inc. (POS)

Sigma Chemical Company (SIG)

The chemicals (and the companies) are listed below in the order as they appear in the text:

HEPES (SIG), NaCl (BKC), KCl (BKC), $CaCl_2.2H_2O$ (MAL), $MgCl_2.6H_2O$ (MAL), $CaSO_4.2H_2O$ (ACN), Trizma Maleate (SIG), Na acetate (BKC), NaOH (MAL), phenylglyoxal (SIG), NEM (SIG), $HgCl_2$ (ACN), PCMB (SIG), glutaraldehyde (POS), formaldehyde (MAL), Uranyl nitrate (BKC), L-α-lysophosphatidylcholine (SIG), phospholipase C (SIG), phospholipase D (SIG), sodium dodecyl sulfate (BRL), triton X-100 (SIG), acetylcholine chloride (SIG), choline chloride (ALH), carbamylcholine chloride (SIG), tetramethylammonium chloride (EAK), tetraethylammonium chloride (ALH), hemicholinium-3 (ALH), hexamethonium chloride dihydrate (MAL), decamethonium iodide (KNK), d-tubocurarine chloride (CBI), nicotine HCl (KNK), eserine salicylate (SIG), neostigmine bromide (SIG), procaine HCl (SIG), tetracaine HCl (SIG), dibucaine (KNK), barbital sodium (MRK), pentobarbital sodium (SIG), ATP disodium (SIG), ouabain (CBI). Most of the chemicals were stored desiccated in the freezer. The lobsters were kept in aquarium tanks filled with cold artificial seawater ("Instant Ocean", Aquarium Systems Inc.).

C. Results

When a voltage pulse is applied at the electrodes, the optical retardation of the nerve changes. The associated change in light intensity was averaged from 400 sweeps at a frequency of 2.5 per second. Equal hyperpolarizing and depolarizing pulses were applied to the nerve about a DC offset of -70 mV inside negative. Fig. 5 shows typical optical signals (top and bottom) produced by trains of 50 mV hyperpolarizing and depolarizing pulses (middle). An increase in light intensity is shown as an upward deflection. The length of the vertical calibration bars corresponds to the stated value of $\Delta I_r/I$ for a single sweep. I is the intensity of the background light and ΔI_r is the change in light intensity caused by a change in optical retardation. $\Delta I_r/I$ for a particular voltage pulse varied from preparation to preparation due to the differing thickness and transparency of the tissues; (but the variation rarely exeeded more than 50% from the average value). In Fig. 5 (top) the slow axis of the quarter-wave plate was oriented parallel to the nerve's longitudinal axis, so that the total retardation phase angle $\Theta=\Theta_0+\pi/2$. The signal could be reversed in sign when the quarter-wave plate was rotated by 90° (bottom), which made $\Theta=\Theta_0-\pi/2$. $\Delta\Theta$ is calculated from the geometric average of both signals according to the formula derived before (see page 21). An average of $\Delta\Theta = 5*10^{-5}$ radian can be elicited by a 50 mV applied voltage, while the resting retardation of a good nerve bundle averaged about 30 nm, which correspond to a phase angle of about 0.3 radian.

A sample of how $\Delta\Theta$ (induced by a depolarizing pulse) is calculated is shown below (for symbols see section A):

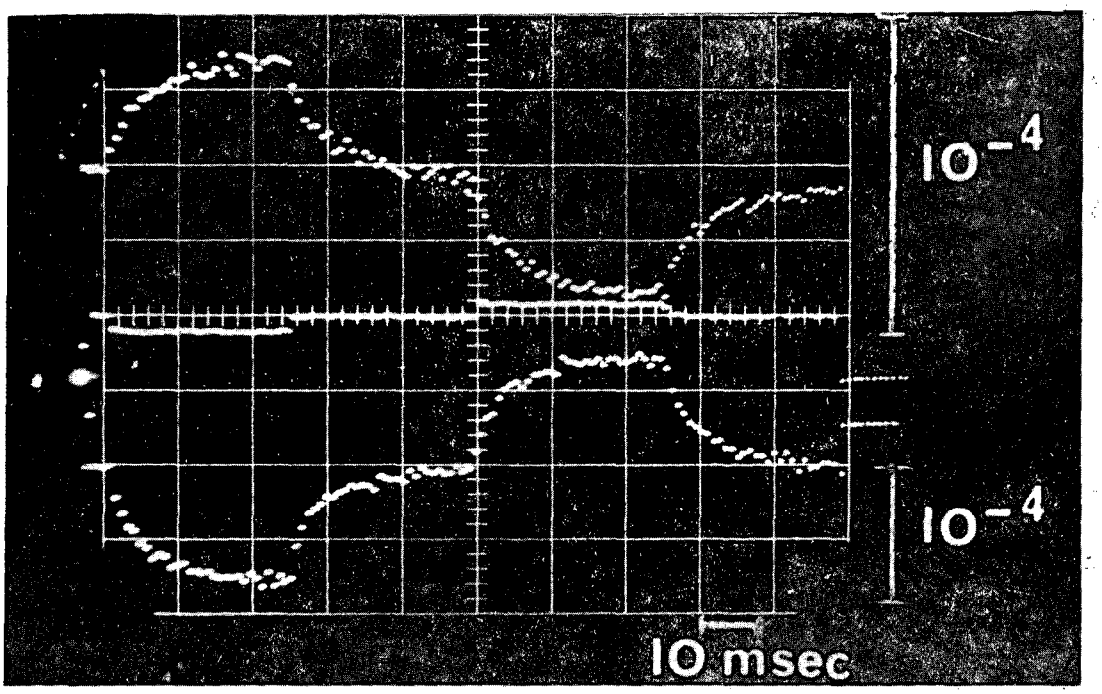


Fig. 5.
Light intensity changes of the nerve with $\Theta = \Theta_o + \pi/2$ (top) and $\Theta = \Theta_o - \pi/2$ (bottom) caused by 50 mV hyperpolarizing (HP) and depolarizing (DP) pulses (middle). Optical calibration shows the relative changes of light intensity, $\Delta I_r/I$, per sweep (total 400 sweeps accumulated).

$I_{total}\ (\Theta = \Theta_0 + \pi/2) = 313$ mV

$I_{total}\ (\Theta = \Theta_0 - \pi/2) = 151$ mV

$I_{leak} + I_{dark}$ at $\Theta = 0$ is 35 mV

Θ is zero when $\psi = 6.5^\circ$. Thus the static retardation of this nerve bundle is 30.93 nm which corresponds to a phase angle of $\Theta_0 = 0.35$ radian.

$I(\Theta = \Theta_0 + \pi/2) = 278$ mV

$I(\Theta = \Theta_0 - \pi/2) = 116$ mV

When the averaging experiments are finished, the stored maximum and minimum deflection of the light intensity changes are measured:

$$dI_1(\Theta = \Theta_0 + \pi/2) = -640 \text{ mV}$$

$$dI_2(\Theta = \Theta_0 - \pi/2) = 600 \text{ mV}$$

Thus $dI_r = \frac{1}{2}(dI_1 - dI_2) = -620$ mV at $\Theta = \Theta_0 + \pi/2$

or $dI_r = +620$ mV at $\Theta = \Theta_0 - \pi/2$

After dividing by the total gain of the system (157.5) and the total number of sweeps ($n = 400$), one gets

$$\left[\frac{dI_r(\Theta)}{I(\Theta)}\right]_{\Theta=\Theta_0+\pi/2} = -3.54 * 10^{-5}$$

and $$\left[\frac{dI_r(\Theta)}{I(\Theta)}\right]_{\Theta=\Theta_0-\pi/2} = 8.48 * 10^{-5}$$

Thus $\Delta\Theta = -5.48 * 10^{-5}$ radian

The observed changes in light intensity usually have rise and decay times of about 7 msec. Using single giant axons of the squid and the standard voltage clamp technique, Cohen et al. (1971) have observed three components of the optical retardation change with relaxation times ranging from about 40 μsec to about 20 msec. In our case, although we apply rectangular voltage pulses to the electrodes,

the cable properties of the nerve fibers as well as the nonuniform fiber diameters in the bundle may prevent an instantaneous change in membrane potential at the central region of the nerve. This seems to limit the fast response of our technique. Although this simple technique suffers slightly in its kinetic behavior, it suffices for our studies since we are comparing only the saturation values before and after the chemical modification.

We have determined that the normalized change in optical retardation exhibits an almost linear voltage dependence (Fig. 6). We can see that large depolarizing pulses produce smaller responses than those produced by hyperpolarizing pulses. In squid giant axons, the slow component also has an almost linear voltage dependence (Cohen et al., 1971). We should note that the cable properties of the nerve in our experiments will attenuate the applied voltages by a certain factor. Thus the actual change in membrane potential at the central region is less than the applied voltage pulses at the electrodes. With a 50 mV voltage pulse the change in membrane potential at the central region can be estimated to be about 20 mV, which is small compared to the amplitude of the action potential ($\approx$ 100 mV). The compound action potential of the fibers can give rise to $\Delta\Theta$ of about 10^{-4} to 10^{-3} radian. Fig. 7 shows the light intensity changes detected at $\Theta = \Theta_0 - \pi/2$ (top) and at $\Theta = \Theta_0 + \pi/2$ (bottom). The relative changes $\Delta I_r/I$ are $42.3 * 10^{-5}$ and $-13.7 * 10^{-5}$ respectively. This experiment was done using a nerve chamber similar to the one used by Kaplan (1972), as shown in Fig. 4. The compound action potential was elicited by an 11 V pulse of 0.5 msec duration.

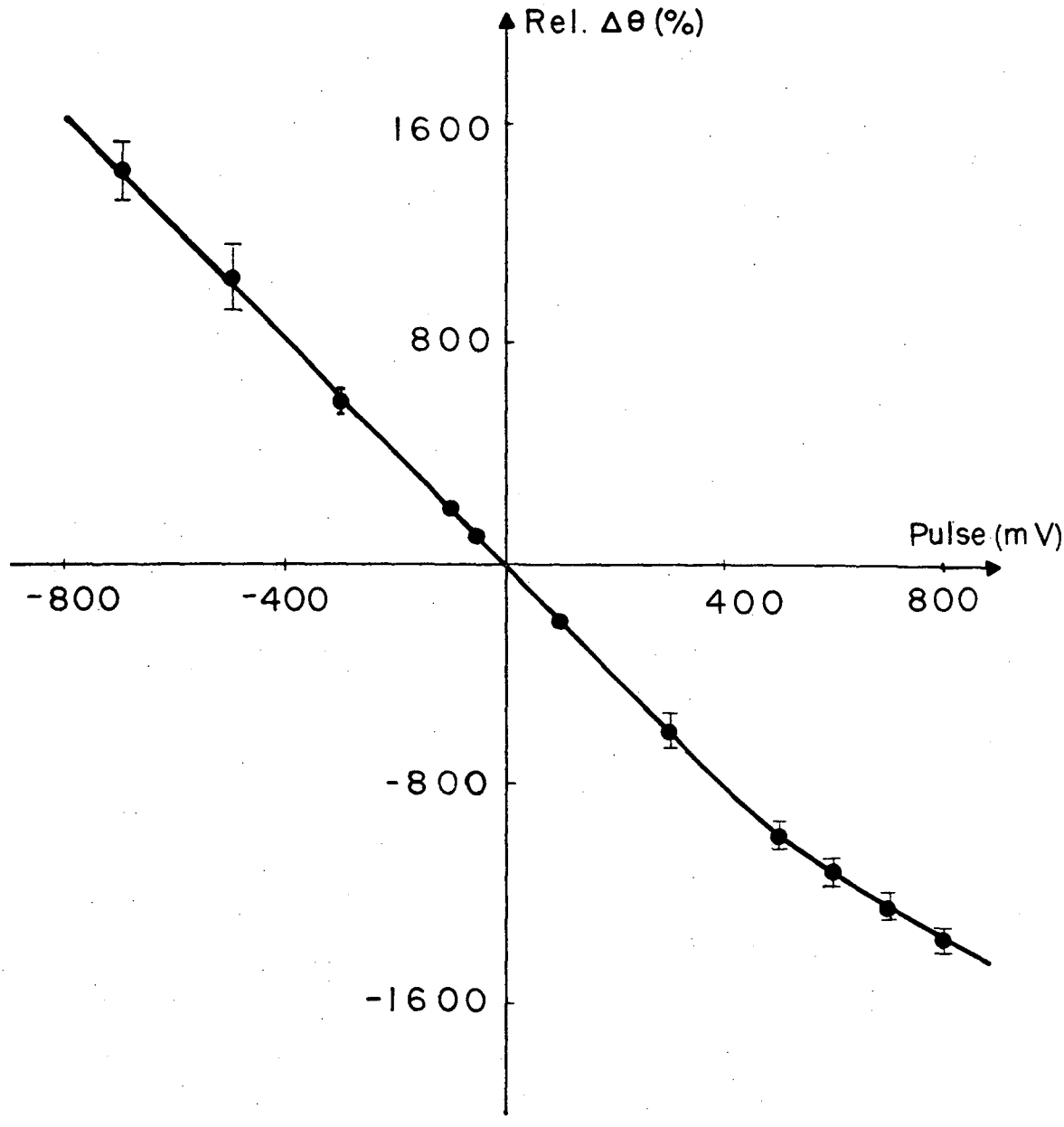


Fig. 6 .
XBL 766-5941
The voltage dependence of the optical retardation change. The data are normalized to the value of $\Delta\Theta$ induced by 50 mV hyperpolarizing (negative) pulse.

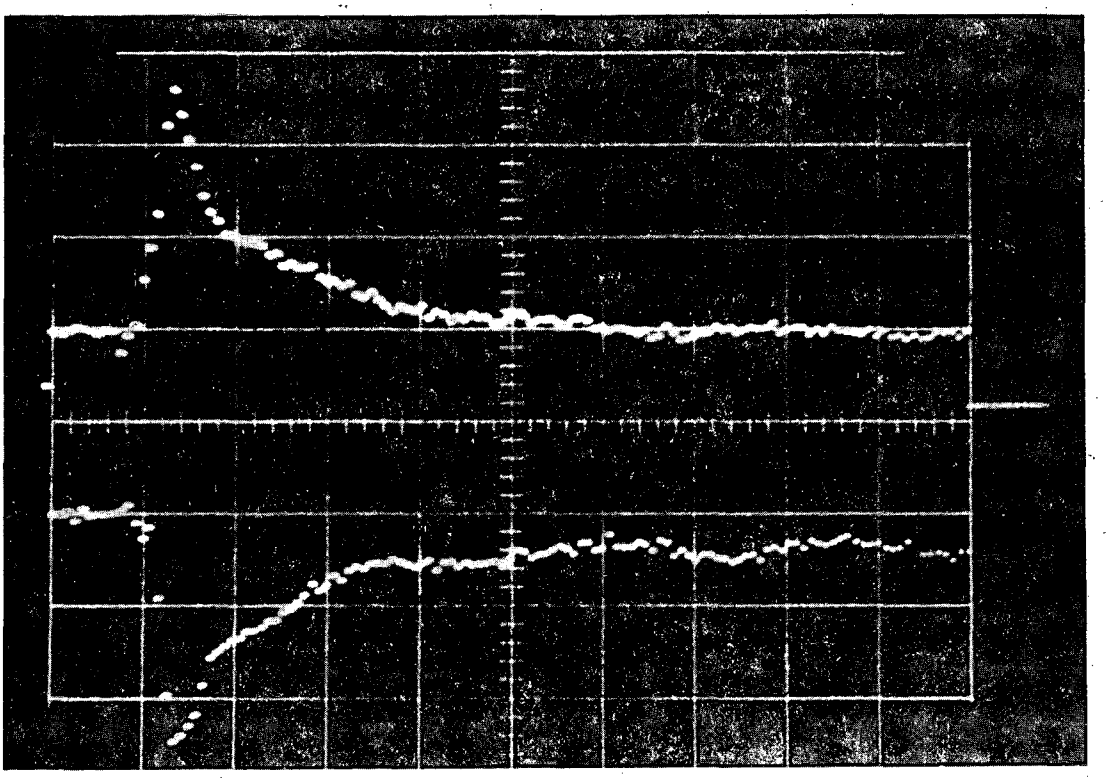


Fig. 7.
The changes in light intensity due to action potential (not shown). At $\Theta = \Theta_o - \pi/2$ (top), $\Delta I_r/I$ at the peak is $42.3*10^{-5}$. At $\Theta = \Theta_o + \pi/2$ (bottom), $\Delta Ir/I = -13.7*10^{-5}$.
Time calibration: 6.4 msec per large division.
400 sweeps averaged.

The values of $\Delta\Theta$ and of the resting retardation Θ_o of different preparations are usually not equal, since the thickness and the general condition of the tissues are not the same. Both $\Delta\Theta$ and Θ_o decrease slowly in time, as shown in Fig. 8. Here and in subsequent figures, we plot the relative values of $\Delta\Theta$ and Θ_o normalized to the value at the beginning of the experiment. The graphs are plotted as averaged values and standard deviations from at least three different preparations. In each experiment $\Delta\Theta$ is calculated from the average of the optical signals induced by 50 mV pulses in both hyperpolarizing and depolarizing directions.

The decrease of the optical signals are believed to be due to a general deterioration of the fibers with time, perhaps due to entry of calcium ions into the axoplasm (see discussion). Fig.8 also shows the effect of the pulse duration (32 msec and 64 msec). Pulses of longer durations tended to cause faster decreases in $\Delta\Theta$ and Θ_o. Pulses with large amplitudes also accelerated the degradation of the nerve, and for these reasons the voltage perturbations were kept short (32 msec) and small (50 mV). Some of the early data were obtained using 64 msec pulses. The effect of the chemical modification will be described now.

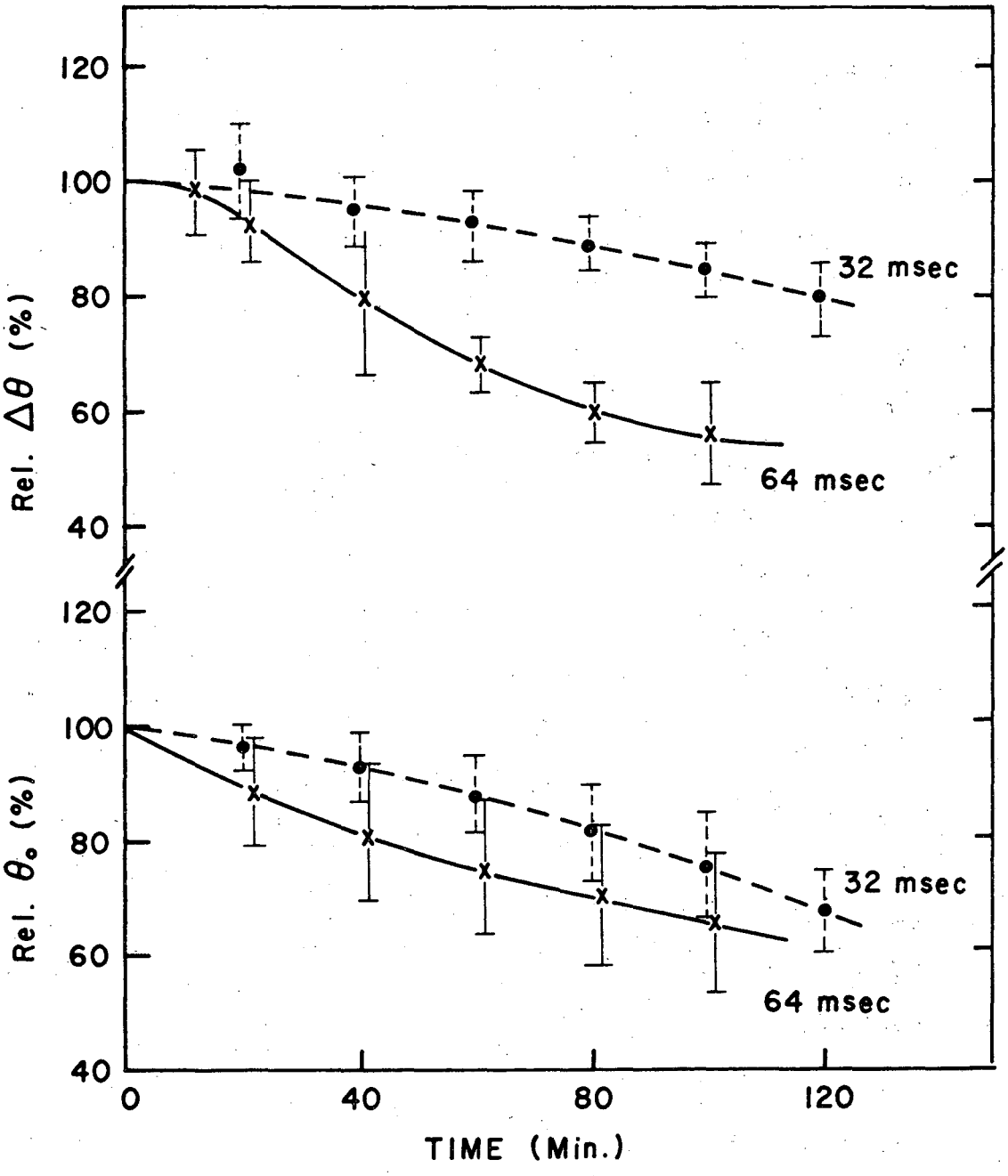


Fig. 8 XBL 764-5832

The time dependence of $\Delta\Theta$ and Θ_0 at two different pulse durations (32 msec and 64 msec). The data are normalized to the values at time zero. Standard deviations are calculated from 3 different preparations.

IV. CHEMICAL MODIFICATION STUDIES

In order to understand the molecular mechanism underlying the excitation process, which is in general the ultimate goal for many researchers in the field of membranes, one needs first to understand the meaning of the spectroscopic signals exhibited by the nerve. The primary objective of this thesis is to investigate the molecular species responsible for the voltage dependent retardation changes of the nerves. The chemical modification technique is used, with the rationale that a chemical perturbation or the interaction of a specific drug with those molecular species may perturb the structure which eventually would cause changes in the optical retardation characteristics. The word specific will have to be used with great caution, because as we will see later, a chemical perturbation may have multiple actions and the effects may be interfered by other components normally present in the medium. Therefore, we will begin by describing first the effects of those cations that are usually present in the control medium. In many cases the chemical modification can be too severe for the nerve and usually the action potential is blocked. However, even in cases like these, the voltage perturbation still permits the detection of optical retardation changes. Since this is the main interest of this thesis, the discussion will be focused mostly on the optical retardation rather than on the electrophysiological aspects of the chemical modifications. Wherever possible, references describing the electrophysiological effects of the drugs will be quoted in each section.

A. Changes in the Ionic Composition of the Bathing Medium

1) Potassium (K^+) and Calcium (Ca^{++})

Increasing the external K^+ concentration will depolarize the membrane potential as described by the Goldman-Hodgkin-Katz-equation (page 4). It will be interesting to study the nerve optical retardation in the depolarized state, as compared to the normal state of the membrane. However, increasing the potassium concentration of the external solution will also cause an increase in calcium influx (Hodgkin and Keynes, 1957). This increase could be due to the electrical or chemical effect of potassium. For this reason the effect of calcium is also included in this section. Fig.9 shows the effects of KCl and $CaCl_2$ on the voltage induced retardation change $\Delta\Theta$ and the resting retardation Θ_0. Here and in subsequent figures, at time zero the control medium bathing the nerve in the central chamber is replaced with a freshly prepared solution containing the modifying compounds. The control experiments were done in the Dalton solution containing 10 mM K^+ and 25 mM Ca^{++} (curves 1a and 1b for $\Delta\Theta$ and the corresponding Θ_0 respectively). Increasing the potassium concentration to 500 mM (osmolality was balanced by substituting potassium for sodium) almost completely abolished $\Delta\Theta$ and Θ_0 (curve 2). However, this effect was less drastic when the experiments were performed in a calcium-free medium (Na^+ replaces Ca^{++}). Curve 3 shows the effects of the potassium induced depolarization on $\Delta\Theta$ and Θ_0 in the absence of calcium ions. A typical example of the effect of potassium on ΔI, the voltage induced changes of light intensity, is shown in Fig. 10. Curves a and c are ΔI in control saline solution at $\Theta = \Theta_0 + \pi/2$ and $\Theta = \Theta_0 - \pi/2$ respectively. Curves b and d are the corresponding ΔI about 10 minutes

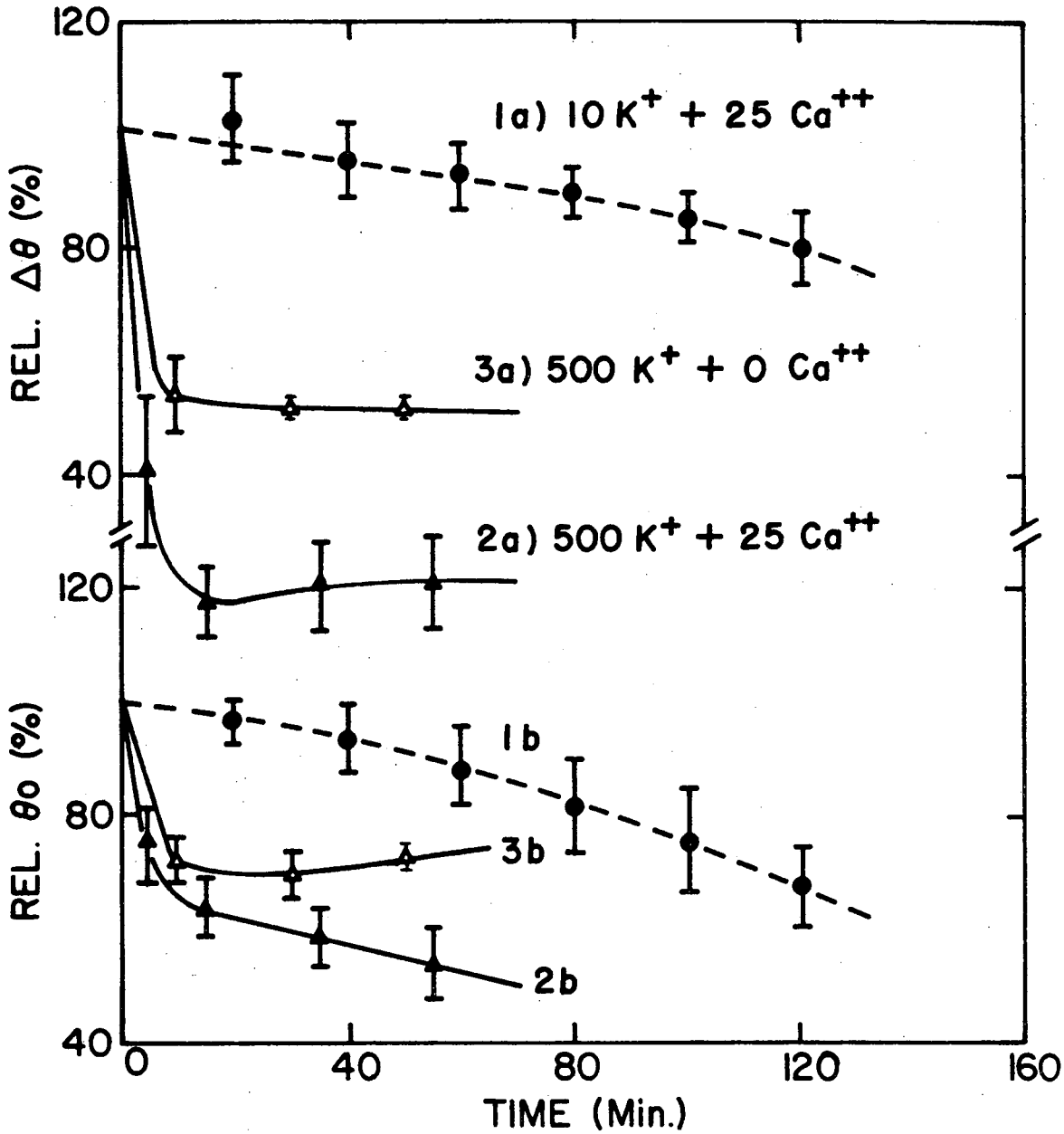


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Fig. 9.
The effects of potassium and calcium on $\Delta\Theta$ and Θ_o:
1) in control solution containing 10 mM K^+ and 25 mM Ca^{++}
2) in 500 mM K^+ and 25 mM Ca^{++}
3) in 500 mM K^+ and 0 mM Ca^{++}

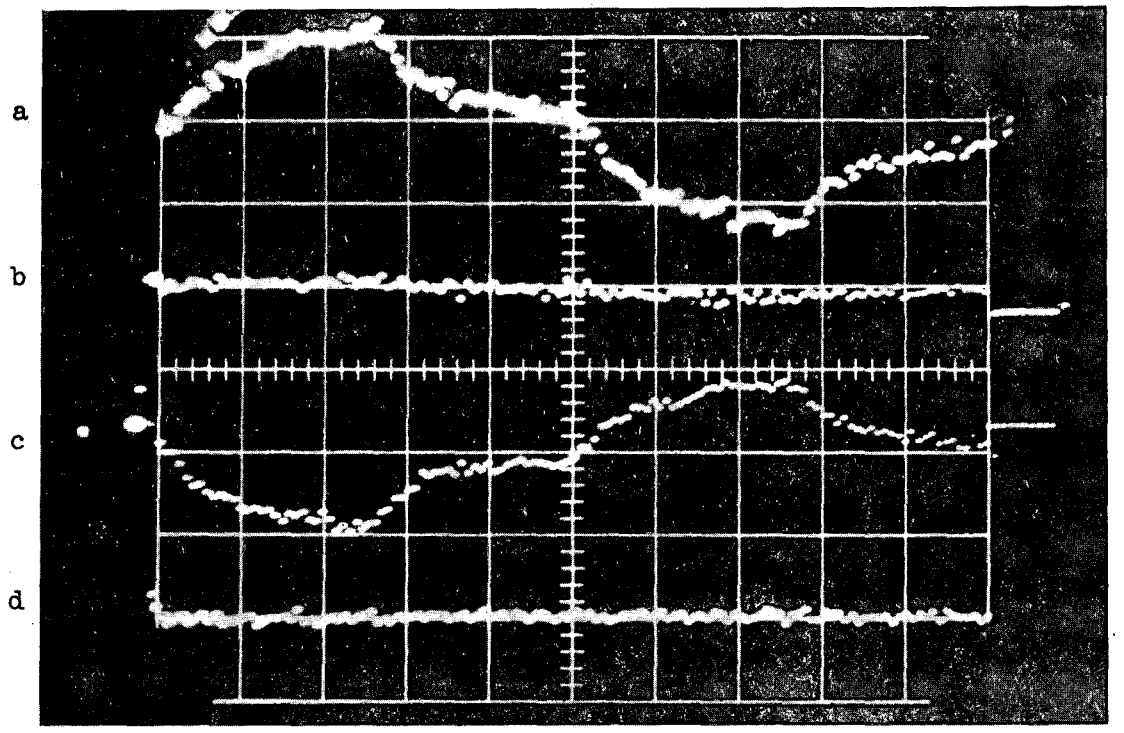

Fig. 10.
Voltage induced changes in light intensity (arbitrary units). Control experiments at $\Theta = \Theta_0 + \pi/2$ (trace a) and at $\Theta = \Theta_0 - \pi/2$ (trace c). Traces b and d are the corresponding results in 500 mM K^+ solution. Time calibration: 12.8 msec per large division. 400 sweeps averaged per experiment.

after the medium was changed to a high potassium solution. The resting light intensity I was also reduced as the nerves became less transparent. The calculated decrease of $\Delta\Theta$ and Θ_o was reversible upon return to the control medium with the low potassium content. An example is shown in Fig. 11. At time zero the control medium was changed with a saline solution containing 60 mM K^+. $\Delta\Theta$ decreased transiently (curve 2) before it equilibrated to a level below control value (curve 1). No significant effect on Θ_o could be seen. When 500 mM K^+ was introduced, both Θ_o and $\Delta\Theta$ decreased sharply, and increased again as soon as the control medium (10 mM K^+) was reintroduced. In some cases the reversibility was not complete, e.g. after prolonged exposure to high potassium solution and especially if the solution contained high Ca^{++}.

In internally perfused squid axons, divalent ions in the perfusate tend to block excitability at concentrations lower than the normal external Ca^{++} concentration (Tasaki, 1968). To the contrary, Begenisisch and Lynch (1974) have reported that internal concentrations of calcium up to 10 mM have little, if any, effect on the time-course, voltage dependence, or magnitude of the ionic currents of voltage-clamped squid giant axons. It is known that microinjection of calcium liquifies the axoplasm and tends to block conduction (Hodgkin and Keynes, 1956). Thus the conduction block may be caused by a slow deterioration of the internal environment of the nerve.

In the external media, small concentrations of divalent cations are necessary for, but large amounts reduce, excitability, or "stabilize" the resting state whereas low Ca^{++} concentration frequently leads to repetitive spontaneous activity. In a voltage clamp experiment, reducing the external Ca^{++} increases the early inward and

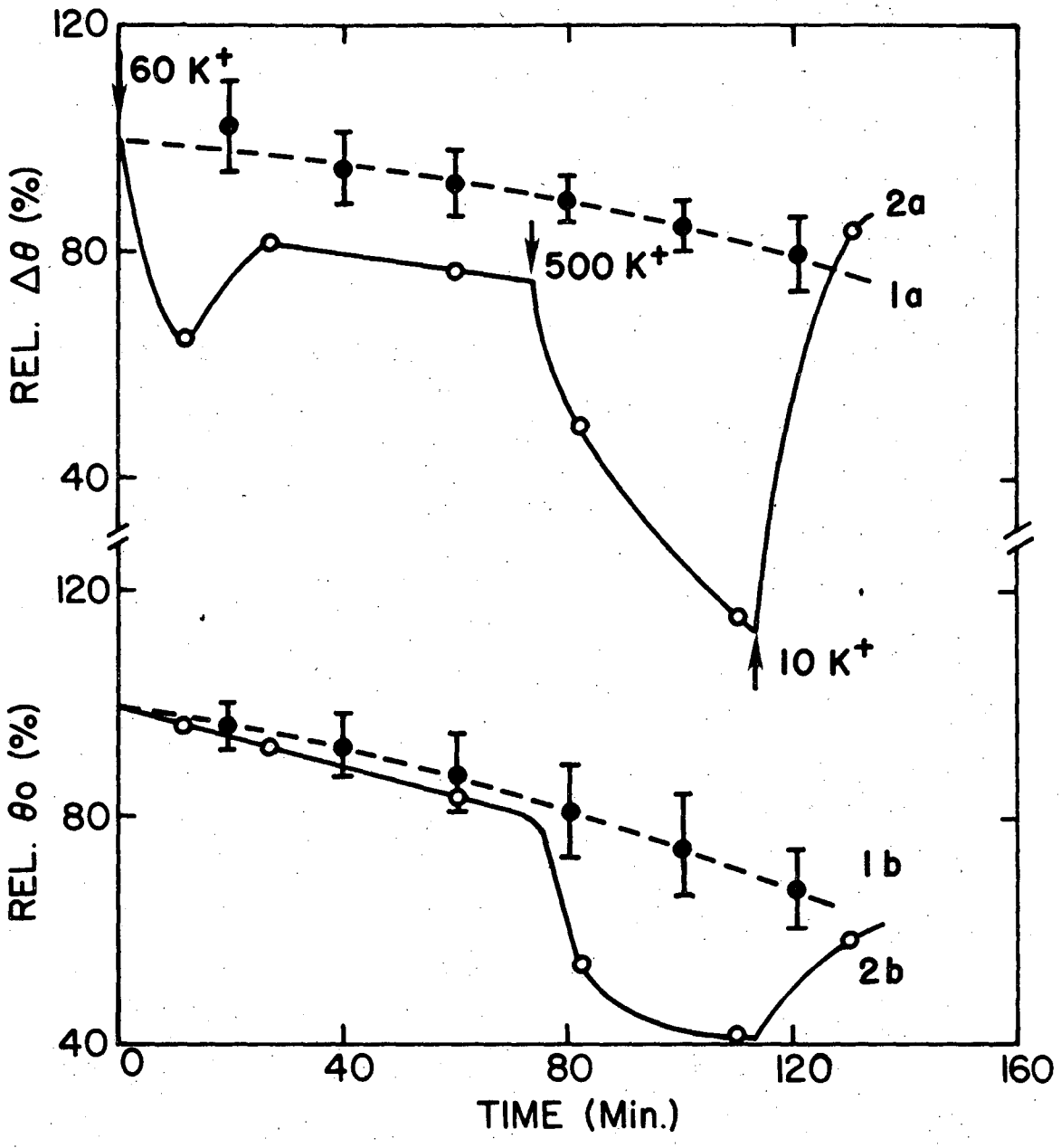

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Fig. 11.
The effects of potassium concentration on $\Delta\Theta$ and Θ_o (curve 2). At time zero (arrow) the concentration was changed from 10 to 60 mM. Second arrow shows change to 500 mM K^+, and later (upward arrow) back to 10 mM K^+. Control experiments (curve 1) in regular saline

the late outward currents and decreases their rise times (Frankenhaeuser and Hodgkin, 1957). The current voltage curve obtained by plotting the maximum inward current vs. applied clamping voltage is uniformly shifted so that a smaller depolarization is required to yield a given size inward current. This shift could be due to a surface charge effect (Frankenhaeuser & Hodgkin, 1957; McLaughlin, Szabo and Eisenman, 1971). Watanabe et al. (1973) believed that at least one component of the birefringence signal of a crab nerve is derived from a change in axoplasm. They concluded that the birefringence signal is probably produced by an increased Ca^{++} influx during excitation. A transient rise in intracellular Ca^{++} concentration could disturb the ordered structure of the axoplasm and decreases its anisotropy. The process of diffusion makes the time course of the signal slow and long - lasting.

The effects of calcium on $\Delta\Theta$ and Θ_o are shown in Fig. 12. The control experiments were done in a medium with 25 mM Ca^{++}. Both $\Delta\Theta$ and Θ_o decrease slowly in time at a rate of about 10% per hour (curve 1). Increasing the calcium concentration to 100 mM (and reducing Na^+ appropriately) results in a faster decrease of $\Delta\Theta$ and Θ_o (curve 2). The nerve also became opaque faster than in control experiments. When the calcium level was further increased up to 350 mM, the effect on $\Delta\Theta$ and Θ_o was not much different than what is shown in curve 2. On the other hand, elimination of the calcium ions from the control medium results in a reduction of $\Delta\Theta$ to a level of about 60 - 70% of the control value, while Θ_o remains higher than control for a long period (curve 3). We should note again that the values of $\Delta\Theta$ and Θ_o in the figures are plotted relative to their magnitudes at time zero when the

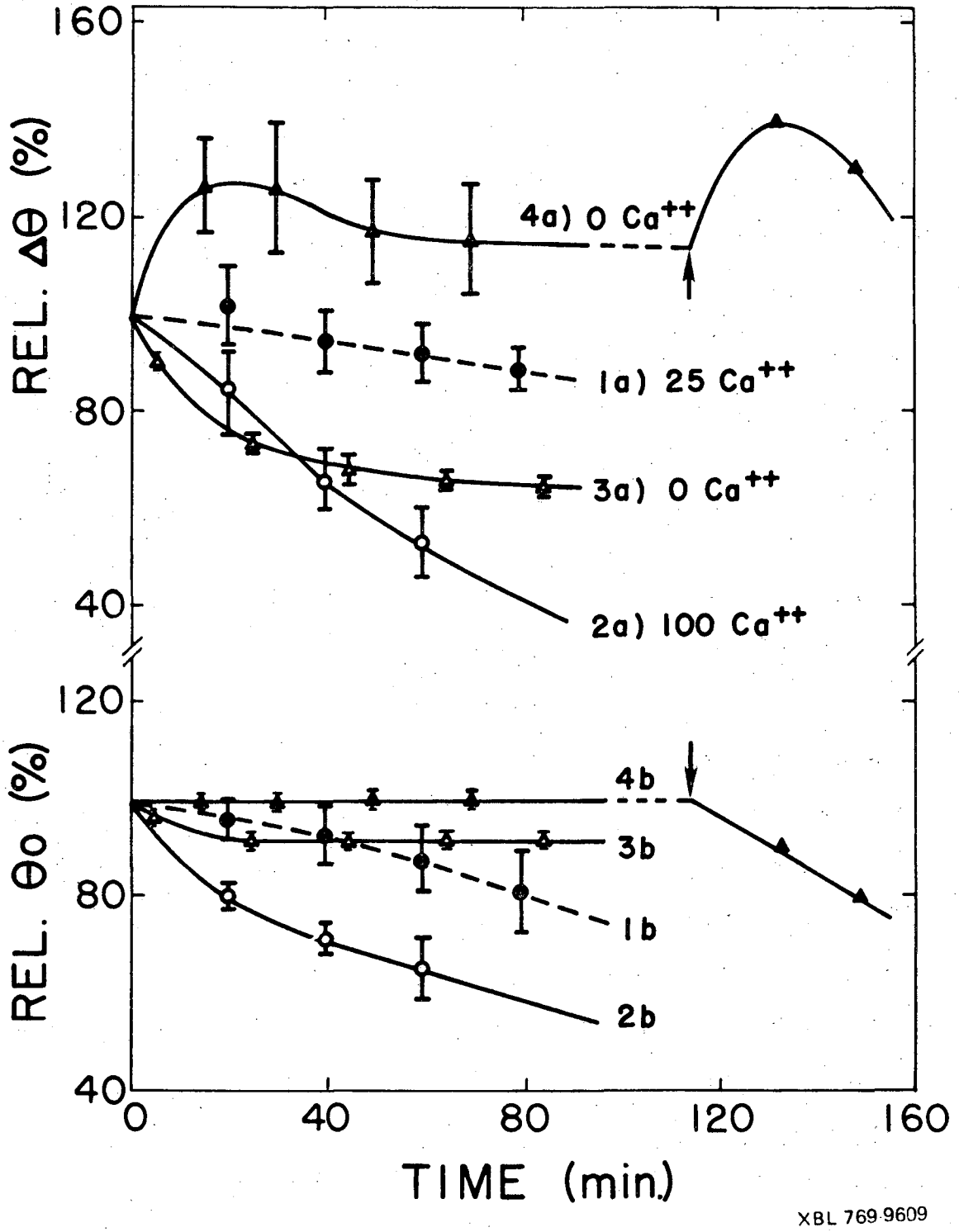

Fig. 12.
The time dependence of $\Delta\Theta$ and Θ_o at different calcium concentrations: 1) 25 mM, 2) 100 mM, 3) and 4) 0 mM with and without prior incubation in 25 mM Ca^{++} (control medium) respectively. Arrow indicates solution change from 0 to 25 mM Ca^{++}.

bathing medium is changed. Thus the absolute magnitude of $\Delta\Theta$ in Ca^{++}-free medium is always lower than in the control medium. However, when the nerves had never been exposed to the control medium (containing 25 mM Ca^{++}), i.e., they were immediately bathed with Ca^{++}-free saline after the dissection, they behaved differently and had the $\Delta\Theta$ and Θ_o time behaviour shown in curve 4. Here time zero corresponds to about ten minutes after the dissection. This ten minute delay was necessary for positioning the nerves in the experimental chamber and then mounting the chamber on the microscope stage etc. We can see from curve 4 that $\Delta\Theta$ rises momentarily (about 20%) before it equilibrates and stays at an almost constant level. The change could be due to the transition from lobster blood to the artificial solution. Θ_o stays always constant at the relative 100% level. It will also be interesting to find out what happens if the calcium level is increased from 0 to 25 mM, the value in the control medium. This is shown in the later part of curve 4 (start at arrows). Usually $\Delta\Theta$ rises relatively about 30 to 40% and then decline slowly, while Θ_o is immediately reduced. All these experiments indicate that high Ca^{++} concentration tends to reduce Θ_o and $\Delta\Theta$ faster than normal. Therefore, in the coming sections it is found necessary to do the chemical perturbations in the normal medium (with 25 mM Ca^{++}) as well as in the Ca^{++}-free saline.

2) pH dependence

It is assumed that the voltage dependent retardation changes may be caused by some kind of field dependent movement of a charged molecule. If one can "titrate" the optical retardation change by

studying its pH-dependence, one may obtain some informations about the nature of the charged particle. However, as the pH is changed, some characteristics of the nerve may also be changed. The pH-dependence of the electrophysiological properties of the nerves will be reviewed briefly.

Hille (1968) has found that as the pH is lowered below 6, the maximum sodium conductance decreases quickly and reversibly in a manner that suggests that protonation of an acidic group with a pK_a of 5.2 blocks individual sodium channels in the frog node. It has been shown that divalent ions, monovalent ions and H^+ ions influence the Hodgkin-Huxley (1952) parameters m, h and n (Chandler et al., 1965; Hille, 1968; Gilbert and Ehrenstein, 1969; Mozhayeva and Naumov, 1970, 1972; Brismar, 1973). To a first approximation, the functions relating m, h and n to voltage are simply shifted along the voltage axis. These shifts arise through the influence of available counterions on the surface potentials, which are set up by the fixed negative surface charges. Cations screen the surface charge by forming an ionic, diffuse double layer at the surface and may also physically neutralize some of the surface charge by forming complexes (binding). Both effects can reduce the negative surface potential. The Gouy-Chapman-Stern theory (Gilbert, 1971) has been applied to measured voltage shifts in axons by choosing values for the fixed surface charge density and, in some cases, assigning acid dissociation constants (pK_a) or dissociation constants for surface complexes with various divalent ions (Gilbert and Ehrenstein, 1969, 1970; Mozhayeva and Naumov, 1970, 1972; Woodhull, 1973; Brismar, 1973). The results from current measurements

suggest an internal negative fixed charge with a separation of 27 Å (Chandler et al., 1965) or 40 Å (Rojas and Atwater, 1968) and external fixed charges with a separation of 11 to 18 Å (Gilbert and Ehrenstein, 1970; Mozhayeva and Naumov, 1970).

Woodhull (1973) also recognized that the description of low pH effects as a voltage shift of the peak sodium permeability-voltage curve and a superimposed block of a given percentage of channels was inadequate. The block of channels by protons depends on membrane potential; fewer channels are blocked for large depolarizations than for small depolarizations as if depolarization drives protons out of their binding site. Thus the sodium permeability titrates away at low pH as if controlled by a negatively charged carboxylic acid with a voltage dependent apparent pK_a in the range between 5 and 6. (Hille, 1975).

The potassium gating components are closely associated with a group with a pK_a on the order of 6.3 (Shrager, 1975), which is close to the pK_a value of histidine imidazole groups. According to him, the slowing and inhibition of potassium current by diethylpyrocarbonate gave further evidence for the participation of histidine residues. Diethylpyrocarbonate is a reagent with a reportedly high selectivity for histidine.

Clark and Strickholm (1971) have reported impedance transitions near pH 6.3 and pH 8. These are tentatively identified as resulting from histidine and sulfhydryl groups. Conformational transitions involving histidine groups of surface membrane proteins are believed to be involved in the action potential and ion permeability regulation. Spyropoulos (1972), Bicher and Ohki (1972) have reported that drastic

changes occur in squid giant axon near pH 5. The resting potential is lowered by approximately 30% and the action potential is completely obliterated. Rojas and Atwater (1968) working with isotonic potassium as the only internal and external cation in squid giant axon, have reported a decrease in conductance for hyperpolarizing currents of the membrane at pH below 4.5.

In general, at low pH regions near pH 5 one can see drastic changes in membrane potential, membrane conductance and ionic currents due to protonation of certain charged groups. It may become difficult to do a straightforward determination of the particular charged group that is involved, because of the uncertainty of the local or surface pH. From the Boltzman distribution law, the surface pH follows as:

$$(pH)_s = (pH)_b + \frac{\mathbf{J}}{2.3RT/ZF}$$

Here $(pH)_s$ is the pH at the surface of shear and $(pH)_b$ is the bulk pH, while $\mathbf{J}$ is the zeta potential, i.e. the value of the potential at the surface of shear. The external surface potential for the squid axon is about -46 mV (Gilbert, 1971), so that the predicted difference between $(pH)_b$ and $(pH)_s$ is about 0.5 pH units. It is also likely that electrostatic interactions with other charged groups may shift the pK_a value of an ionized group. At extreme unphysiological pH, irreversible damage to the tissue can also occur. These are just a few of the problems that one has to be aware of when studying titration curves of membranes. The pH dependence of $\Delta\Theta$ and Θ_o will be described now.

The pH of the medium in the central chamber was changed at time zero. Prior to this the nerves had been equilibrated in a control medium at pH 7.5 for at least 20 minutes, either with or without calcium depending upon the experiment. This process was necessary since $\Delta\Theta$ usually increases slightly during the first 30 minutes (curve 4 Fig. 12). The solutions were buffered with 10 mM HEPES ($pK_1 = 3$, $pK_2 = 7.5$), Trizma Maleate ($pK_1 = 5.8$, $pK_2 = 8.3$) or acetate buffer ($pK_a = 4.7$). No buffer was used for pH higher than 10. The pH values were checked many times with a Beckman pH meter. The buffers themselves do not have any effect on $\Delta\Theta$ or Θ_o. The time dependence of $\Delta\Theta$ and Θ_o at pH 5 and at pH 9.5 are shown in Fig. 13, both with or without calcium (25 mM). At pH 5.0 and zero calcium, $\Delta\Theta$ rises and almost doubles its value and then declines slowly (curve 1a). The increase is reversible. Θ_o stays almost constant (curve 1b). When the medium contains calcium $\Delta\Theta$ increases slightly, followed by a rapid decrease, which is irreversible (curve 2a). This decrease, which turns out to be an artifact caused by calcium was first noted in a preliminary report (Ang and Klein, 1974). Θ_o also decreases (curve 2b). The decrease of $\Delta\Theta$ and Θ_o was more severe at lower pH values whenever calcium was present. At pH 9.5 $\Delta\Theta$ decreases slightly (curve 3a) and decreases more rapidly if the medium contains calcium (curve 4a). The resting retardation Θ_o remains almost unchanged at the relative 100% level (curves 3b and 4b). Not shown here are $\Delta\Theta$ and Θ_o at pH 7.5 that stay relatively constant with the scale of Fig. 13 (see also Fig. 8 & Fig. 12).

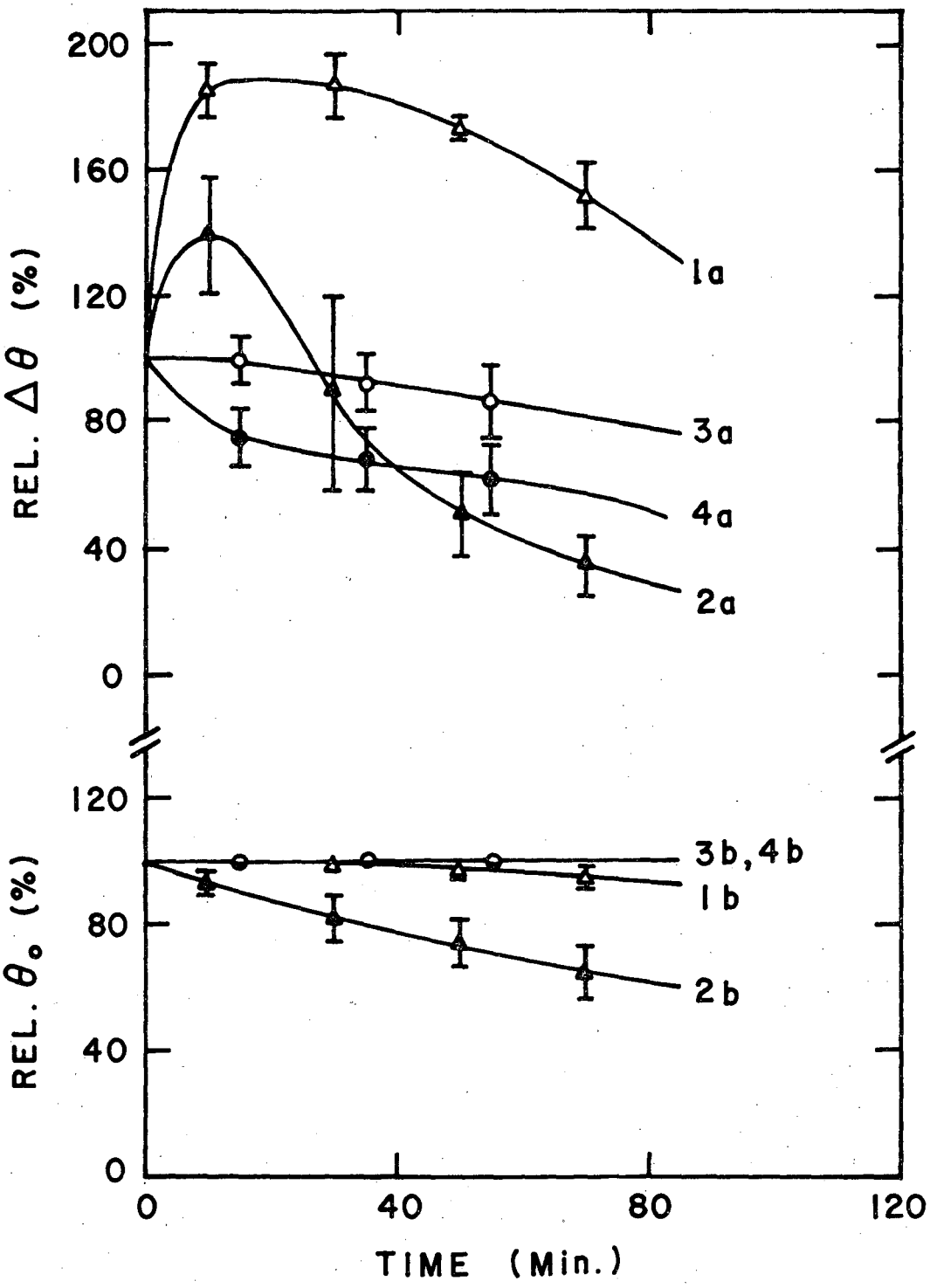

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Fig. 13.
The time dependence of $\Delta\Theta$ and Θ_0 at pH 5 (curves 1 and 2) and at pH 9.5 (curves 3 and 4) The concentration of calcium is 0 mM (curves 1 and 3) or 25 mM (curves 2 and 4).

At pH higher than pH 10.5 both $\Delta\Theta$ and Θ_o decrease very rapidly to zero even if no calcium is present in the medium. An example of the actual oscilloscope record (after 400 sweeps) is shown in Fig. 14. From top to bottom, the first trace is control at pH 7.5 with $\Theta = \Theta_o + \pi/2$, the second is its corresponding record 20 minutes after incubation at pH 11. The third record is also control at pH 7.5 but $\Theta = \Theta_o - \pi/2$ and the fourth is a similar record at pH 11. No more changes in light intensity can be detected in Fig. 14 at pH 11 and the decrease of $\Delta\Theta$ in this case is irreversible.

The increase of $\Delta\Theta$ at low pH values (without Ca^{++}) can usually be seen visually from the changes of light intensity in each sweep. Fig. 15 shows typical records of light intensity changes. The first record (top) is control at pH 7.5 with $\Theta = \Theta + \pi/2$ and the second is its corresponding record at pH 5.0 (after 10 minutes). The third record is also control at pH 7.5 but $\Theta = \Theta_o - \pi/2$. The fourth (bottom) is its corresponding record at pH 5.0. The increase of the voltage induced changes of light intensity is obvious and the increase of $\Delta\Theta$ can be readily calculated. When the pH is further lowered from pH 5 to pH 3, $\Delta\Theta$ increases only slightly as if it saturates. At these low pH values it was often observed that hyperpolarization pulses induced about 25% larger $\Delta\Theta$ than depolarization pulses. However, the relative values of $\Delta\Theta$ were always calculated from the average of $\Delta\Theta$ induced by hyperpolarizing and depolarizing pulses. The increase of $\Delta\Theta$ at very low pH was usually followed by an irreversible decrease. Similarly Θ_o was also reduced and the nerve turned opaque. This could very likely be due to some kind of denaturation after the long incubation period in unphysiological pH.

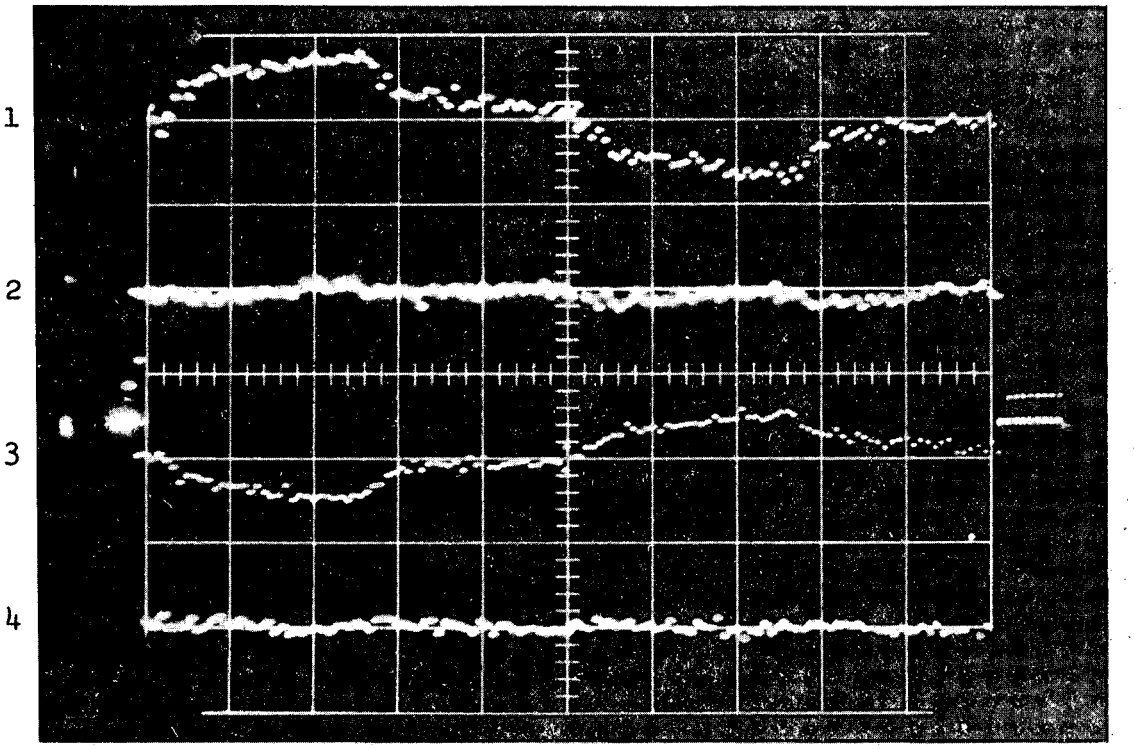

Fig. 14.
The effect of high pH on the voltage induced changes in light intensity. From top to bottom: 1) and 2) at pH 7.5 and pH 11 respectively ($\Theta = \Theta_0 + \pi/2$). 3) and 4) at pH 7.5 and pH 11 respectively ($\Theta = \Theta_0 - \pi/2$). Calibrations of the large divisions are 12.8 msec (horizontal) and arbitrary units (vertical).

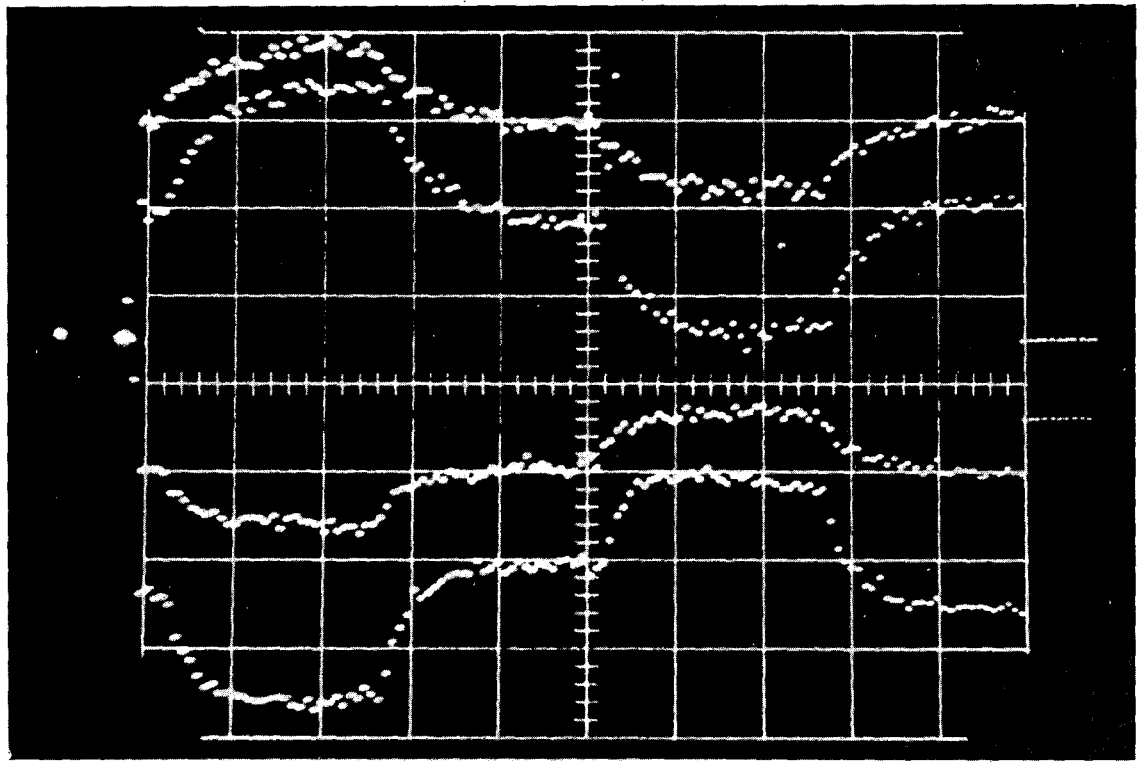

Fig. 15.
The effect of low pH on the voltage induced changes in light intensity. 1) and 2) at pH 7.5 and pH 5 respectively ($\Theta = \Theta_0 + \pi/2$) 3) and 4) at pH 7.5 and pH 5 respectively ($\Theta = \Theta_0 - \pi/2$). Calibrations of the large divisions are 12.8 msec (horizontal) and arbitrary units (vertical)

The pH dependence of $\Delta\Theta$ is summarized in Fig. 16. Here the values of $\Delta\Theta$ were obtained from the experiments in the Ca^{++}-free medium and each point represents the calculated $\Delta\Theta$ twenty minutes after a pH change. It is assumed that within twenty minutes the nerves have been well equilibrated with the new medium and that no severe denaturation has yet occurred. All values of $\Delta\Theta$ in Fig. 16 are normalized to their control values at pH 7.5. We can see clearly that at low pH there is a sharp increase of $\Delta\Theta$. It saturates at about 200% and the mid-point is at about pH 5.5. There is a sharp decrease between pH 10 and 11. This decrease could be due to denaturation of the nerve or due to neutralization of a strong positively charged group. The latter is supported by the increase of $\Delta\Theta$ at low pH, where probably protonation of some moeities may take place, thus reducing the negative charges. A candidate for the positively charged group could be either amino group (pk = 10) or the guanidino group of arginine ($pK \approx 12$). The absence of $\Delta\Theta$ decrease before pH 10 supports the guanidino group as the possible source of $\Delta\Theta$. We will test this hypothesis further in the next section by probing protein side chains with chemical modifiers.

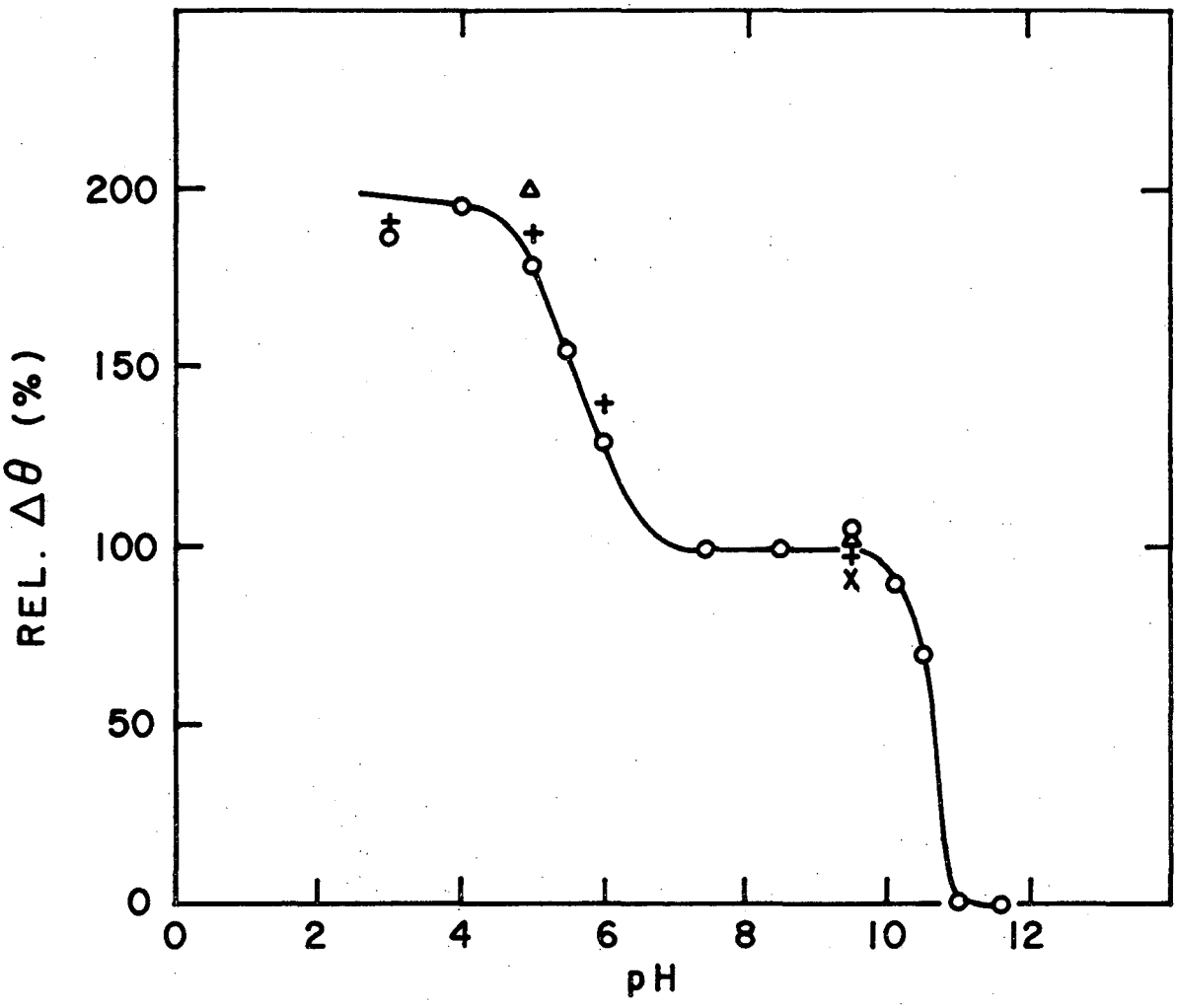

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Fig. 16.
The pH dependence of $\Delta\Theta$, normalized to $\Delta\Theta$ at pH 7.5. The data are the results obtained with many different nerves (one preparation per point)

B. Protein Reagents

1) Phenylglyoxal

The data from the pH dependence of $\Delta\Theta$ (Fig. 16) have suggested that at neutral pH a strongly basic group, presumably a guanidino, may be responsible for the voltage induced retardation change. One would expect also to see some changes of $\Delta\Theta$ upon chemical modification of this group. Phenylglyoxal is a reagent which reacts with guanidino groups of proteins near neutral pH (Takahashi, 1968). The reaction (Fig. 17) gives derivatives containing two phenylglyoxal moieties per guanidino group (Means and Feeney, 1971). Upon extensive treatment with phenylglyoxal, ε-amino groups may also react.

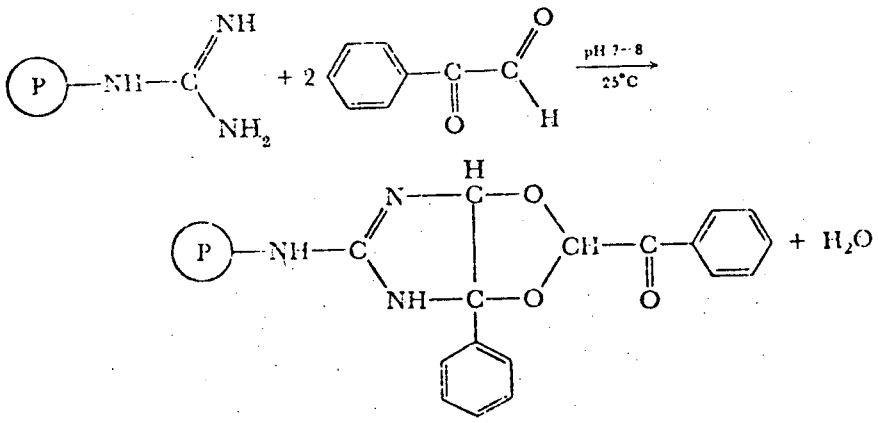

Fig. 17 : Reaction of the guanidino group of a protein with phenylglyoxal (from Means and Feeney, 1971, by permission of Holden-Day Inc.).

The effect of 10 mM phenylglyoxal on the nerve optical retardation is shown in Fig. 18. The experiment was performed in Ca^{++}-free solution to avoid the side-effects of calcium. We can see that $\Delta\Theta$ is quickly reduced, thus supporting the idea that a guanidino group may be the source of $\Delta\Theta$. The effect is however irreversible and a slow decrease of Θ_o also occured, suggesting some kind of denaturation. For comparison, the actions of other protein denaturants were also tested, e.g. sulfhydryl reagents and protein crosslinking agents. These are described below.

2) Sulfhydryl Reagents

Several reagents have been used to study the consequences of modification of the sulfhydryl groups of proteins. N-Ethylmaleimide (NEM) is a widely used sulfhydryl reagent. However, it is also able to react with amino groups at high pH values. At pH 7, its reaction rate with simple thiols is approximately 1000-fold greater than that with corresponding simple amino compounds (Means and Feeney, 1971). Mercurials are reagents that react rapidly and specifically with the sulfhydryl groups of proteins. The most commonly used mercurials are mercuric chloride ($HgCl_2$) and para chloro mercuribenzoate (PCMB). The reactions of NEM and mercurials with sulfhydryl groups are shown in Fig. 19.

The action potential of squid giant axon is blocked by internal or external application of N-Ethylmaleimide (NEM) and mercuric chloride (Huneeus-Cox et al., 1966). The inward (sodium) and outward (potassium) currents are also reduced by mercuric acetate (Gilbert and Lipicky, 1975). NEM inhibits the sodium current almost completely while potassium current is only slightly reduced (Shrager, 1975).

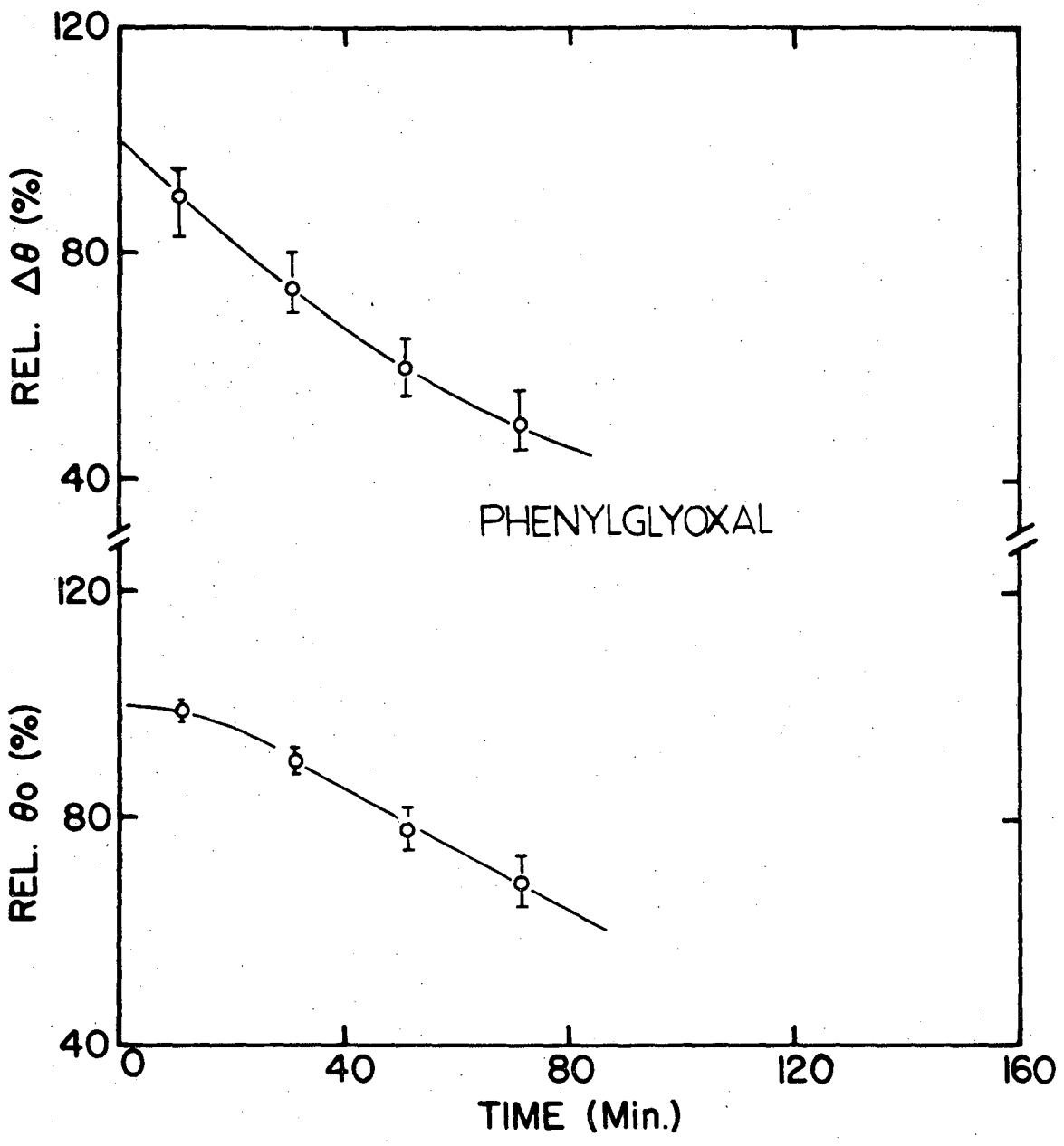

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Fig. 18.
The effect of 10 mM phenylglyoxal on $\Delta\Theta$ and Θ_o.

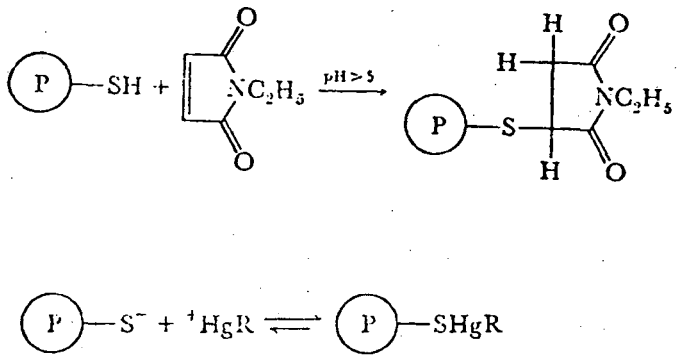

Fig. 19 : Reactions of a sulfhydryl group with N-Ethylmaleimide and with mercurials (from Means and Feeney, 1971, by permission of Holden-Day Inc).

The effects of NEM (2 mM) and $HgCl_2$ (10^{-4} M) on the optical retardation of the nerve are shown in Fig. 20. Both $\Delta\Theta$ and Θ_o are strongly reduced. The experiments with NEM were performed in a calcium-free medium while the experiments with $HgCl_2$ were performed in a regular saline. However, a few experiments with $HgCl_2$ in a Ca^{++}-free medium also showed a strong reduction of $\Delta\Theta$, although Θ_o was reduced at a slower rate. The effects of these drugs on $\Delta\Theta$ and Θ_o were irreversible.

PCMB at a concentration of 10^{-4} M in the regular saline medium also reduced $\Delta\Theta$ and Θ_o, but compared to the effect of $HgCl_2$ the decrease was not as strong (Fig. 20). Typically after one hour $\Delta\Theta$ was only reduced by 20% and Θ_o by 30%. This drug was initially dissolved in a drop of NaOH solution before it was diluted with saline. The solubility at neutral pH is limited. This fact and may be also the presence of the negative charge of the carboxyl group could cause difficulty for the drug to penetrate the membrane.

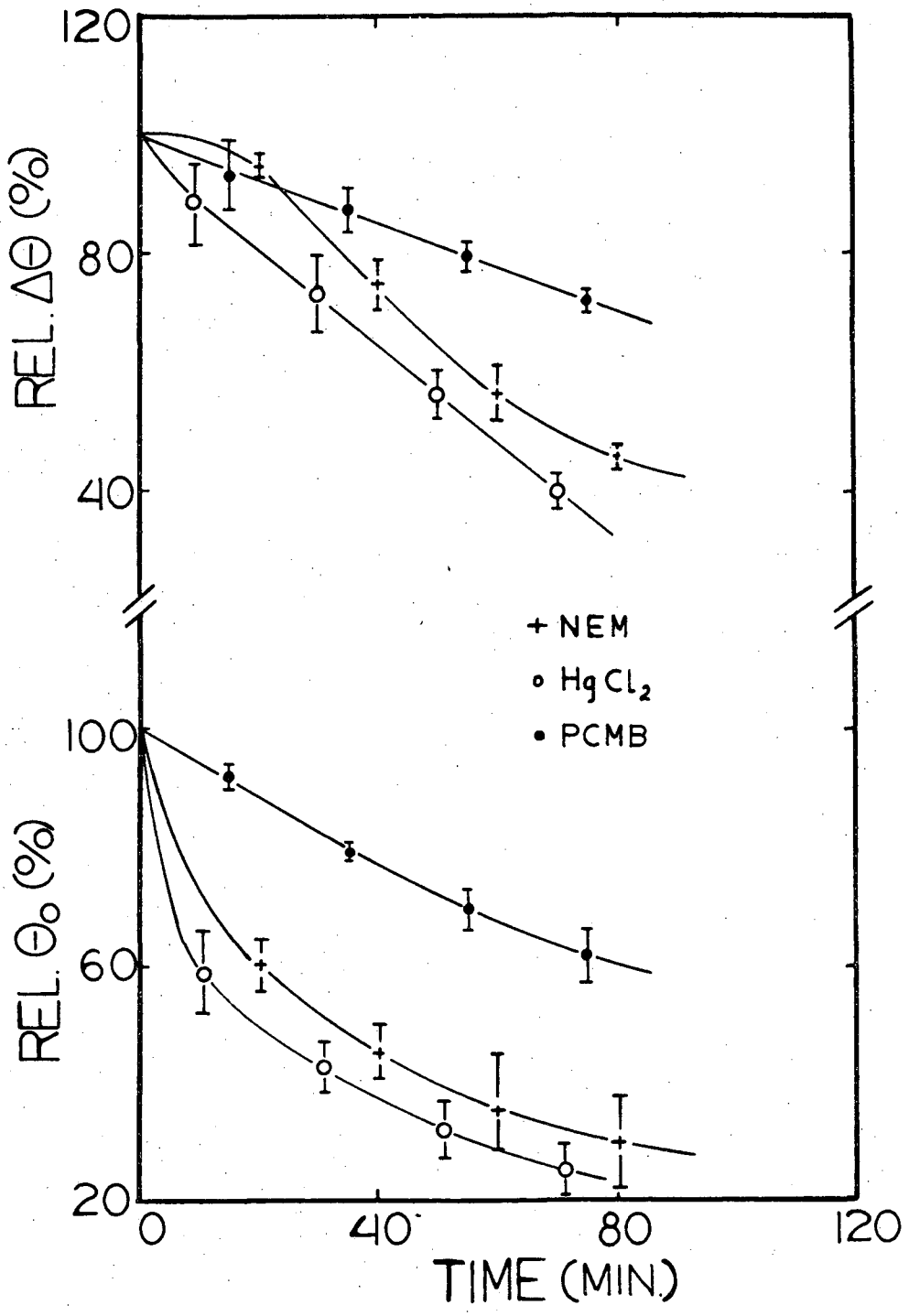

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Fig. 20.
The effects of NEM (2 mM), $HgCl_2$ (10^{-4} M) and PCMB (10^{-4} M) on $\Delta\Theta$ and Θ_o.

3) Glutaraldehyde

Glutaraldehyde, a protein crosslinking agent, was first introduced as a fixative by Sabatini et al. (1963). This is such a gentle fixative that it can leave many cellular enzymes undisturbed as to function, and is therefore of great value in studies with the electron microscope. The crosslinking reaction (Fig. 21) between polymeric glutaraldehyde and two primary amino groups was proposed by Richards and Knowles (1968). The amino groups are converted to secondary amines connected by a five-carbon bridge. The effect of glutaraldehyde on rhodopsin has been reported by Brown (1972) and Cone (1972). Following glutaraldehyde fixation, the photoinduced dichroism remains, probably as the result of crosslinking. No dichroism will remain if the rhodopsin has not been fixed previously, since the molecules are free to rotate and undergo random Brownian motion. Glutaraldehyde treatment also reduces the motion of maleimide spin labels covalently attached to proteins (Jost et al., 1973).

Shrager et al. (1970) have shown in crayfish axons, that small amounts of glutaraldehyde reduce and widen the action potential, especially during the falling phase. Voltage clamped squid axons are also affected by glutaraldehyde. The membrane currents decrease and the onset of the outward current is delayed (see appendix). This suggest that if glutaraldehyde forms molecular bridges, then the molecular motions are greatly restricted, or in other words the membrane becomes immobilized. If this is the case, one can expect that the voltage induced change in retardation should also be greatly reduced by glutaraldehyde.

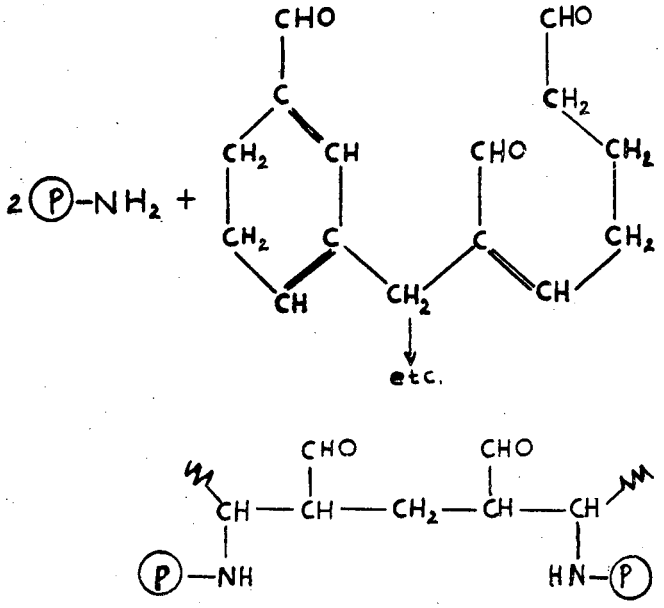

Fig. 21 : Reaction between two amino group and glutaraldehyde (adapted from Richards and Knowles, 1968).

Fig. 22 shows the effect of 0.1% by volume glutaraldehyde (in Ca^{++}-free solution) on the nerve optical retardation. $\Delta\ominus$ decreases strongly while $\ominus_o$ increases slightly. This effect is irreversible and usually the nerve becomes rather stiff and slightly brown-colored at the end of the experiment. No decrease of $\Delta\ominus$ and $\ominus_o$ could be detected when formaldehyde was given to the nerve at 0.1%. Compared to glutaraldehyde, formaldehyde is a weaker crosslinking agent. However, when it was given at a higher concentration (about 1%), then a decrease was seen.

The results of the experiments with protein reagents give a strong suggestion that proteins may be involved in the generation of $\Delta\ominus$. This is also supported by the fact that glutaraldehyde treatment has little or no effect on the fluid bilayers (Jost et al., 1973). They observed no changes in the electron spin resonance spectra of a lipid spin label in nerve bundles upon exposure to glutaraldehyde.

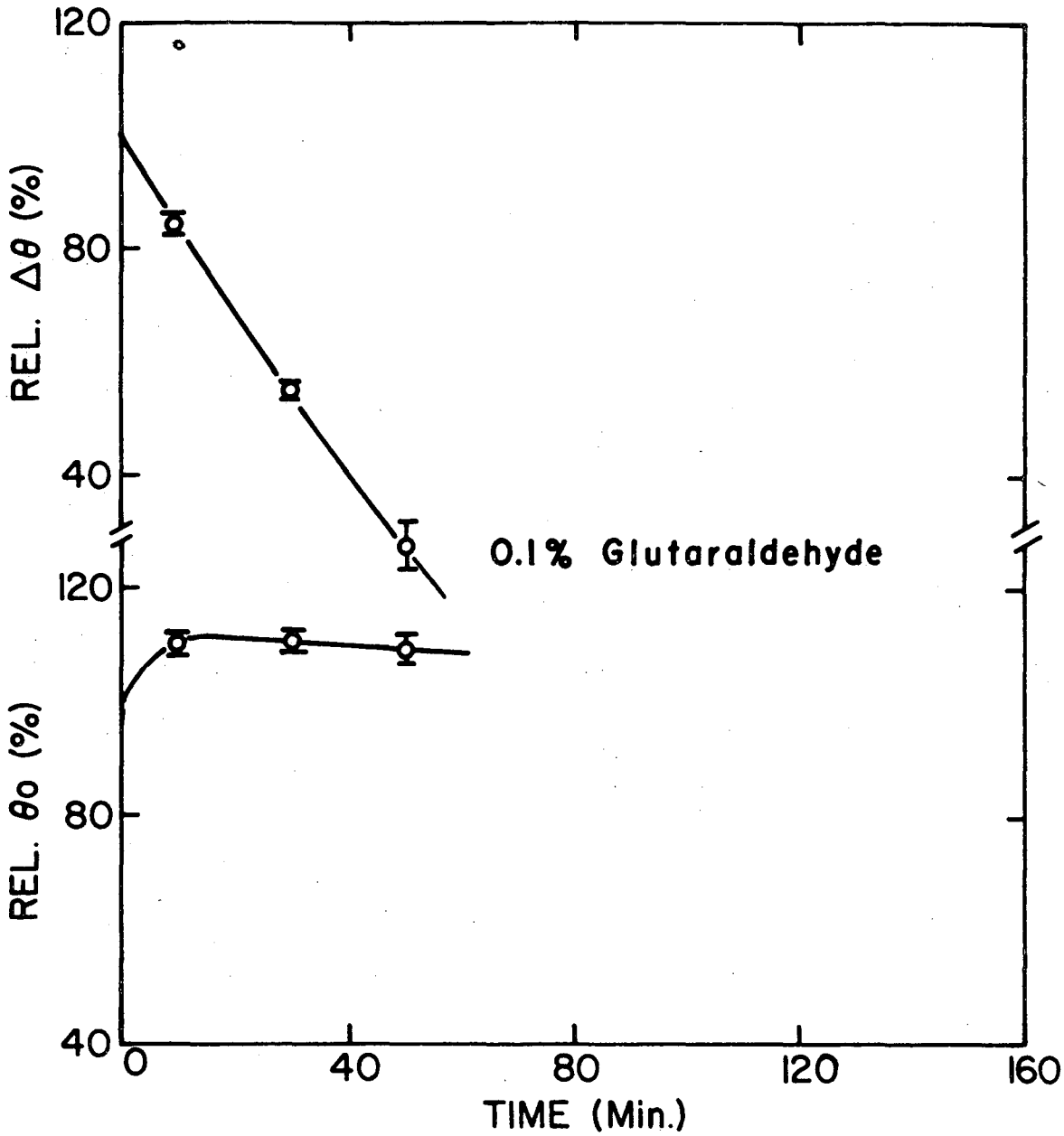

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Fig. 22.
The effect of glutaraldehyde (0.1% by volume) on $\Delta\Theta$ and Θ_o.

Further tests with lipid reagents will be described in the next section to check the role of lipids on the generation of $\Delta\ominus$.

C. Modification of Phospholipids

1) Uranyl Ion (UO_2^+)

UO_2^+ has been shown to bind very effectively at extremely low concentrations (10^{-6} to 10^{-5} M in the bulk aqueous phase) to negative surface charges on phosphatidyl serine bilayers, whereas much higher concentrations (10^{-3} to 10^{-1} M in the bulk phase) of the alkaline earths bind minimally and predominantly screen these charges (McLaughlin, Szabo and Eisenman, 1971). An extensive uranyl ion binding can cause the negative surface charge density on the axon to be decreased considerably. This was evidenced by the fact that equal concentrations of alkaline-earth cations did not have the same effect on the threshold potential (D'Arrigo, 1975). Thus uranyl ion can be used to study the role of the phosphate headgroups of phosphatidyl serine on the voltage induced retardation change. When uranyl nitrate was added to the medium, no significant changes of $\Delta\ominus$ or $\ominus_0$ could be detected. After one hour incubation, the relative value of $\Delta\ominus$ was 95% and that of $\ominus_0$ was 100%. The drug was given at a concentration of 10^{-3} M, but the effective concentration was only on the order of 10^{-4} M since it was not completely soluble at physiological pH. The effect on the threshold potential was significant at about 10^{-5} M although uranyl ion did not have any detectable effect on the actual shape of the action potential itself (D'Arrigo, 1975). The absence of an effect of uranyl ion on $\Delta\ominus$ suggests that the negative charge of phosphotidyl serine is not the primary origin of $\Delta\ominus$.

Other reagents that perturb the phospholipids are phospholipases and detergents. The effects of these drugs will be described below.

2) Phospholipases

Phospholipases are able to disrupt the lipid bilayer of membranes. The sites of action of phospholipases on phosphoglycerides are shown in Fig. 23 (Lehninger, 1970). Phospholipase A (found in some snake venoms) cleaves one fatty acid linkage, leaving the polar heads of the phospholipid molecules intact. However, the product, lysophosphatide, is toxic and injurious to membranes. Phospholipase B is a mixture of phospholipase A and lysophospholipase. The lysophospholipase removes the fatty acid from lysophospholipids. Phospholipase C hydrolyses the ester linkage between the α-carbon of glycerol and the phosphate group in some phospholipids. Phospholipase D hydrolyses the linkage between the phosphate and the rest of the head group.

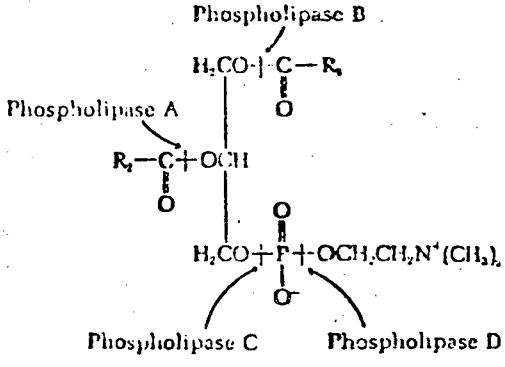
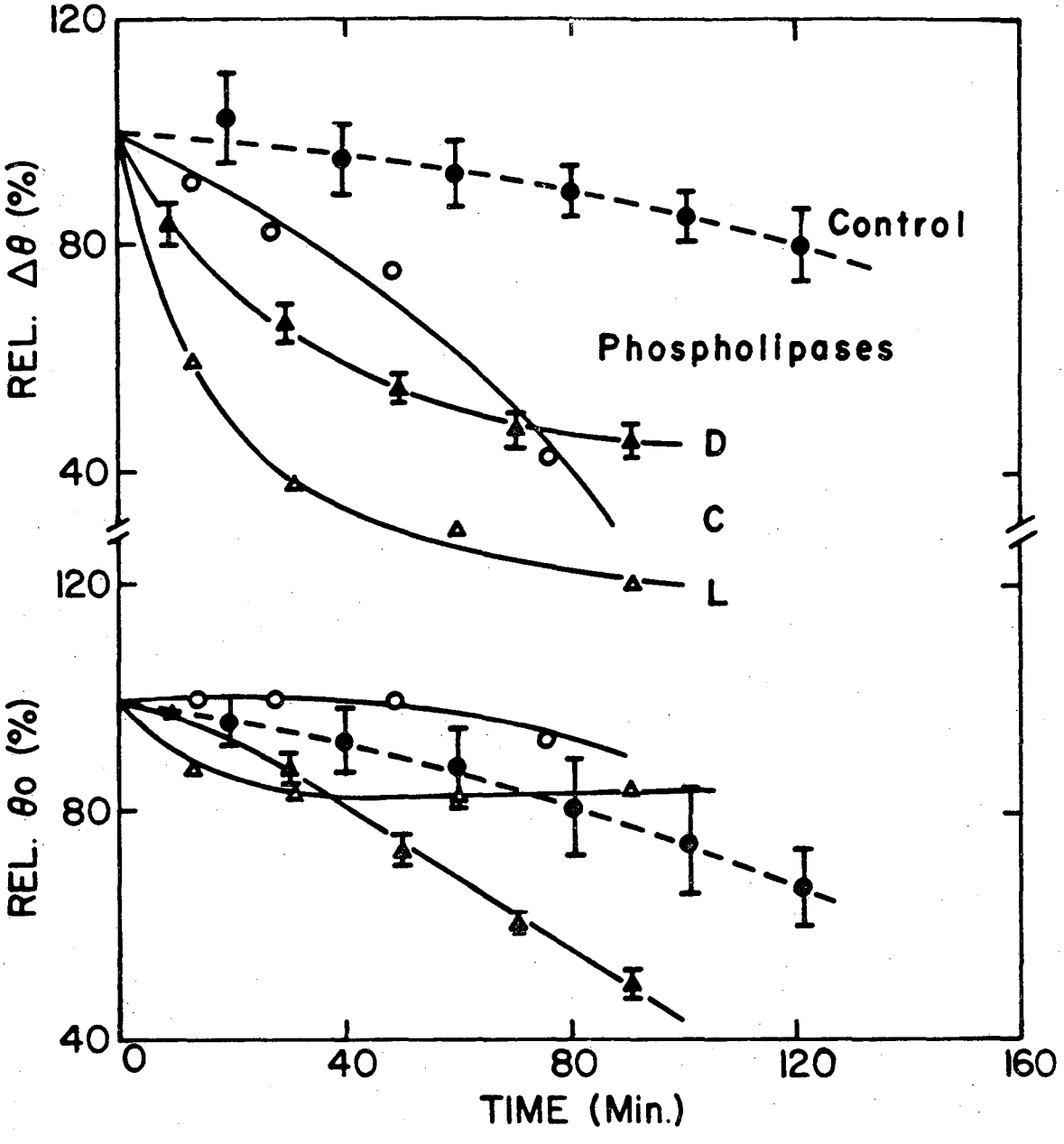


Fig. 23 Sites of action of phospholipases on phosphatidyl choline (from Lehninger, 1970)

The effects of phospholipases on the excitability of the nerve have been investigated by many workers. In general, internal application of phospholipases A, C or D suppresses the excitability (Tasaki and Takenaka, 1964; Abbott et al., 1972). The effects of externally administered phospholipases are still controversial, since the purities of the enzymes used by different groups are not identical. In this study we will focus our attention mostly to the effects on the optical retardation. Fig. 24 shows typical effects of 0.5 mg/ml lysophosphatidylcholine (product of phospholipase A and phosphatidyl choline), phospholipase C (1 mg/ml) and phospholipase D (1 mg/ml). These phospholipases in general reduce $\Delta\Theta$ irreversibly. This effect was also seen in a few experiments where calcium was absent. Phospholipase D also reduces Θ_o quite significantly. In the experiments with the other phospholipases, Θ_o stays almost constant and in many cases it is larger than the values of the control experiments. It is possible that the membrane is disrupted by the action of the phospholipases. This possibility will be investigated by studying the effects of detergents on the nerve.

3) Detergent

A detergent is usually a surface-active agent containing a hydrophilic moiety at one end and a fairly long chain lipophilic group at the other end. Kishimoto and Adelman (1964) have studied the effects of detergents on squid giant axon resting and action potentials as well as membrane conductances in the voltage clamp. Sodium lauryl sulfate or sodium dodecyl sulfate (an anionic detergent) at 0.1 to 1 mM causes a temporary increase and a later irreversible decrease of



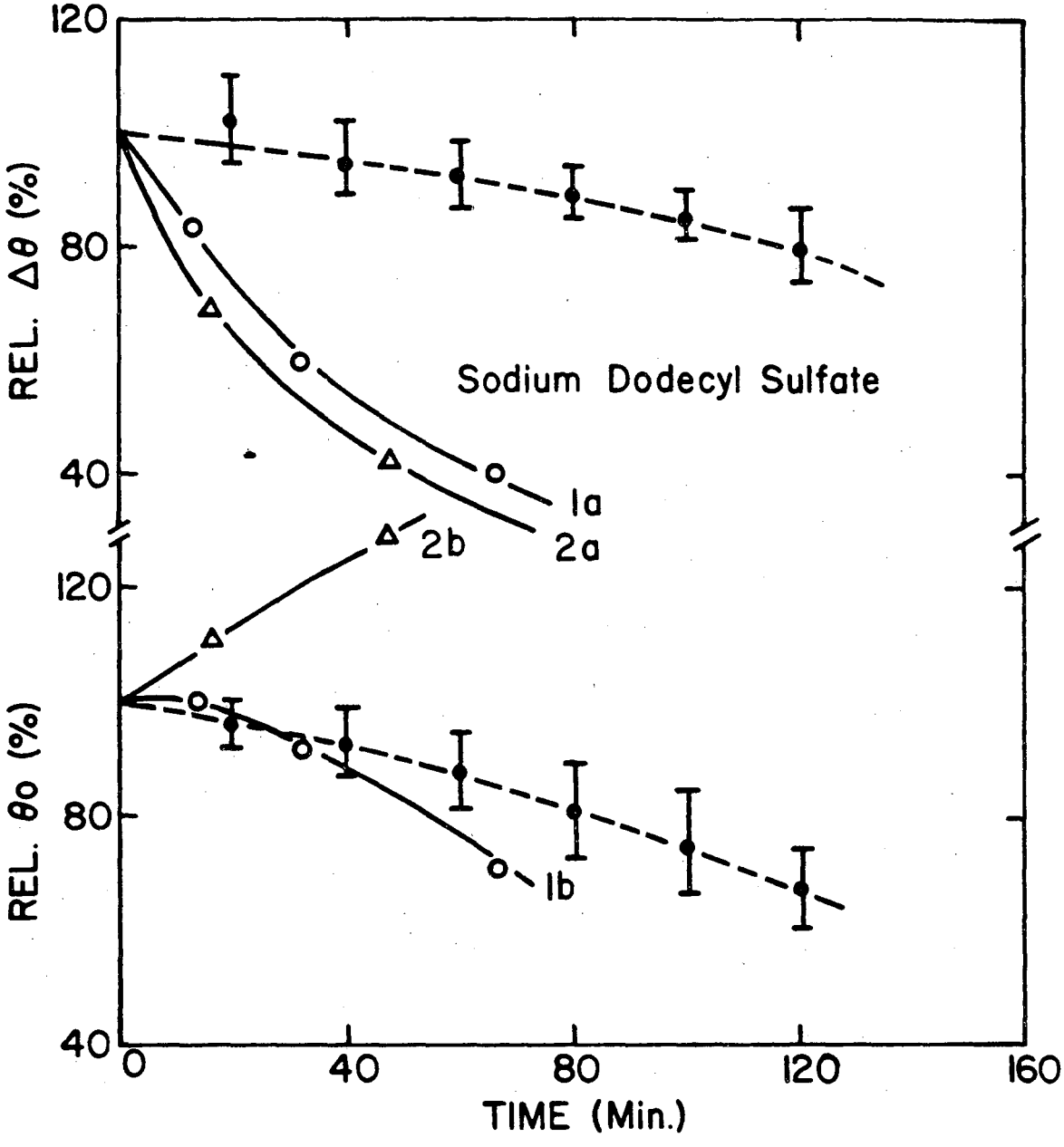
XBL 769-9618

Fig. 24.
The effects of 1 mg/ml phospholipase D, 1 mg/ml phospholipase C and 0.5 mg/ml lysophosphatidylcholine (L) on $\Delta\Theta$ and Θ_o.

action potential height and the value of the resting potential. The sodium and potassium conductances are also reduced irreversibly.

The typical effects of 10^{-4} M sodium dodecyl sulfate on the optical retardation are shown in Fig. 25. One experiment was done in a regular saline solution, containing calcium (curves 1a and 1b), and one other experiment was done is a calcium-free medium (curves 2a and 2b). In both cases $\Delta\Theta$ decreases, while Θ_o decreases slightly (1b) or even increase (2b). The increase of Θ_o will be discussed further in the Discussion section.

Triton X-100, a non-ionic detergent, showed almost similar effects as sodium dodecyl sulfate. At a concentration range between 10^{-3} and 10^{-1}% (by volume) $\Delta\Theta$ was reduced strongly but Θ_o decreased only slightly (Fig. 26). In this case the experiments were done using just the normal saline solution. The decrease of Θ_o could be due to entry of calcium ions. Sodium lauryl sulfate also causes a considerable increase in leakage current for hyperpolarized potentials (Kishimoto and Adelman, 1964). It was assumed that this phenomenon implies a non-specific leak or an increased general permeability of the membrane to all ions and may in part be responsible for the degradation of the nerve. The decrease of $\Delta\Theta$ caused by the detergents (even in calcium-free medium) indicates that the intact structure of the membrane is necessary for the generation of $\Delta\Theta$. The general actions of the phospholipases resemble those of the detergents and thus they may reflect a non-specific disruption of the lipid matrix. Based on these results and on the strong effects of protein reagents described earlier, it seems beneficial to look for specific proteins that may be associated with the change in the optical retardation.



XBL 769-9612

Fig. 25.
The effect of 10^{-4} M sodium dodecyl sulfate on $\Delta\Theta$ and Θ_o. 1) experiment in regular saline, 2) experiment in Ca^{++}-free saline. Broken lines are control experiments in regular saline.

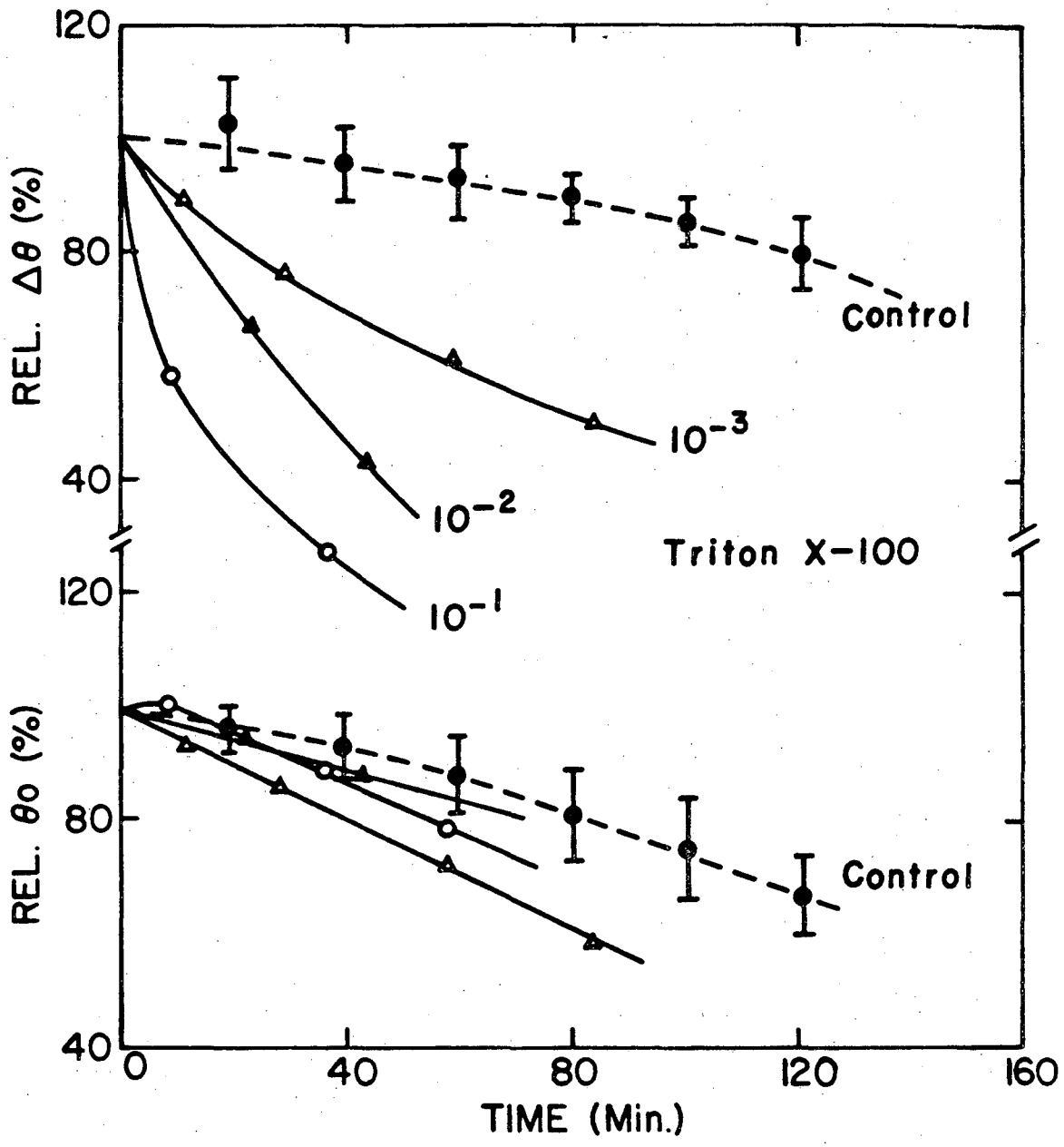


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Fig. 26.
The effects of Triton X-100 on $\Delta\Theta$ and Θ_o.
Numbers correspond to concentration (volume %).

D. Cholinergic Compounds

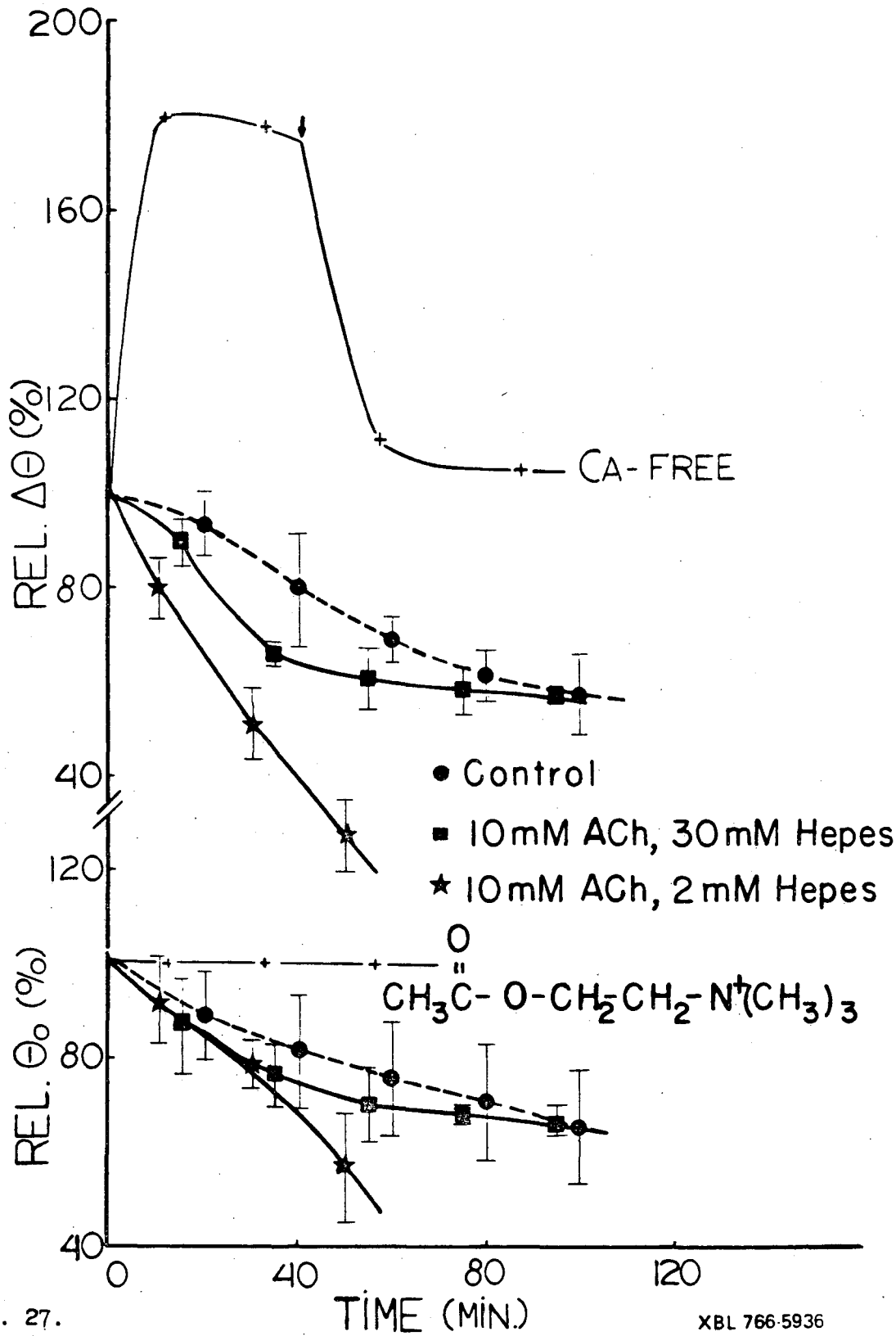
Nerve axon membranes are known to exhibit considerable acetylcholinesterase activity (Nachmansohn, 1959; Neumann and Nachmansohn, 1975; Villegas and Villegas, 1974; Soeda et al., 1975; Denburg et al., 1972, Balerna et al., 1975). Acetylcholinesterase inhibitors and acetylcholine block excitation in several types of nerve fibers (Dettbarn and Bartels, 1968; Hoelman and Dettbarn, 1971). At present there are some conflicting ideas about the role of acetylcholine in nerve conduction. The localization of acetylcholinesterase in the giant nerve fiber of the squid was established by histochemical electron microscopic determination and it was found to be distributed along the axolemma and attached to its axoplasmic leaflet (Villegas and Villegas, 1974). At these regions the trilaminar substructure of the axoplasm and a narrowing of the axon-Schwann cell interspace were also present. Recently, Villegas (1975) has also shown that a cholinergic system is involved in the genesis of the long-lasting Schwann cell hyperpolarization in the squid giant nerve fiber. However, the presence of acetylcholine and its related enzymes in the peripheral nerves was once thought to be related to their transport towards the active physiological sites of the nerve terminals at the synapses and neuromuscular junctions (Koenig and Koelle, 1961). On the other hand, a complex, multi-reaction model suggesting that the permeability changes of excitable membranes are controlled in vivo by specific reactions involving the acetylcholine system has been proposed by Nachmansohn (1959) and Neumann et al. (1975). Although the concept is supported by a number of biochemical and biophysical data, more definitive tests are still required. Denburg et al. (1972, 1973) isolated macromolecules

from lobster axon membranes that bind cholinergic ligands and local anesthetics. The exact roles of the acetylcholinesterase and of the cholinergic binding macromolecule in the axon membrane deserve further experimental studies. The effects of some cholinergic compounds, anticholinesterase and local anesthetics on $\Delta\Theta$ and Θ_o will be described next.

1) Acetylcholine Group

Acetylcholine in concentrations of about 10 mM depolarize the membrane, prolong the action potential, and with progressive depolarization reversible block of conduction occurs (Dettbarn and Bartels, 1968). The response of a cholinergic membrane to acetylcholine might involve several sites of action, and the active site of acetylcholinesterase might be one. Acetylcholine is hydrolyzed at an appreciable rate by the membrane bound acetylcholinesterase. An acetylcholine induced depolarization response in lobster giant axon has been demonstrated to be due to localized acidification of the membrane caused by the hydrolysing activity of acetylcholinesterase (Hoekman and Dettbarn, 1971). A similar depolarization phenomenon was reported for eel electroplax in which the receptor sites had been modified by hypertonic solution, leaving the cholinesterase activity unaltered (Podleski and Changeux, 1967).

Adding 10 mM acetylcholine to the solution bathing the nerve in the central chamber markedly reduced the voltage induced retardation change $\Delta\Theta$ and the resting retardation Θ_o (Fig. 27). The decrease of $\Delta\Theta$ and Θ_o was much faster in a medium containing low buffer concentration (2 mM) than in a medium with a high buffer concentration (30 mM).



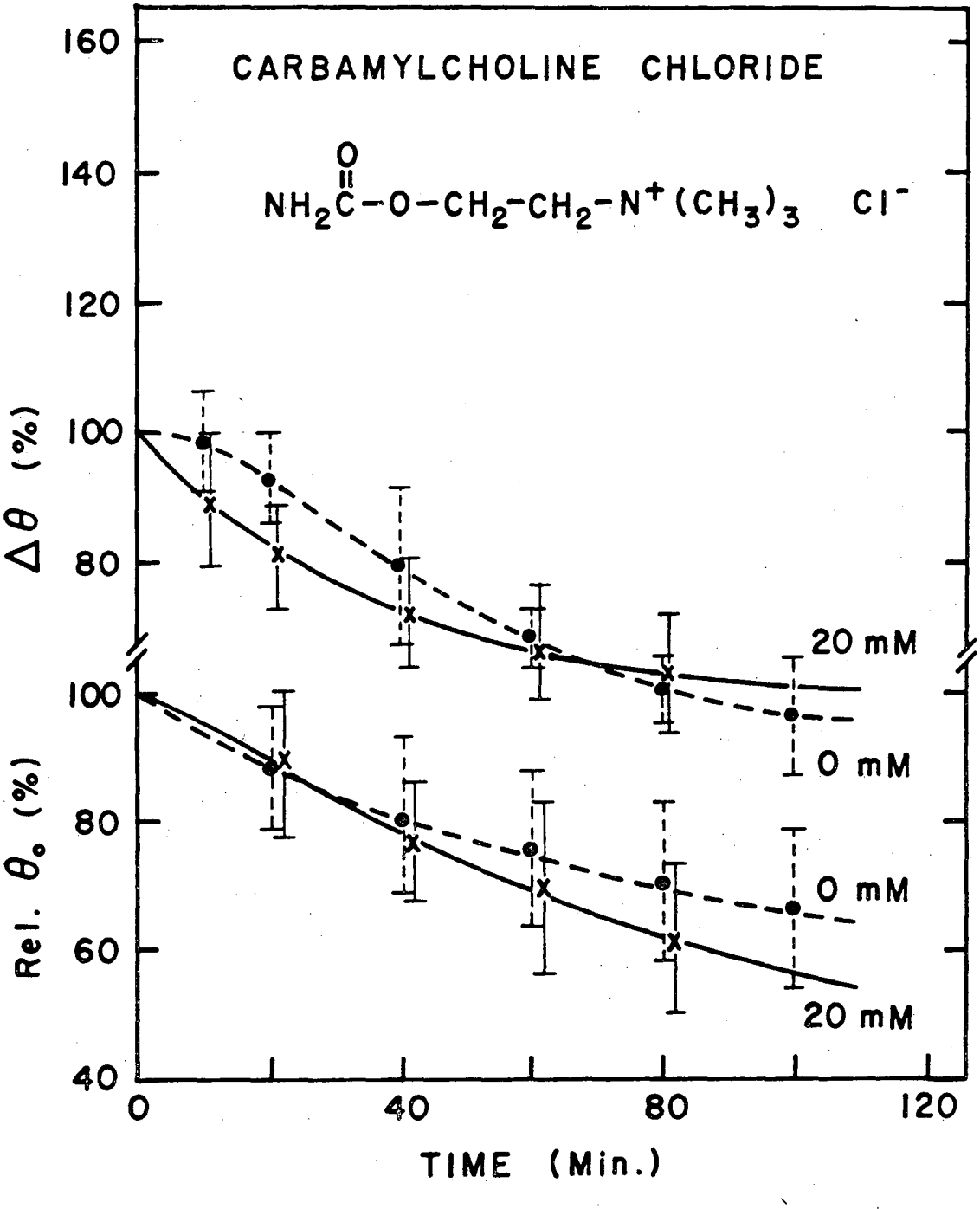
XBL 766-5936

Fig. 27.
The effects of 10 mM acetylcholine (ACh) on $\Delta\Theta$ and Θ_o at different buffer concentrations (2 mM or 30 mM Hepes) in the regular solution. The curves at the top (+ —— +) show results in Ca^{++}-free solution with 10 mM ACh. At the downward arrow this solution was changed with a Ca^{++}-free solution without ACh.

The control experiments were done with 10 mM Hepes buffer. When lower or higher concentrations of buffer were used in the control experiment, no significant changes were detected. Similarly no effect was seen when 20 mM choline or acetic acid, the hydrolysis products of acetylcholine , was added to the medium at a carefully maintained pH.

When the experiment with acetylcholine was performed in a calcium-free medium, the result was different. As shown in Fig. 27 (top), $\Delta\Theta$ increased by about 80% while Θ_o stayed constant at 100%. The effect was reversible upon return (arrow) to the calcium-free control medium. In this case $\Delta\Theta$ went back to near 100% level. Thus the data in Fig. 27 suggest that acetylcholine induced a change in local pH, probably due to a local release of acetic acid when acetylcholine was hydrolysed by the acetylcholinesterase of the tissue. The pH change was also seen clearly when the nerve bundle was placed on top of a pH indicator paper. Adding a drop of solution that contains acetylcholine (10 mM) buffered with Hepes (10 mM) at pH 7.4, changed the color of the paper near the tissue to yellow-orange (pH 5) in 10 minutes, although the color of the surrounding liquid still remains light green (pH 7).

On the basis of the above observations it was therefore of interest to test the effects of cholinergic compounds that are not hydrolysed by the acetylcholinesterase. This kind of study can determine whether there are any other structures with which the acetylcholine can interact and cause the changes as shown in Fig. 27. Some cholinergic compounds such as carbamylcholine, tetramethylammonium, tetraethylammonium, hemicholinium-3, hexamethonium, decamethonium, d-tubocurarine induced no significant effect. These experiments were performed using the regular saline. The results are shown in Fig. 28

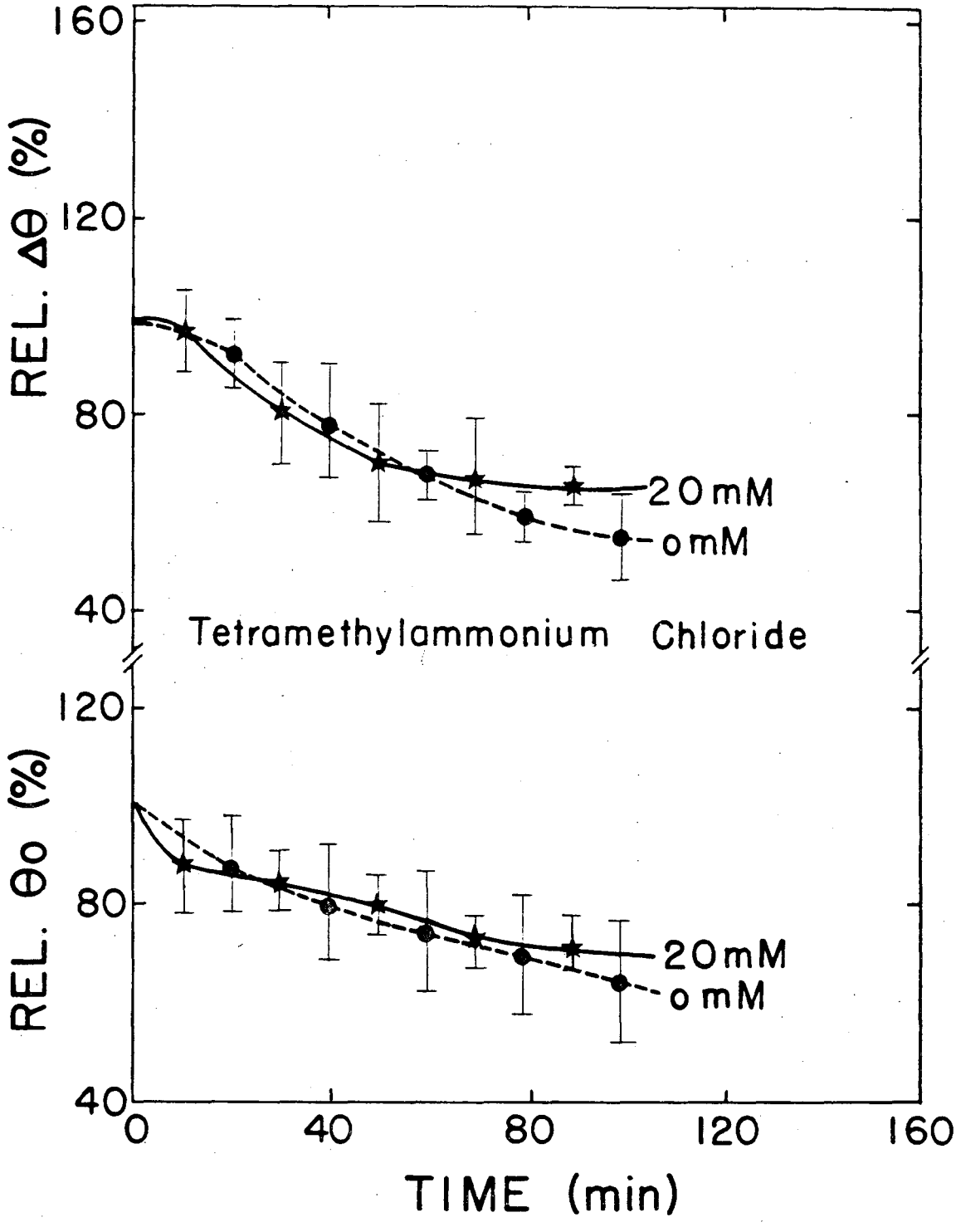


XBL 764-5830

Fig. 28.
The effect of carbamylcholine (20 mM or 0 mM) on $\Delta\Theta$ and Θ_o. $\Delta\Theta$ was induced by pulses of 64 msec duration.

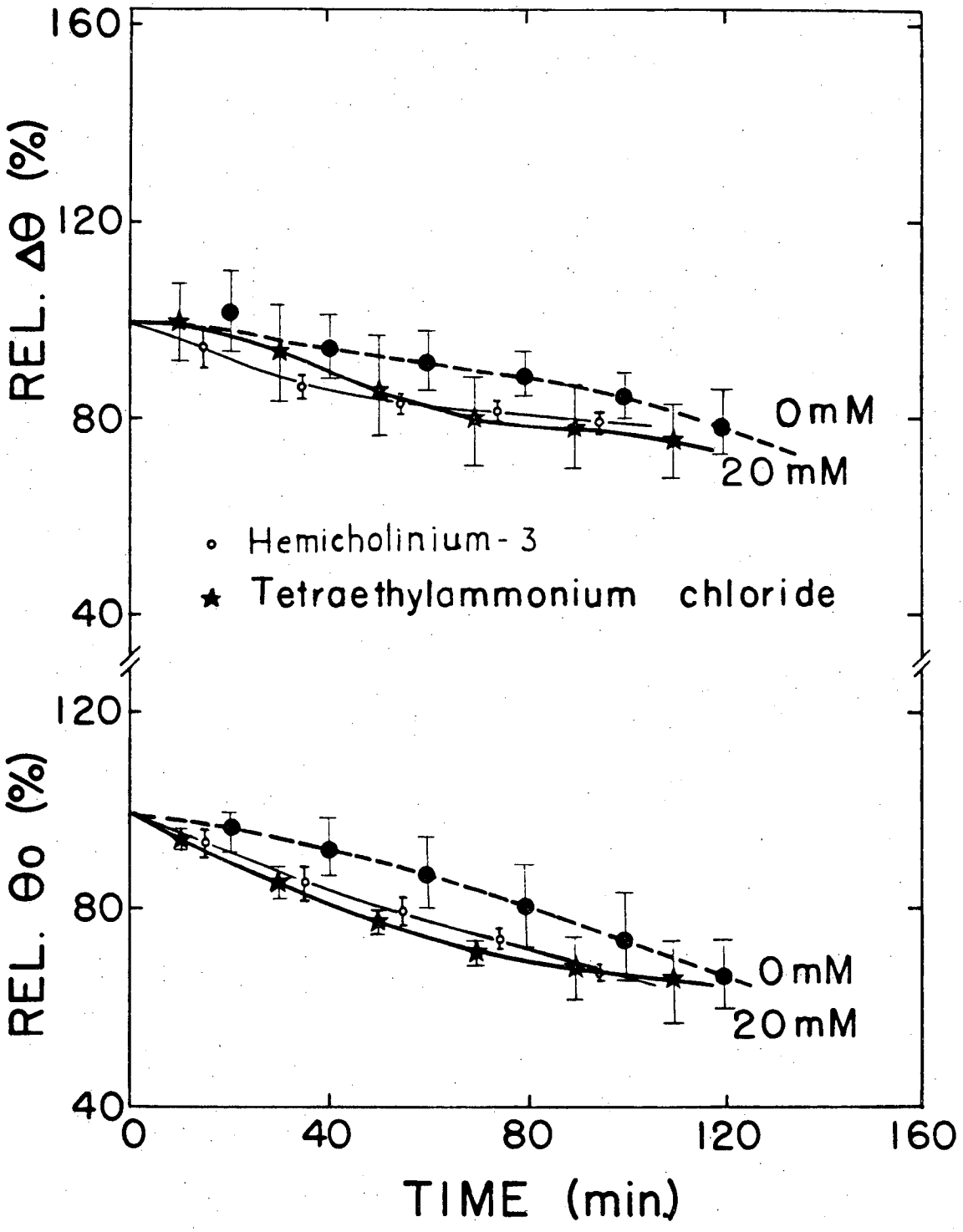
for carbamylcholine, in Fig. 29 for tetramethylammonium, in Fig. 30 for tetraethylammonium and hemicholinium-3, in Fig. 31 for hexamethonium and decamethonium, and in Fig. 32 for d-tubocurarine. The experiments with carbamylcholine and tetramethylammonium were done using 64 msec pulses which caused the values (also in control experiments) to decrease faster (see Fig. 8). The lack of an effect of the previously listed compounds may indicate that they have difficulties penetrating the membrane, perhaps due to their charged quarternary amine nature. Yeh and Narahashi (1974) have reported that external application of carbachol, acetylcholine, hexamethonium, decamethonium, d-tubocurarine have little or no effect on resting and action potentials. However, when perfused internally, they decrease the height of the action potential and prolong its duration. Ionic conductances are also suppressed.

External application of 10 mM nicotine results in a 30-40% reduction of the steady state current (Frazier et al., 1973). Like the other cholinergic ligands, nicotine is also more potent in blocking membrane ionic conductances when applied from the inside of the membrane than from the outside. It is known that nicotine binds to a component from a preparation of an axon-plasma membrane with a dissociation constant of 0.42 ± 0.04 mM (Denburg et al., 1972). Nicotine has an interesting effect on $\Delta\Theta$, as shown in Fig. 33. Given at 20 mM (pH 7.5) it gradually lowered the values of $\Delta\Theta$ and Θ_0 below the control experiment in the regular saline solution. However, when the experiment with nicotine was performed in the calcium-free medium, $\Delta\Theta$ was increase 20% temporarily while Θ_0 stays close to 100% level. Care was taken that the nerves were equilibrated long enough



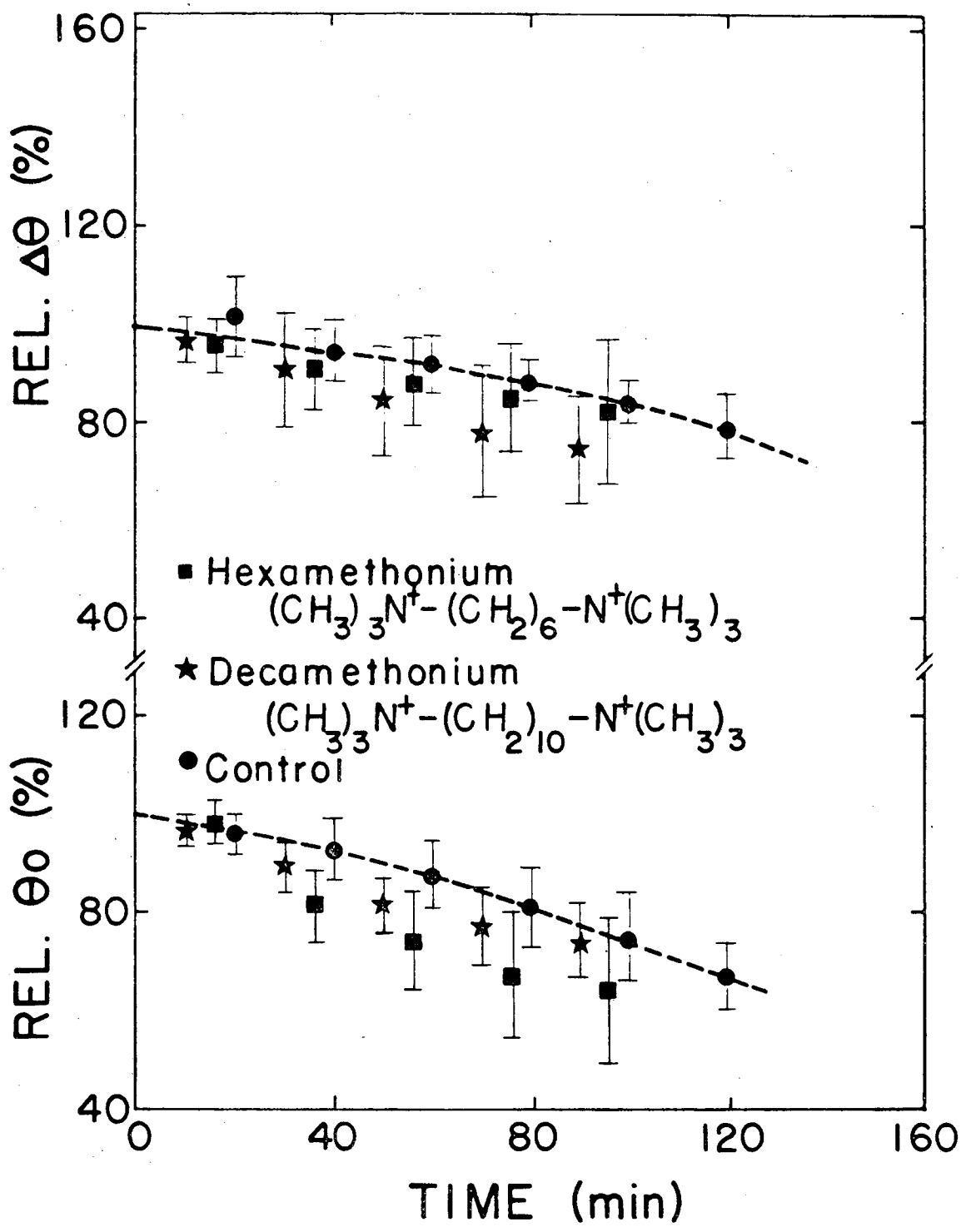
XBL 766-5935

Fig. 29.
The effect of tetramethylammonium (20 mM or 0 mM) on $\Delta\Theta$ and Θ_o. $\Delta\Theta$ was induced by pulses of 64 msec duration.



XBL 766-5938

Fig. 30.
The effects of 20 mM tetraethylammonium and hemicholinium-3 on $\Delta\Theta$ and Θ_o. Control experiment (0 mM) in regular saline.



XBL 766-5940

Fig. 31.
The effects of 20 mM hexamethonium and decamethonium on $\Delta\Theta$ and Θ_o.

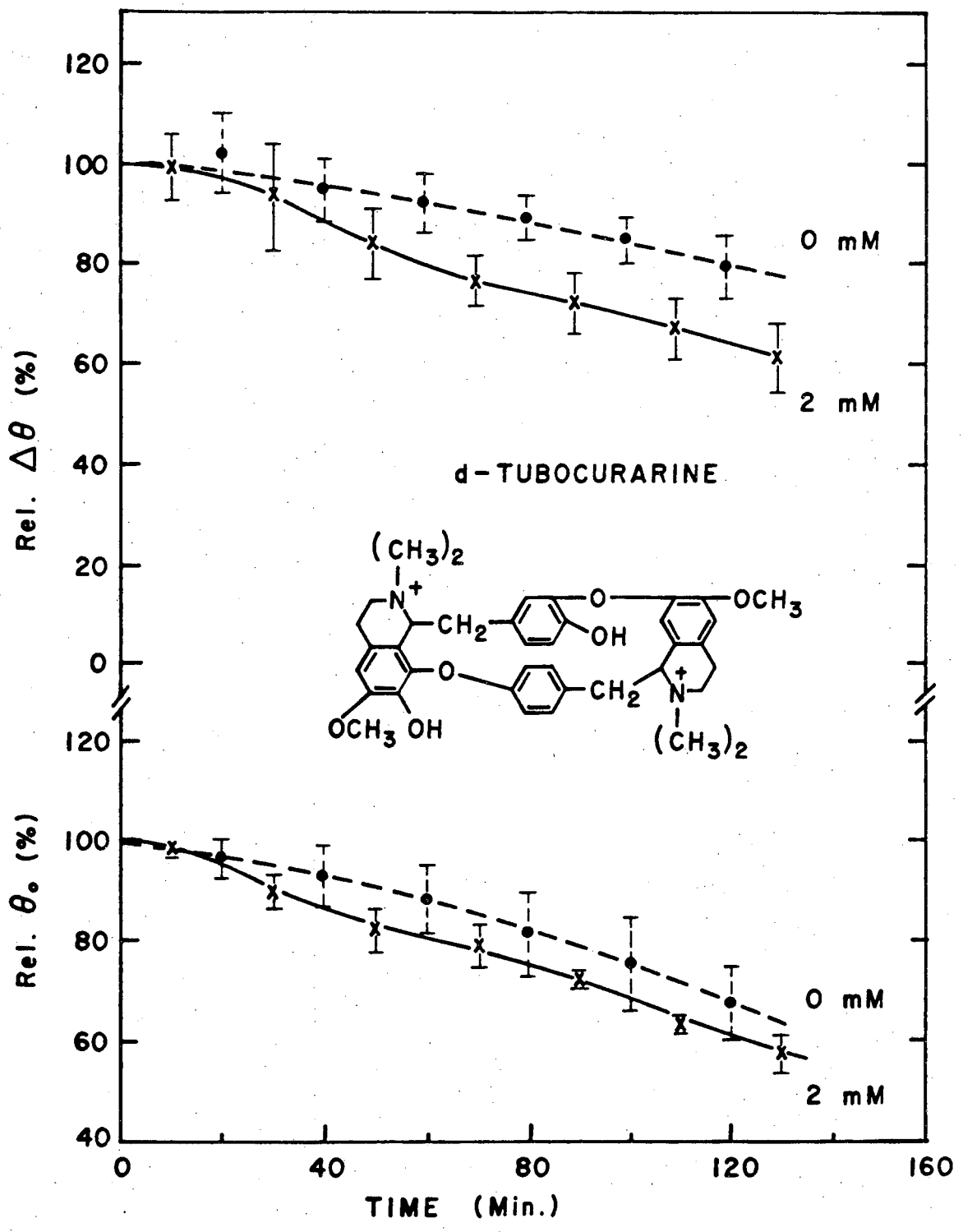


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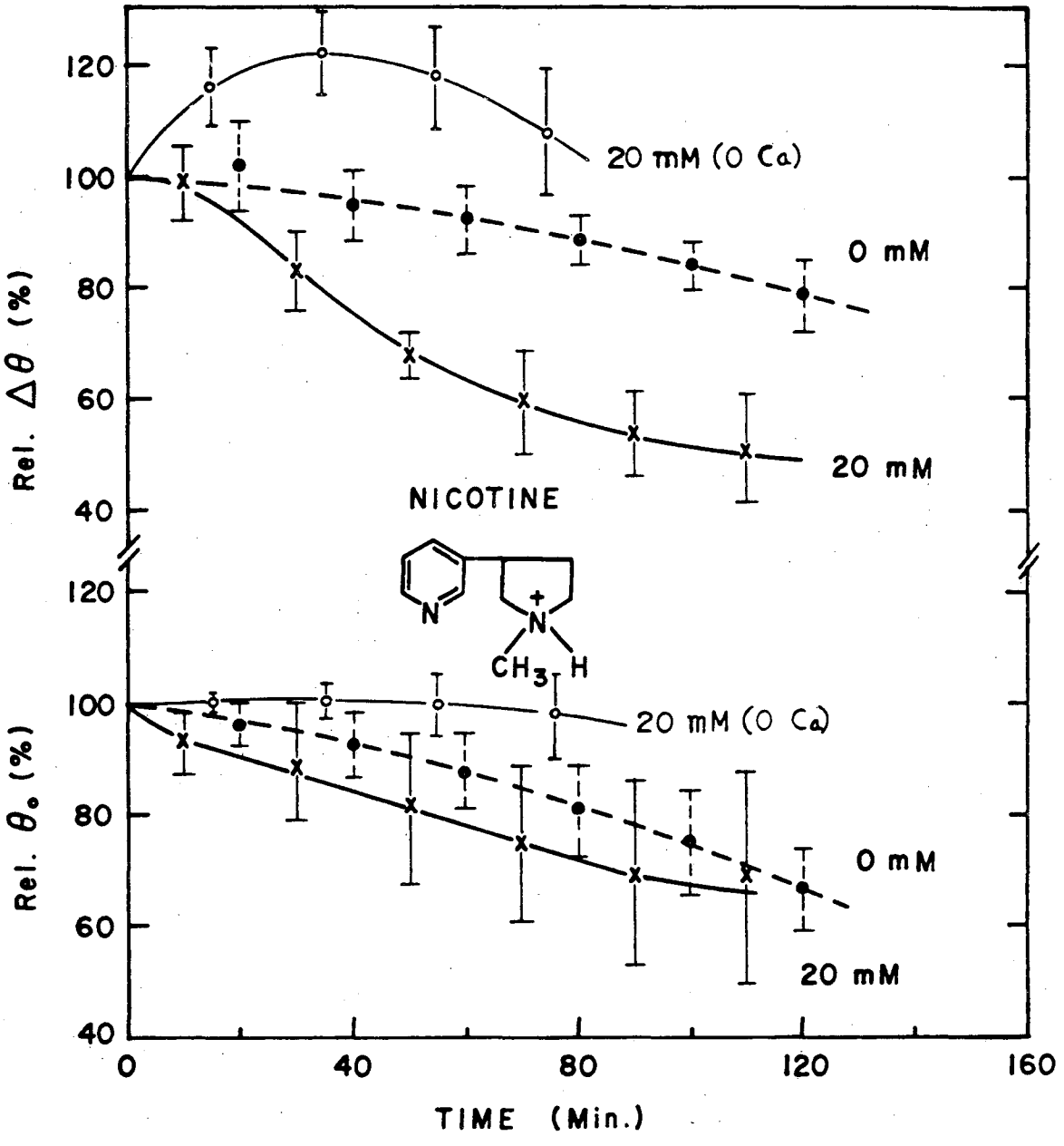
Fig. 32.
The effect of 2 mM d-Tubocurarine on $\Delta\Theta$ and Θ_o.



XBL 764-5831

Fig. 33.
The effect of nicotine (20 mM) on $\Delta\Theta$ and Θ_0: with Ca^{++} (x——x) or without Ca^{++} (o——o). Control experiment (0 mM nicotine) with Ca^{++} (●——●).

(at least 20 minutes) with a Ca^{++}-free solution, before the nicotine was added.

2) Anticholinesterase: Eserine (physostigmine) and neostigmine (prostigmine)

Both eserine and neostigmine are known to be potent inhibitors of acetylcholinesterase extracted from many different tissues (Nachmansohn, 1959; Soeda et al., 1975). In monocellular electroplax preparations, eserine at $5*10^{-5}$ M inhibits the acetylcholinesterase, while at $5*10^{-3}$ M it prevents the usual acetylcholine induced depolarization (Higman and Bartels, 1962). Eserine also inhibits the binding of nicotine to the axonal cholinergic binding macromolecule (Denburg et al., 1972). Dettbarn and Bartels (1968) have studied the electrophysiological effects of eserine and neostigmine on the giant axons of the lobster. Eserine at 10 mM caused a 15 mV depolarization and the action potential was prolonged before it was blocked. Neostigmine did not have an effect on the membrane or on the action potential although it prevented the depolarizing effect of acetylcholine. The effect of 10 mM eserine on the optical retardation is shown in Fig. 34. Care was taken to minimize the exposure of the eserine solution to heat, light and air. Solutions that showed a pink color, probably due to hydrolysis, were discarded. $\Delta\Theta$ was reduced significantly and Θ_o was slightly lower than in control. In this case the side effects of calcium were also checked. When the same experiments with eserine were performed in a Ringer solution that contained no calcium, different results were obtained (top curves). Both $\Delta\Theta$ and Θ_o increased for the first 40 minutes and then decline. This kind of phenomenon

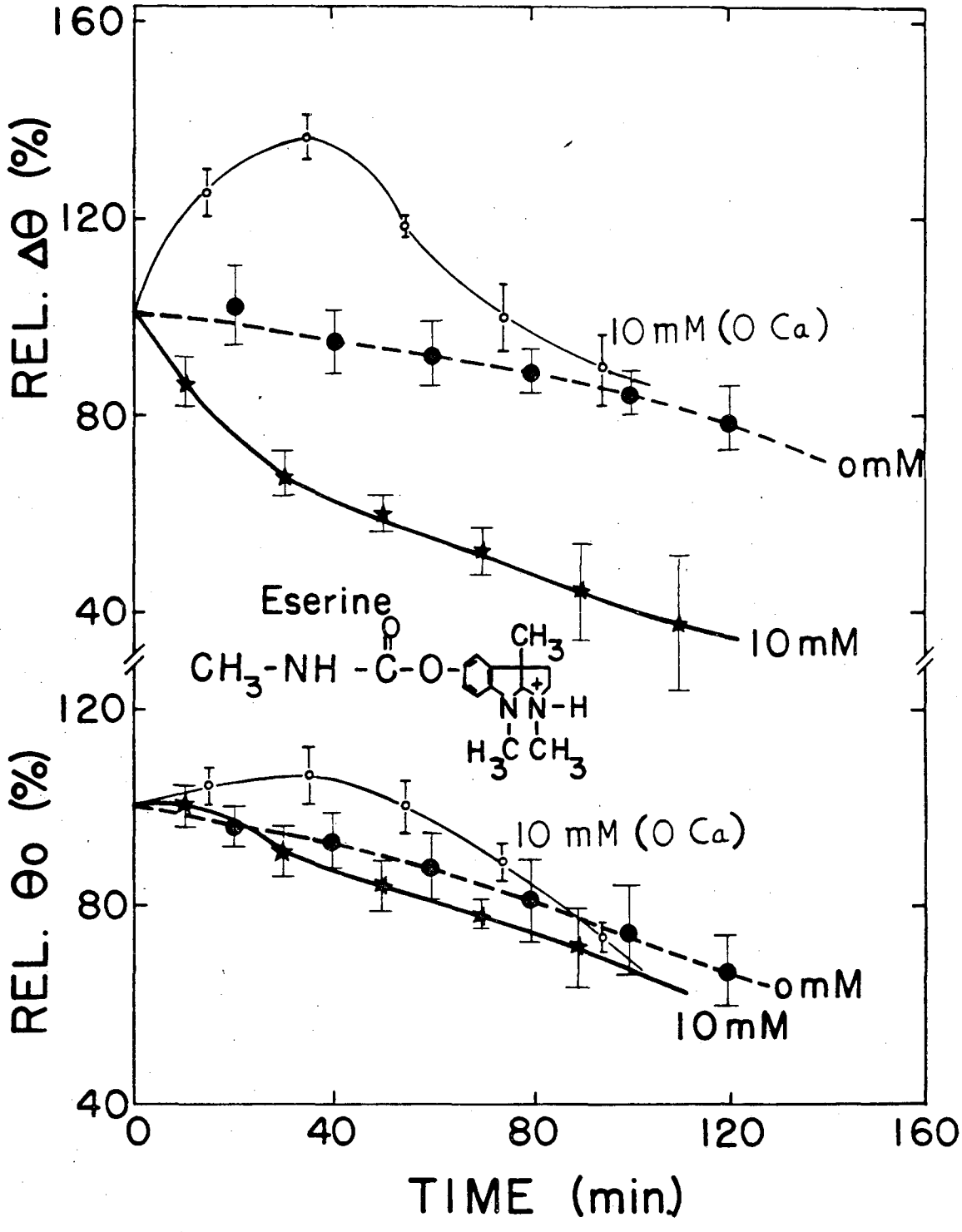


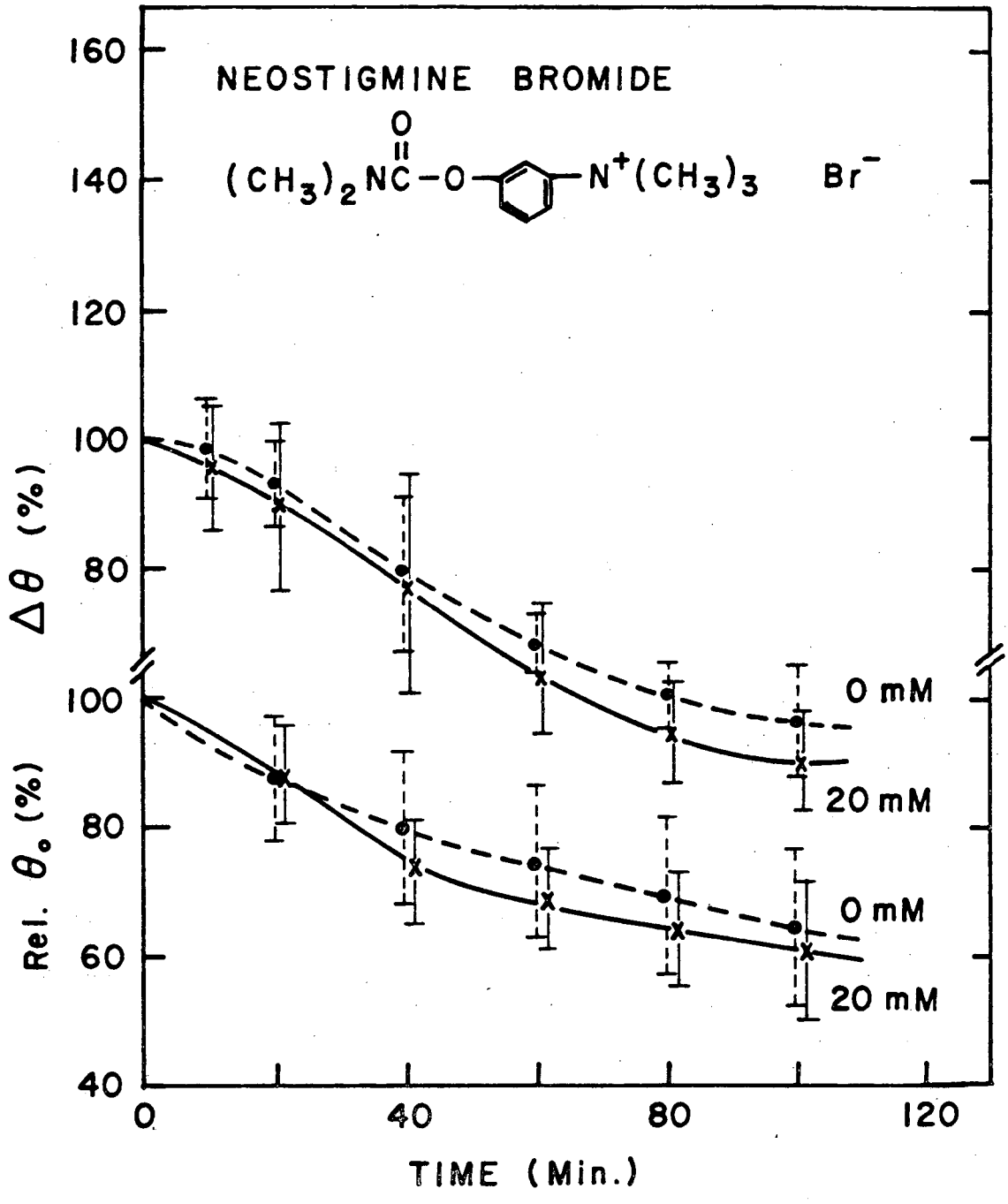
Fig. 34. XBL 766-5939
The effect of eserine (10 mM) on $\Delta\Theta$ and Θ_o, with Ca^{++} (★—★) or without Ca^{++} (o—o). Control experiment (0 mM eserine) with Ca^{++} (●—●).

has been observed many times also with different kinds of chemical modification. The possible mechanisms for the increase or decrease of $\Delta\ominus$ will be described in detail in the discussion section.

Neostigmine at 20 mM did not show any significant effect on the optical retardation (Fig. 35). However, a few additional experiments have indicated that pretreatment of the nerves with neostigmine decreased the effectiveness of acetylcholine in reducing the optical retardation signals. This is very likely due to inhibition of the acetylcholinesterase activity by neostigmine.

E. Local anesthetics and Barbiturates

The generation of action potentials in the nerve can be blocked by local anesthetics such as procaine, tetracaine or dibucaine, and by barbiturates such as barbital sodium and pentobarbital. The active component of local anesthetics is the charged form (positive) acting from the inside of the axon. However, due to their lipid solubility, most tertiary amine local anesthetics can block the action potential from both sides of the membrane. Barbiturates are derived from pyrimidine with 3 keto oxygen and some hydrocarbon chains. Barbiturates and local anesthetics bind to phospholipids in vitro. The binding of calcium to the phospholipids are increased by barbiturates, while local anesthetics decrease it (Blaustein and Goldman, 1966). Local anesthetics are dissociated into cations but barbiturates are dissociated into anions. Blaustein and Goldman (1966) suggested that the polar heads of cationic drugs would tend to neutralize the negatively charged site on the phospholipid so that fewer sites would be available for the binding of divalent cations. Conversely, if the



XBL 764-5829

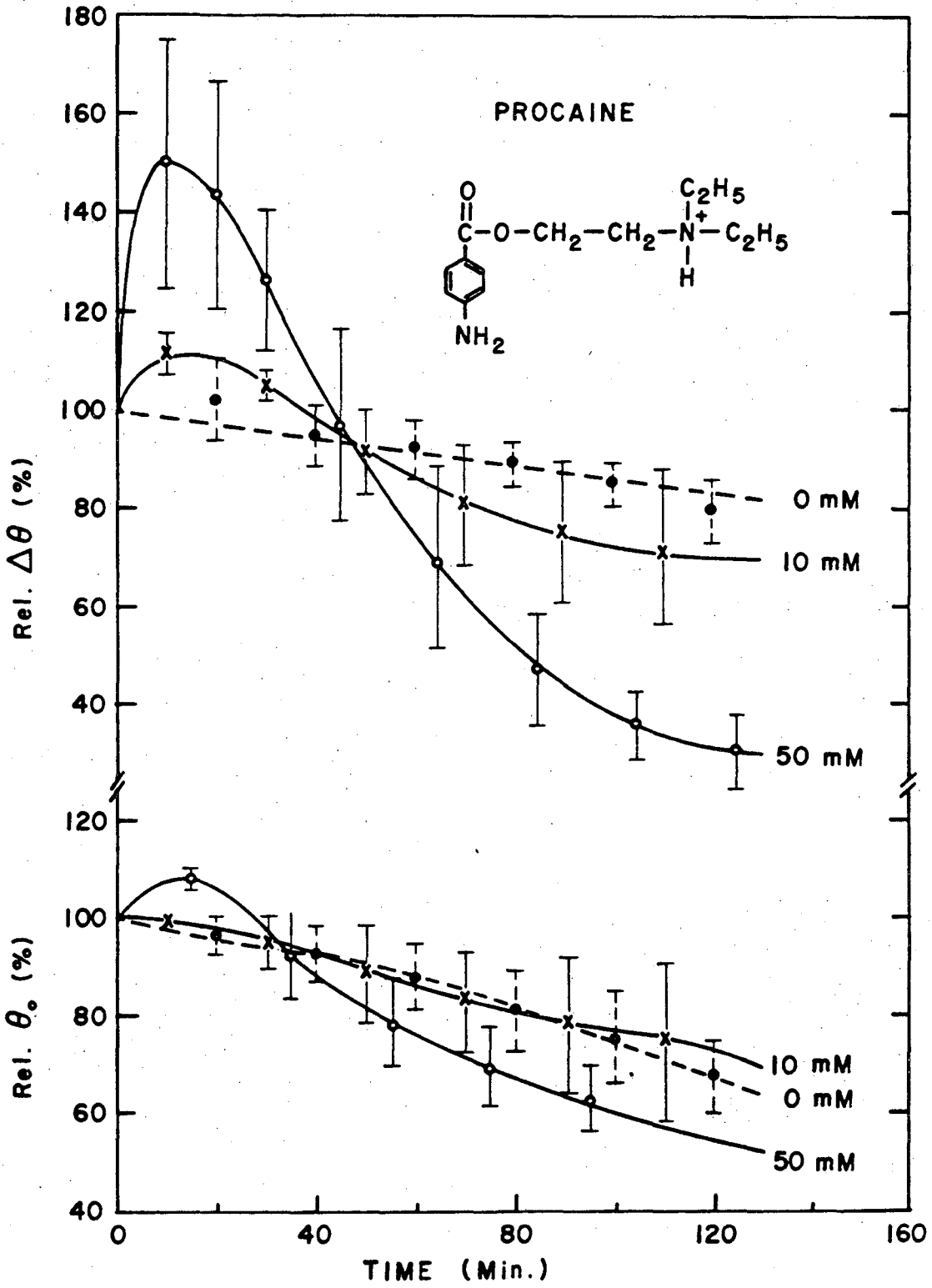
Fig. 35.
$\Delta\Theta$ and Θ_o with neostigmine (20 mM) and control (0 mM).

drug were an anion its negatively charged head would tend to neutralize the cationic nitrogen of the phospholipid, so that there would be an increased tendency for divalent ions to bind to the phospholipids. In voltage clamp experiments, both types of drugs inhibit the peak transient and late steady state currents, and increase the time for the transient current to reach its peak (Narahashi, 1971). The mechanism of the current inhibition by anesthetics and tranquilizers still remains obscure. Previous interpretations of the effects of local anesthetics on cellular membrane have focused largely on the interaction of these drugs with membrane lipids. Local anesthetics are known to produce molecular disordering in lipid bilayers and to enhance the fluidity of phospholipids in membranes (Seeman, 1972; Hubbell et al., 1969, 1970; Papahadjopoulos, 1975). Hubbell et al. (1969) have found that anesthetics cause a melting or disordering of the membrane such that more hydrophobic molecules enter the membrane's hydrophobic regions. The electron spin resonance spectra of nitroxide molecules within the membrane phase indicate that the anesthetics increase the molecular freedom of motion in the membrane. More recent experiments suggest that anesthetics also interact with membrane proteins (Boggs et al., 1976; Seeman, 1972, 1975). It is known that local anesthetics expand biological membranes. At concentrations of drugs (such as ethanol) known to block the nerve fibers, the membrane expands on the order of 2% to 3%, and significantly the expansion is something like ten times greater than predicted from the amount of drug known to enter the membrane. When the same experiments were done with liposomes, in which no protein was present, no such amplification occurred. Since anesthetics are able to produce conformation

changes in proteins (Balasubramanian et al., 1966), it is possible that the membrane expands as a result of modification of the membrane proteins.

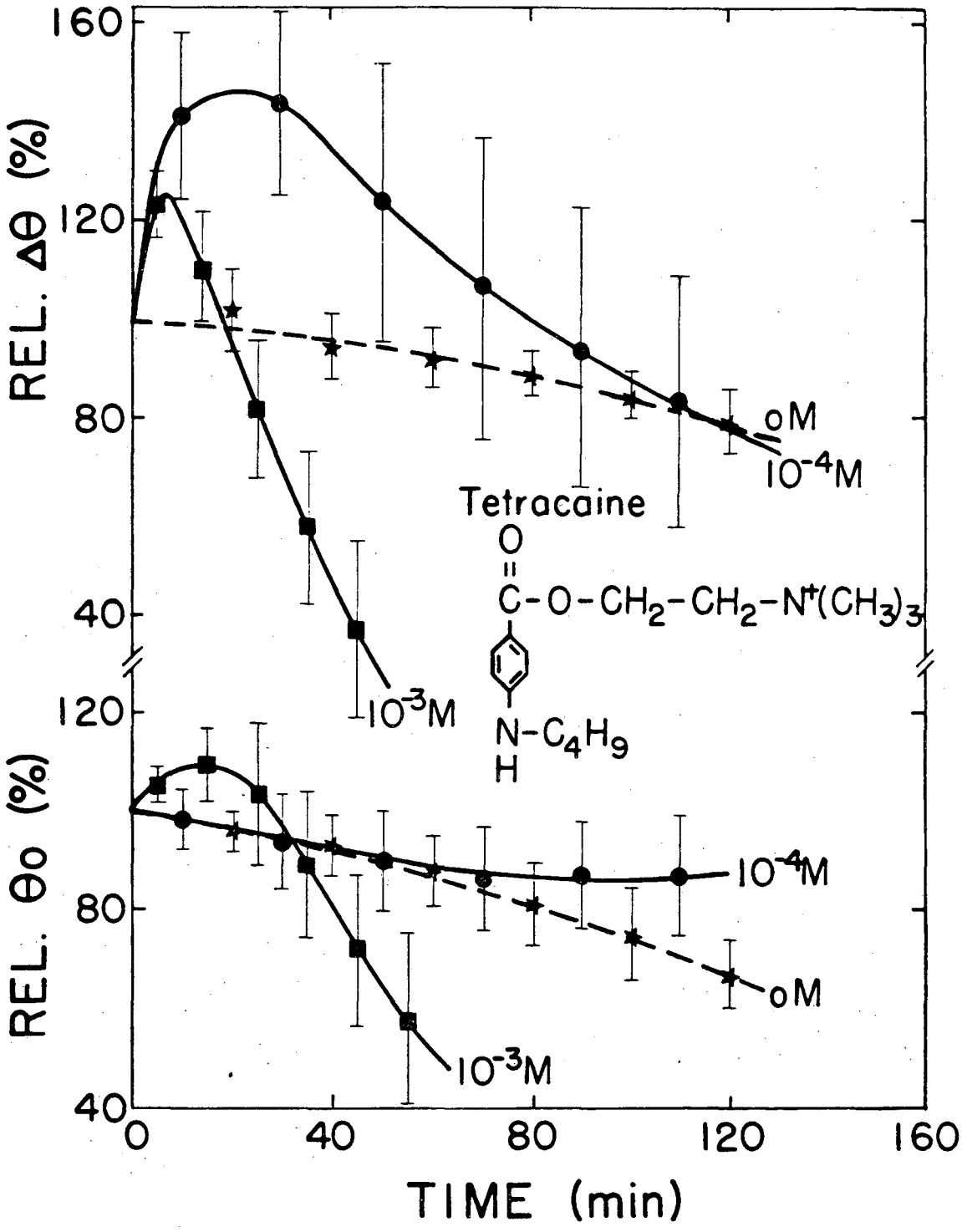
Since anesthetics can perturb the lipid and/or the protein structures in the membrane, it will therefore be interesting to study their effects on the optical retardation. Cohen et al. (1971) found no effect of 10 mM procaine. At 10 mM the effect may not be seen easily because it is still within the standard deviation range of the control experiment. However, when we used higher concentrations (50 mM), a significant effect was observed (Fig. 36). Both $\Delta\Theta$ and Θ_0 increase for a while and then decrease. Similar effects were induced by tetracaine at submillimolar concentrations (Fig. 37). The transient increase of $\Delta\Theta$ was reversible and in addition, it was not affected by pretreatment of the fibers with eserine or neostigmine. The transient increase of Θ_0 and the late decrease of $\Delta\Theta$ were more pronounced at high concentrations.

The effect of dibucaine, a local anesthetic that is more potent than procaine or tetracaine, was investigated at 3 different concentration ranges (Fig. 38). At 10^{-5} M there were no significant effects, while at 10^{-4} M $\Delta\Theta$ was reduced and Θ_0 was increased. At 10^{-3} M there was a rapid increase of Θ_0 which was then followed by a sharp decrease. At this concentration $\Delta\Theta$ was irreversibly reduced immediately after the addition of the drug. However, when the experiments were done in a calcium-free solution, different results were obtained, as shown in Fig. 39. At 10^{-3} M we can see an increase in $\Delta\Theta$ and Θ_0 which is then followed by a decrease. When lower concentrations (10^{-4} M) were used, the onset of the decrease was usually delayed. Tetracaine at 10^{-3} M



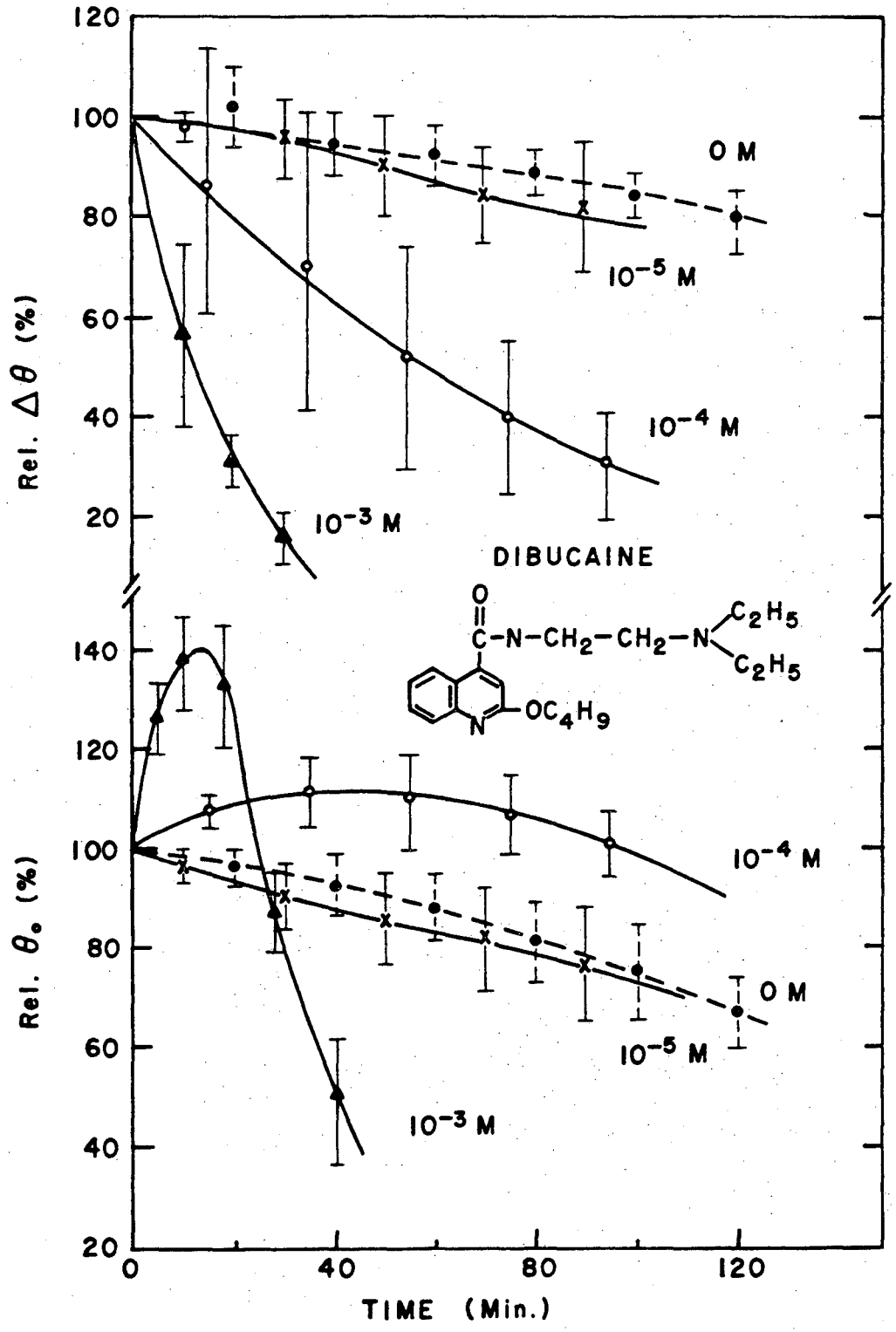
XBL 764-5834

Fig. 36.
The effect of procaine (10 mM and 50 mM) on $\Delta\Theta$ and Θ_o.
Control experiment (0 mM) in regular saline.



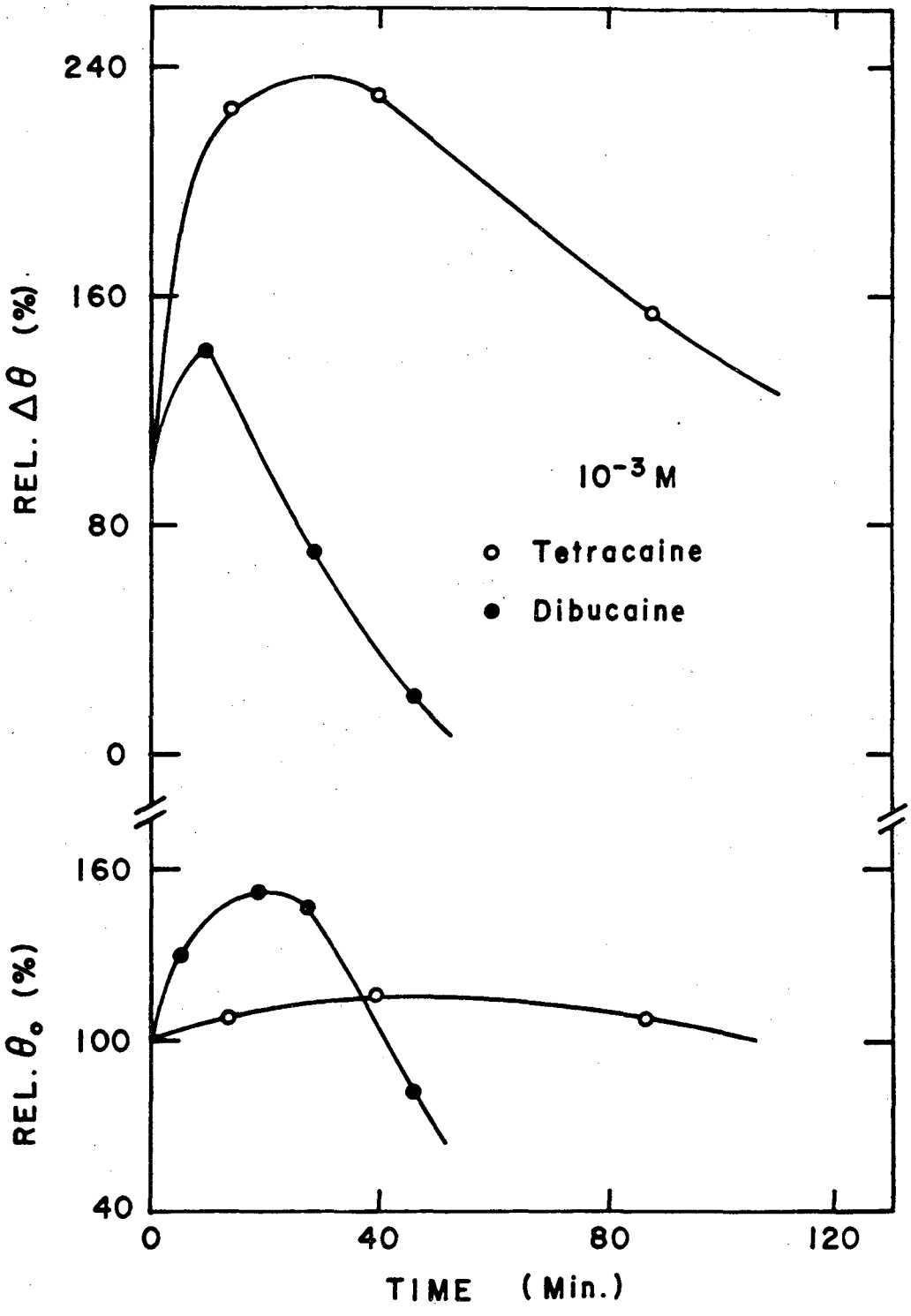
XBL 766-5937

Fig. 37.
The effect of tetracaine (10^{-4} M and 10^{-3} M) on $\Delta\Theta$ and Θ_o.
Control experiment (0 M) in regular saline.



XBL 764-5835

Fig. 38.
The effects of dibucaine (10^{-5} M, 10^{-4} M and 10^{-3} M) on $\Delta\Theta$ and Θ_o. Control experiment (0 M) in regular saline.



XBL 769-9607

Fig. 39.
The effects of 10^{-3} M tetracaine (o—o) and dibucaine (●—●) in Ca^{++}-free medium.

also induced almost the same effect (Fig. 39). The early increase of $\Delta\Theta$ was very large (note the change in scale) while Θ_o increased slightly. Both $\Delta\Theta$ and Θ_o decreased gradually later. At higher concentration (10^{-2} M) this decrease was usually more rapid and irreversible. By comparing Fig. 39 with the previous figures (Fig. 37, 38) one can see that in calcium-free solutions the local anesthetics induced larger and longer lasting increase of $\Delta\Theta$ and Θ_o. An example of the huge increase of $\Delta\Theta$ is shown in Fig. 40. Here the increase in light intensity changes (Δ I) at both orientations of the quarter wave plate is shown, before, and 10 minutes after the addition of 10^{-3} M tetracaine. This increase is usually reversible upon rinsing with tetracaine-free medium. No change in the kinetics of the signals could be detected after the addition of tetracaine.

The barbiturates also have strong effects on the optical retardation, as shown in Fig. 41 for barbital sodium. At 20 mM or 50 mM, $\Delta\Theta$ increased transiently and then decreased, an effect similar to that induced by procaine. However, Θ_o showed only a decrease without a transient increase. The decrease of $\Delta\Theta$ and Θ_o was more severe at high concentration (50 mM). A few experiments were also performed with other barbiturates, e.g. pentobarbital (nembutal). This drug decreased $\Delta\Theta$ and Θ_o already at a concentration of 1 mM (Fig. 42). All the experiments with the barbiturates were done in the normal saline solution.

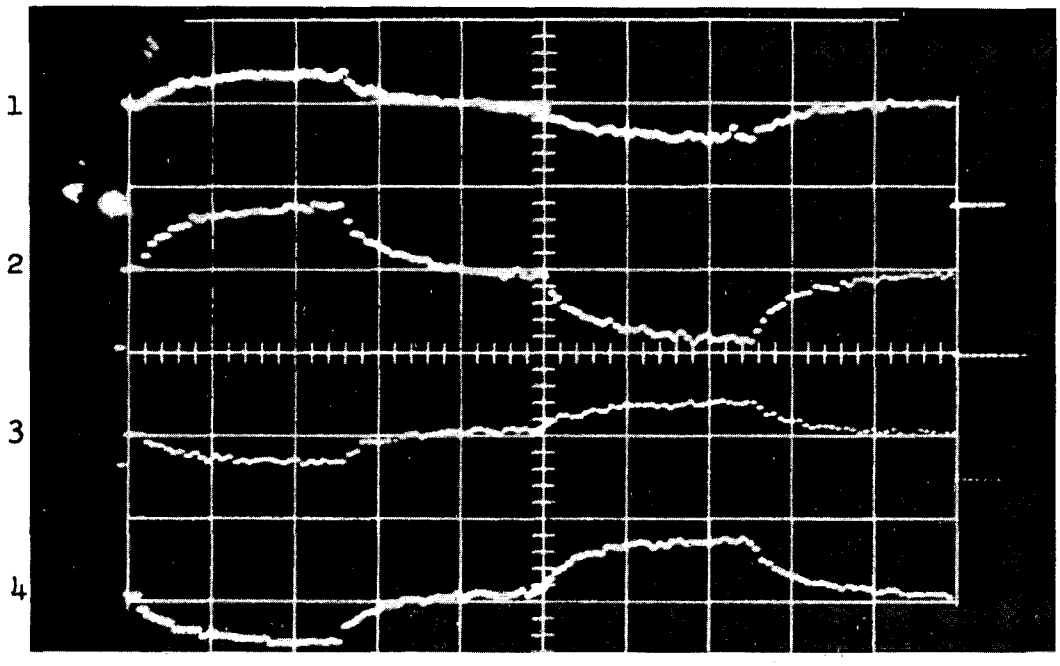


Fig. 40.
The changes in light intensity of the nerve (arbitrary units).
1st and 2nd: at $\Theta = \Theta_0 + \pi/2$, before and after the addition of tetracaine.
3rd and 4th: at $\Theta = \Theta_0 - \pi/2$, before and after the addition of tetracaine respectively.
Time calibration: 12.8 msec per large division.

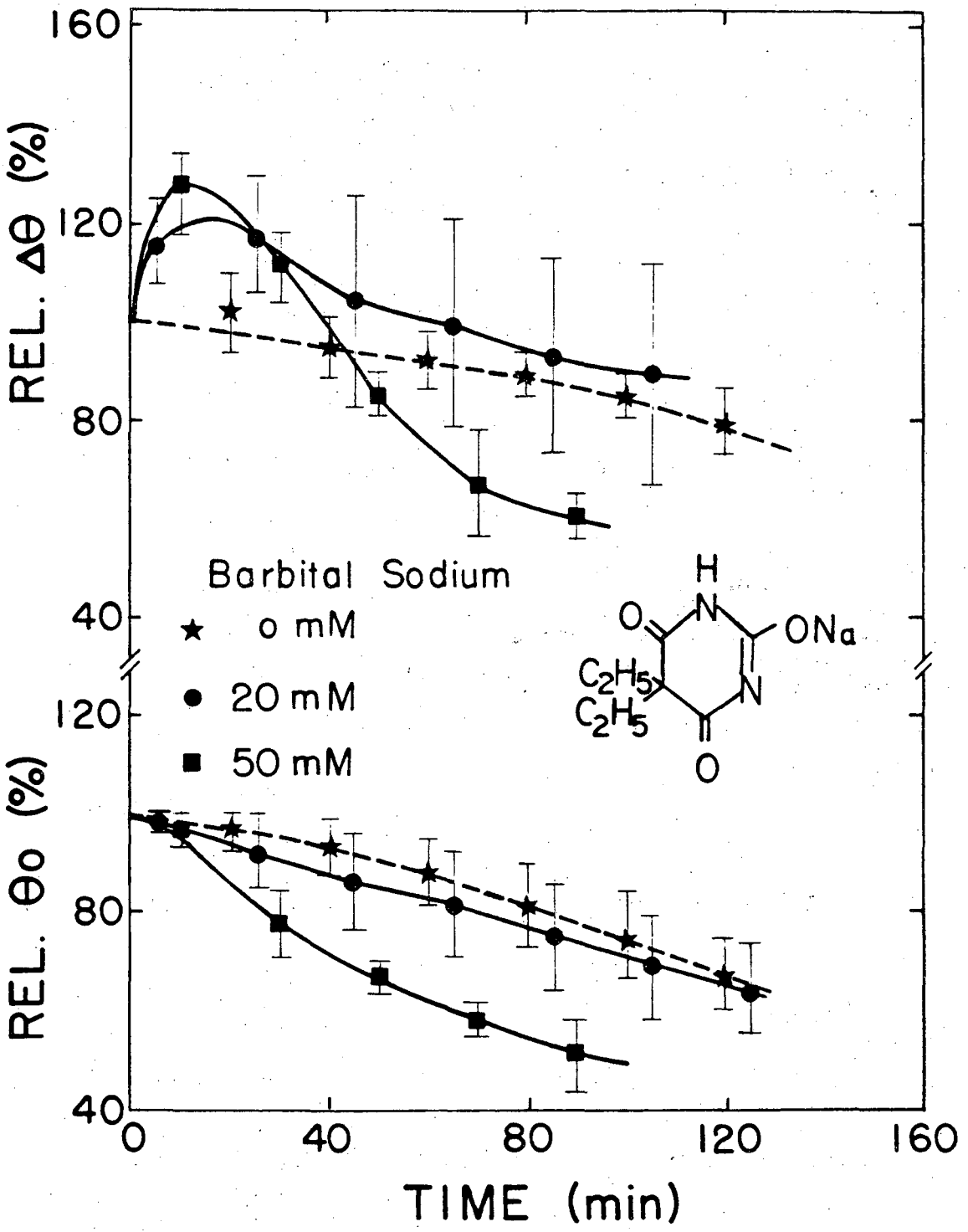
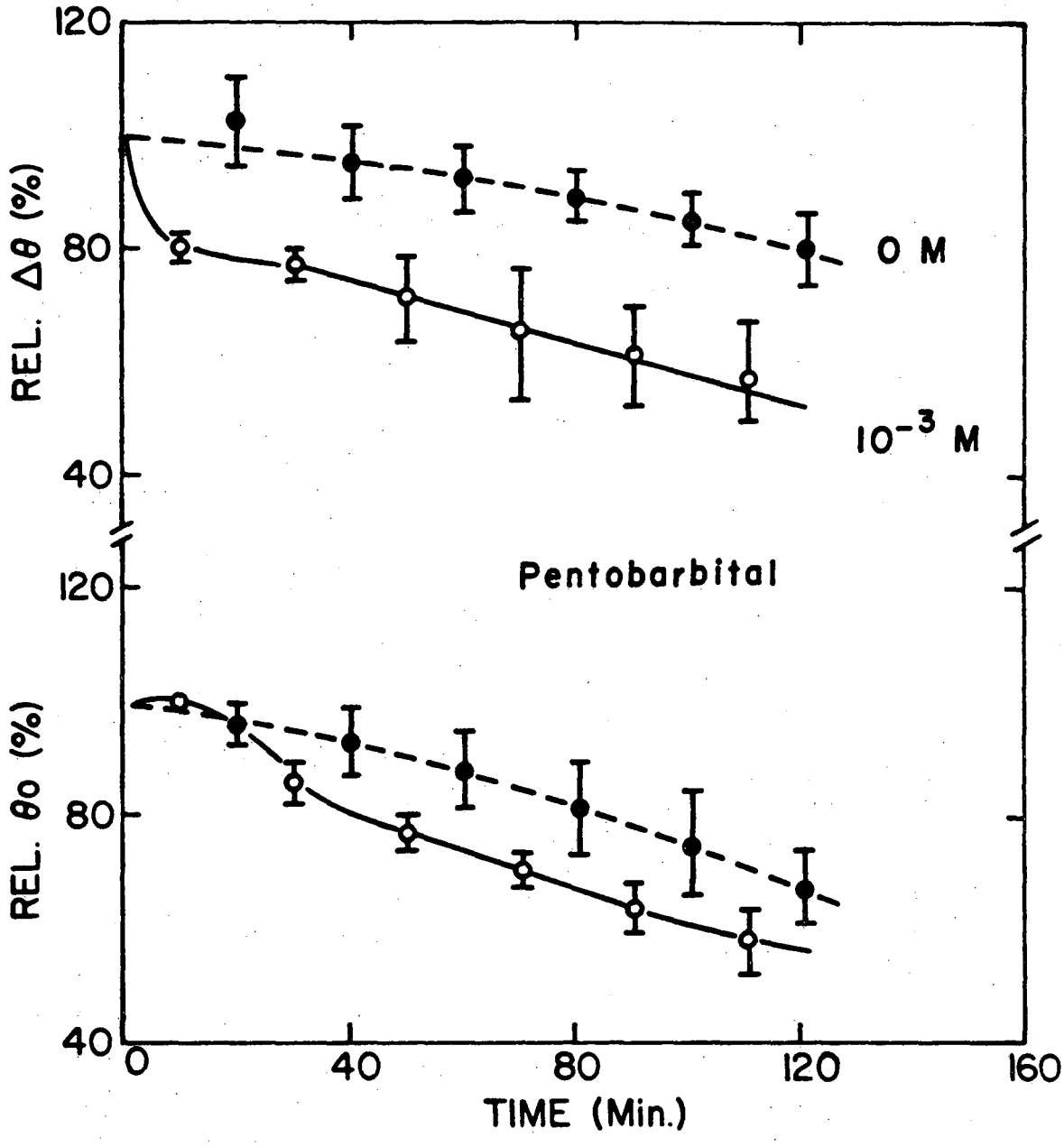


Fig. 41. XBL 766-5934
The effect of barbital sodium (20 mM and 50 mM) on $\Delta\Theta$ and Θ_o. Experiments in regular solution.



XBL 769-9615

Fig. 42.
The effect of pentobarbital (1 mM) on $\Delta\Theta$ and Θ_o.

F. Modification of ATPase

It is known that the sheath from giant axons contain an ATPase enzyme that is activated by magnesium ions and hydrolyzes ATP to ADP, liberating inorganic phosphate (Sabatini et al., 1968; Sjodin, 1971). ATP is the energy source for the "pump" that actively transports sodium out of the cell. It is of considerable interest to find out whether the changes in the optical retardation can be associated with the ATPase. Some of the drugs that have been used earlier, e.g. tetraethylammonium and hemicholinium-3, are known to be inhibitors of the ATPase (Matsumura and Narahashi, 1971). However, as described earlier, these two drugs did not have any significant effect on the optical retardation $\Delta\Theta$ and Θ_o (see Section D).

In this section, the effects of ATP and ouabain will be described. ATP is the substrate of ATPase, while ouabain, a cardiac glycoside is a well known inhibitor of the enzyme. ATP at a concentration of 20 mM decreased $\Delta\Theta$ and Θ_o only slightly (Fig. 43). At this concentration the drug was not completely soluble in the normal saline solution. On the other hand, ouabain, at a concentration of only 2 mM has a strong effect, as shown in Fig. 44. $\Delta\Theta$ rose continuously while Θ_o stayed constant initially, but a slow decrease was apparent after a long period of incubation in ouabain. The experiments with ouabain were done in a calcium-free saline to avoid the interfering effects of calcium. All the possible mechanisms and intrepretations of the drug effects will be described in the Discussion section.

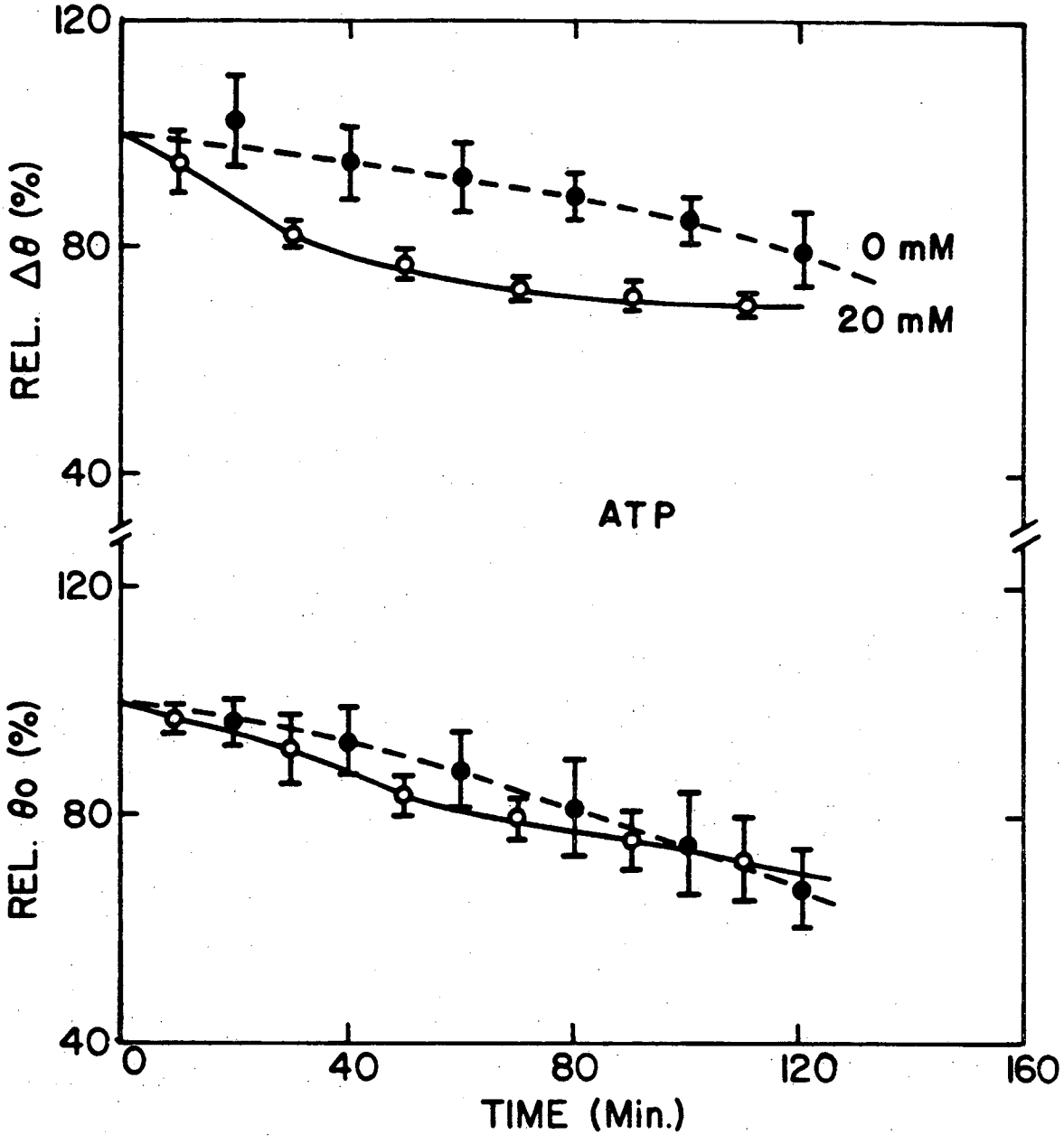


Fig. 43.
The effect of ATP (20 mM) on $\Delta\Theta$ and Θ_o.
Control experiment (0 mM) in normal saline solution.

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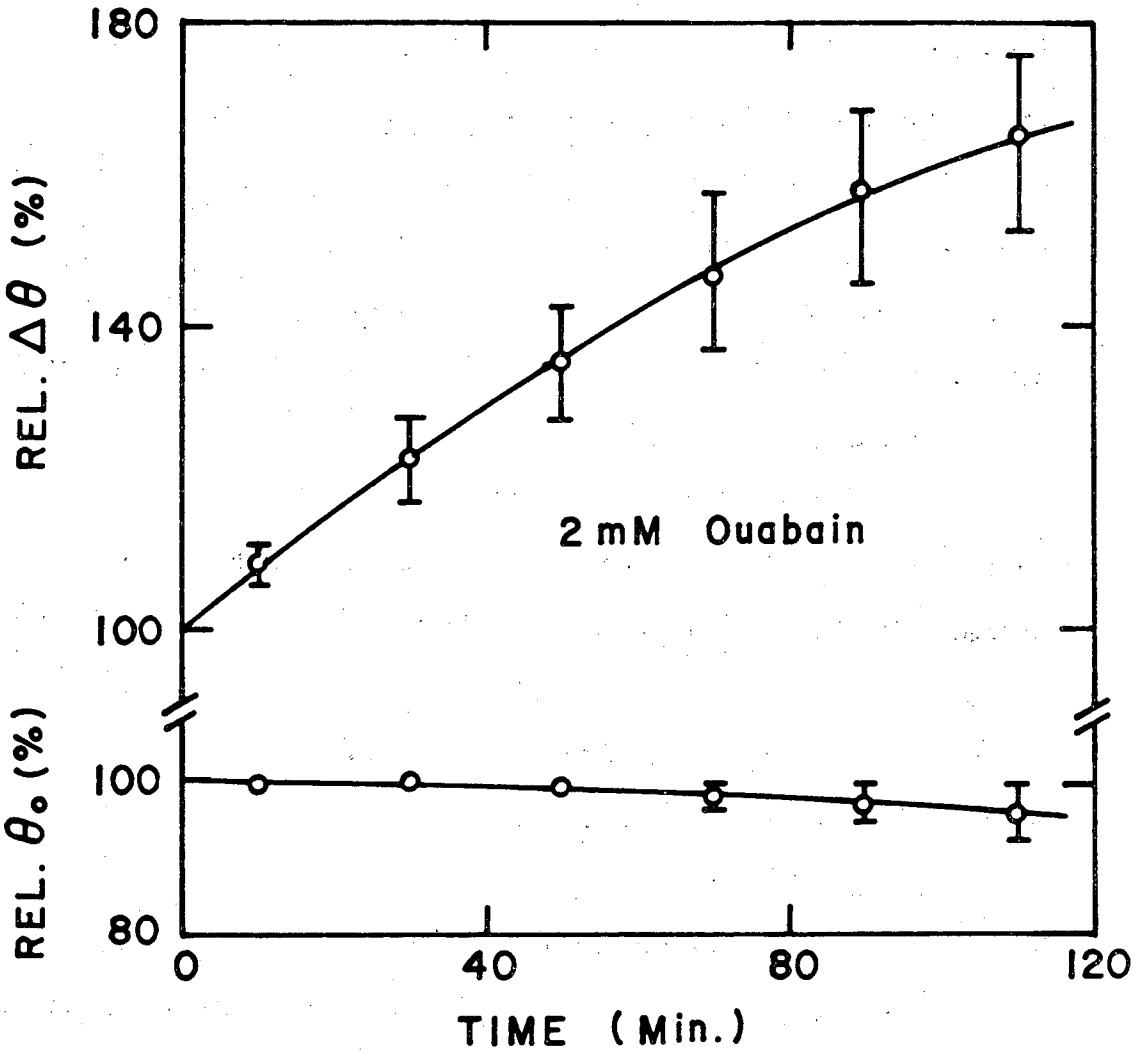

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Fig. 44.
The effect of ouabain (2 mM) on $\Delta\Theta$ and Θ_o.
Experiments in Ca^{++}-free solution.

V. DISCUSSION AND CONCLUSIONS

We have demonstrated that a relatively simple method, not requiring internal electrodes, permits studying the effects of membrane potential perturbations on the optical retardation of nerves. The method offers the advantage that these optical signals can be studied even under conditions in which no action potential can be elicited. Consequently, this kind of study may provide more insight into the molecular origin of the potential-induced retardation changes, since chemical and biochemical modifications and perturbations are compatible with the experiments.

The results of the chemical modification experiments have shown that there are different types of drug effects on $\Delta\Theta$ and Θ_o. The decrease in $\Delta\Theta$ could be due to one or more of the following mechanisms:

1) Calcium entry into the interior of the axon

2) Disruption of membrane lipids

3) Disruption of proteins

4) Neutralization of a positively charged group

The decrease of $\Delta\Theta$ due to calcium can be seen clearly in Fig.9, Fig. 12 and in many other drug experiments. It has often been observed that in Ca^{++}-free media the drug effects on $\Delta\Theta$ are not the same as in the regular Ringer solution (Figs. 13, 27, 33, 34, 39). Thus some of the drugs are capable of inducing an increase in calcium influx. The slow decrease of $\Delta\Theta$ and Θ_o in the control experiments (Fig. 8) are probably also due to calcium entry into the interior of the nerve, because in Ca^{++}-free solution, both $\Delta\Theta$ and Θ_o

are more stable and always stay close to the relative 100% level (Fig. 12). Dipolo et al., (1976) have measured the values for ionized calcium in squid axons by measuring the light emission from a drop of aequorin confined to a dialysis tube located axially. With a freshly isolated axon in 10 mM Ca^{++}-seawater, the aequorin glow invariably increased with time, indicating an increase of ionized calcium inside the nerve. The calcium level was promptly reduced when the axon was bathed in Ca^{++}-free seawater. The axoplasm of a living nerve fiber is solid and anisotropic in the resting state, showing the existence of some molecular organization (Bear and Schmitt, 1937a, 1937c; Metuzals and Izzard, 1969). Hodgkin and Keynes (1956) have shown that the solidity of the nerve axoplasm is destructed with a small amount of calcium in the internal medium. The decrease in Θ_o may reflect a disturbance in the ordered structure of the axoplasm.

The experiments with lysolecithin, phospholipases (C and D) and detergents suggest that the decrease in $\Delta\Theta$ could be due to a disruption of the membrane lipids, which may also lead to a collapse of the membrane structure. The absence of any effect of uranyl ion suggests that the head group of phospholipids is not directly involved in $\Delta\Theta$.

The decrease of $\Delta\Theta$ could also be due to disruption of protein structures. Phenylglyoxal, $HgCl_2$ and NEM are protein reagents that strongly decrease $\Delta\Theta$ and Θ_o. Glutaraldehyde reduces $\Delta\Theta$ probably due to its crosslinking action on proteins. The involvement of proteins in the changes of retardation has been suggested by Sato et al. (1973) and by Kaplan and Klein (1974).

The decrease of $\Delta\Theta$ at pH higher than pH 10.5 suggests the involvement of a positively charged group that is neutralized at high pH. This positive charge could be an amino or a guanidino group. Since no significant decrease of $\Delta\Theta$ was observed between pH 7.5 and pH 10, and since $\Delta\Theta$ was strongly reduced by phenylglyoxal (which is more specific for guanidino group than for amino group), it is believed that this charge is a guanidino group. The almost linear voltage dependence of $\Delta\Theta$ suggest that $\Delta\Theta$ may originate from a simple displacement of the charged guanidino group by the change in the electric field of the membrane.

The increase in $\Delta\Theta$ could be due to one or more of the following mechanisms:

1) Protonation of negative charges

2) Increase in membrane fluidity

3) Interaction with a cholinergic binding macromolecule

Protonation of negative charges can occur at low pH values, and in this region $\Delta\Theta$ increases (Fig. 16). The decrease in the number of the negative charges may result in an increase in the total (net) positive charge, so that the electric field may induce a larger displacement of the positive charge. It is also possible that the positive and negative charges are on different molecules. In this case, protonation of the negative charges may result in a decrease in the attractive electrostatic forces between them. This can also result in a larger displacement of the positive charge.

The increase of $\Delta\Theta$ could also be due to an increase in the fluidity of the membrane, as suggested by the experiments with local anesthetics and barbital sodium (Figs. 39 - 41). It is possible that when

the membrane viscosity decreases, the hydrocarbon chain of arginine or lysine may encounter less frictional forces in the membrane. Thus the electric field could induce a larger displacement of the positively charged guanidino or amino groups which would then cause an increase in $\Delta\Theta$. The decrease in viscosity should also affect the rise and decay times of the optical signal. Unfortunately, our system does not permit us to detect a decrease in the time constant, since the rate limiting step of the system is determined by the cable properties of the nerve bundle. Keynes and Rojas (1974) have shown that 1% procaine roughly halved the time constant of the gating current. This again supports the notion of an anesthetic induced increase in the fluidity of the lipid matrix of the membrane. Some local anesthetics also transiently increase the static retardation Θ_o of the nerve (Figs. 36 - 39). This increase may indicate a loss of the negative retardation component of the nerve, which is very likely caused by the disordering effect of the drug on the lipid structures. What remains is then only the positive retardation component, which mostly originates from the microtubules and microfilaments of the axoplasm. However, prolonged exposure or application of high concentrations of anesthetics rapidly decreased the static retardation Θ_o as well as the retardation change $\Delta\Theta$. The decrease of Θ_o suggests that these drugs may cause a disruption of the axoplasmic microtubular protein structures. Supporting this view is the electron microscope examination by Nicolson et al. (1976) of untransformed BALB/3T3 cells. Treatment with anesthetics revealed significant reductions in plasma membrane-associated microtubules and microfilamenets and/or their plasma membrane attachment. It is believed that calcium may play a

role in the destruction of the microtubular structures.

The interaction of some drugs with a cholinergic binding macromolecule in the membrane is believed to be also one of the causes of the increase in $\Delta\Theta$. This macromolecule could be similar to the one isolated by Denburg et al. (1972) from lobster axon membranes. They have shown that the macromolecule, which is a phospholipoprotein, binds cholinergic ligands (nicotine, eserine etc.) and local anesthetics. Nicotine and eserine induced an increase in $\Delta\Theta$ when the experiments were done in a Ca^{++}-free medium (Figs. 33, 34). As described earlier, local anesthetics also induced an increase in $\Delta\Theta$. The large increase was interpreted as due to a decrease in membrane viscosity. However, a small percentage of the increase of $\Delta\Theta$ could also be due to a direct interaction between the local anesthetics and the macromolecule. Weber and Changeux (1974) have shown that local anesthetics inhibit competitively the binding of (^3H) acetylcholine to the cholinergic receptor site from Torpedo electric tissues. They concluded that local anesthetics bind to the cholinergic receptor site with a low affinity, but that they inhibit the depolarization of the electroplax by binding at different sites situated on or near the cholinergic receptor protein.

We have tested many different kinds of cholinergic compounds. Most of these charged quarternary amine compounds may have difficulty penetrating the membrane due to their poor lipid solubility and thus have no significant effect on the optical retardation. They might also face diffusional barriers exerted by the Schwan cells and connective tissues surrounding the axon It is believed that the nicotine receptor is located on the internal surface of the axon plasma membrane (Denburg et al., 1972; Frazier et al., 1973). The effect

of acetylcholine (Fig. 27) is unique since it resembles the effect of low pH (Fig. 13). The hydrolysis of acetylcholine by the acetylcholinesterase is responsible for the decrease in local pH of the tissue. High buffer concentration or pretreatment with neostigmine decreased the effectiveness of acetylcholine in reducing $\Delta\Theta$. Neostigmine, an acetylcholinesterase inhibitor, did not have any significant effect on $\Delta\Theta$ and Θ_o (Fig. 35). All these results suggest that $\Delta\Theta$ may not be associated directly with the acetylcholinesterase.

The absence of an effect of ATP (Fig. 43) rules out the possibility that ATPase may be associated with the retardation changes. On the other hand, the ouabain induced continuous increase of $\Delta\Theta$ was rather surprising (Fig. 44). However, Denburg and O'Brien (1973) have shown that besides inhibiting ATPase, ouabain is also a noncompetitive inhibitor of (^{3}H) nicotine binding with a $K_i = 7*10^{-5}$ M. Thus the ouabain induced $\Delta\Theta$ increase could be due to the interaction of ouabain with the cholinergic binding macromolecule. The exact nature of interaction between many different drugs and the macromolecule needs further investigation. Denburg et al. (1972) concluded that the nicotine-binding component of the axonal membrane resembles the acetylcholine receptor that exist at cholinergic post-synaptic membranes. Frazier et al. (1969) on the other hand suggested that the two macromolecules may be functionally different. In relation to these views, it is interesting to know that birefringence changes during activity have also been observed in the electric organ of Electrophorus electricus (Cohen et al., 1969). It is known that the electric organ is rich in acetylcholine receptors. The importance of the birefringence changes in the molecular mechanism of excitation is a subject that needs further inves-

tigation. It will be interesting to do the chemical modification experiments by applying the drugs directly on the internal surface of the membrane. This can be achieved easily using the internal perfusion technique on the giant axons of the squid. Large squids are unfortunately not readily available in the West-coast.

Taken together, the data from the effects of pH (Fig.16), protein denaturants (Figs.18, 20, 22) and proteases (Kaplan and Klein, 1974; Sato et al., 1973) suggest that at least part of the electric field induced retardation changes may be associated with a charged group on a protein macromolecule. It is reasonable to assume that such a macromolecule is located within or close to the membrane since $\Delta\Theta$ also responds to changes in membrane fluidity (as shown by the effects of drugs such as local anesthetics and barbiturates). Changes in the membrane properties as induced by the phospholipases and detergents, which are known to modify the integrity of the structure and the dynamics of membranes, may render the macromolecule more or less susceptible to the changes in the electric field. It will be interesting to find out if the charge movement associated with the retardation change can be correlated with the charge movement of the gating current (Armstrong and Bezanilla, 1974; Keynes and Rojas, 1974). This can be done when more data from the effects of chemical modification on the gating current become available.

I hope that the results of my experiments have narrowed down the "band-with" in the search of the origin of retardation changes in nerves. All the interpretations of the results are subject to further testing with other types of spectroscopic technique. It is hoped that the other techniques will be able to give more detailed informations

about the nature of the changes induced by the electric field. At least we are almost certain now which part of the membrane is worth future study.

APPENDIX

Effect of Glutaraldehyde on the Nerve Membrane Currents

Materials and Methods

Giant axons of squid, Loligo pealii, available in Woods Hole, Massachusetts during the summer were used for this study. The axon is dissected free from the mantle by means of very fine microscissors. Both ends of the axon are tied with thread. The major portion of the small nerve fibers surrounding the giant axon is removed under a dissecting microscope. The diameter of the giant axons ranges between 300 and 500 μm.

A plexiglass chamber was built for the nerve. Black platinized platinum electrodes are used for the external current electrodes (center and grounded guard electrodes) as used conventionally. A piggy-back arrangement is used for the internal current injector-voltage sensor wires. The internal current electrode (Pt wire, 100 μm in diameter) is placed next to the internal potential electrode (teflon insulated Pt wire 25 μm in diameter, platinized over 1 mm at the tip). The two wires are adhered together using dental sticky wax. The tip of the voltage electrode is located in the middle of the platinized portion of the current electrode. Only 13 mm of the tip of the current electrode (axial wire) is platinized. The mantle area of the axon surrounding this platinized tip is about 0.25 cm^2 for an axon 300 μm in diameter. A major part of the current injected through the axial wire will flow through the large guard electrodes, while only a small portion will flow to the current detecting (center) electrode. This electrode has a surface area of about 1/8th of the

guard electrodes. Thus the center electrode will utilize only about 0.03 cm^2 of the axon surface.

The internal and external potential electrodes are also made of black platinum wires, which are not as toxic as Ag-AgCl for the nerve. The use of platinum wires for potential measurements has the advantage that it eliminates the necessity to work with the conventional microelectrodes. However, the junction potential of the platinum electrode is not as stable as the potential of the reversible Ag-AgCl electrode. Frequent replating of the platinum electrodes is necessary. As a result, the absolute value of the membrane potential cannot be measured with great certainty.

External fluid medium is delivered from a reservoir to the nerve chamber through polyethylene tubing and a stainless steel cooling coil, which is placed in a box filled with ice. During control experiments, natural sea water (pH 7.9) is circulated around the axon. For the glutaraldehyde experiments, one capsule of glutaraldehyde (10 ml 8%, Electron Microscopy Grade, pH 7) is diluted in sea water to obtain a final concentration of 0.25%.

If the nerve has been mounted in the chamber, an opening on the axon is made. Then under the dissecting microscope, the axial wires are inserted into the axon through the opening, with the help of a micromanipulator. During this procedure, resting and action potentials (with external stimuli) are monitored continuously to see whether the axon is damaged or not. The tip of the current electrode is covered with a dot of epoxy glue to reduce scratching of the inner wall of the axon.

A conventional electrometer preamplifier (unit D) and voltage

clamp device of the excitable membranes class are used for the experiments. The instrument does not have an adjustment for compensation of series resistance. Tektronix pulse generators (type 161 and 162) are used to trigger clamp pulses. The voltage and current density are displayed on a dual beam oscilloscope (Tektronix 5103N) and photographed with a polaroid oscillograph camera.

Results

After inserting the axial wires longitudinally into the axon, rapid flow of the extracellular medium is initiated. Due to the instability of the potentials of the platinum electrodes, sometimes it is necessary at this point to readjust the balance between the internal and external potential electrodes using the D.C. offset of the electrometer preamplifier. Usually a resting potential of -60mV and an action potential of 90 mV can be recorded. Then the instruments are set to the clamp mode. Starting from a holding potential of -60 mV a prepulse is given, followed by the test pulse. Fig. I shows the time course of membrane potential (top) and current (bottom) obtained at different voltages. The prepulse (-20 mV) is followed by the test pulses, (60, 70, 80 mV) which have a duration of several milliseconds. We see the typical membrane current associated with a rectangular depolarization, i.e., a transient initial inward current (negative) followed by an outward current (positive) which reaches a quasi steady state level (Hodgkin and Huxley, 1952). Also tested are hyperpolarizing prepulses of -40 and -60 mV to check the leakage current, which usually is very small.

After the control experiments have been done, the circulating

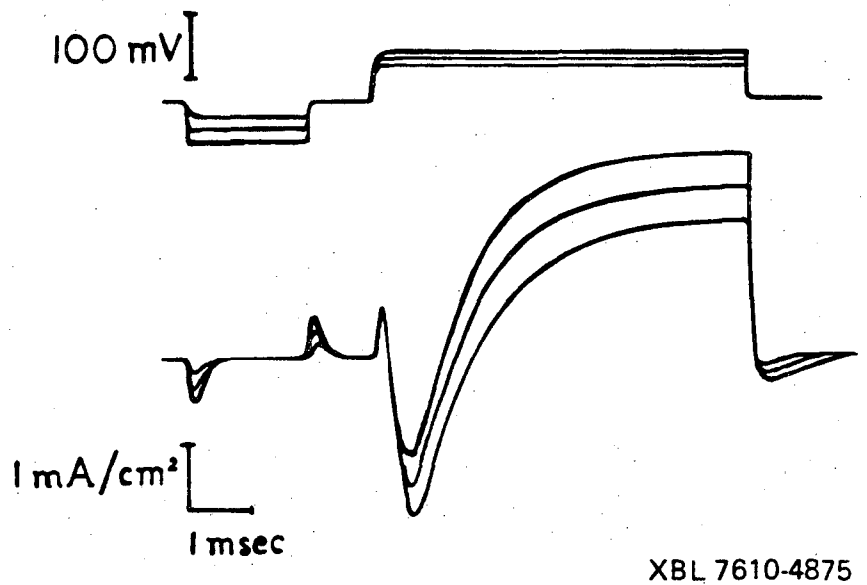

Fig. I.
Membrane potential (top) and current density (bottom)
Voltage clamp experiment in natural sea water.

medium is then changed to a solution of natural sea water and 0.1% glutaraldehyde. Both the inward and outward currents decrease gradually as shown in Fig. IIa which is taken 10 minutes afterwards. Washing the axon with normal sea water does not increase the currents, thus the effect is irreversible. The glutaraldehyde concentration is then increased to 0.25% and the results after 10 additional minutes are shown in Fig. IIb. The currents are now much smaller. We see also that the outward currents rise about 20% slower than during control, while almost no change is seen on the kinetics of the inward currents. At this moment the resting and action potential remain only about 60% of the original values.

Current-voltage relationship can be obtained if we plot the peak value of the inward current (I_p) and the quasi steady state value of the outward current (I_s) versus membrane potential. From the data obtained with another axon we plot in Fig. III the current-voltage relationship during control (line) and also 20 minutes after 0.25% glutaraldehyde has been applied (broken). Again we see the decrease of the currents after glutaraldehyde treatment. The slopes of these currents are also reduced, suggesting an increase in membrane resistance.

Discussion

We have seen that the major effect of glutaraldehyde is to decrease both inward and outward currents, as well as to slow down the turn-on process of the outward current. These effects might be responsible for the prolongation of the action potential especially in the falling phase, as reported by Shrager et al. (1970). Inhibition or delay of the "sodium inactivation" process might also be able to

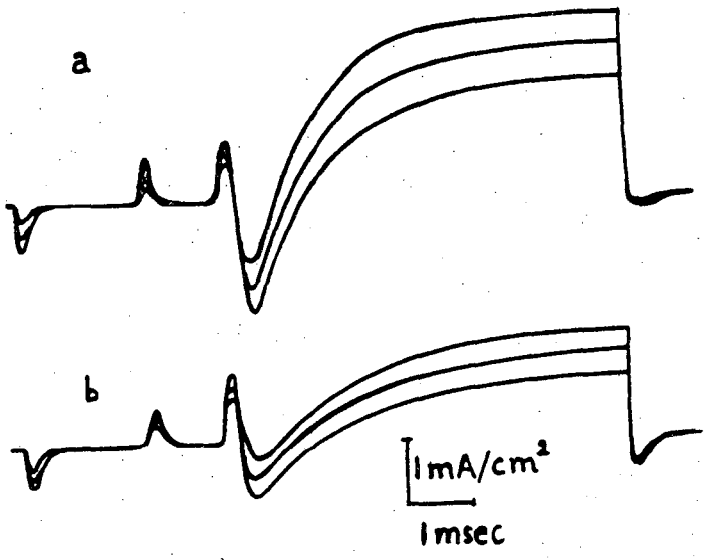

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Fig. II.
a) Membrane current densities after 10 minutes in sea water containing 0.1% glutaraldehyde.

b) Membrane current densities in sea water containing 0.25% glutaraldehyde 10 minutes later.

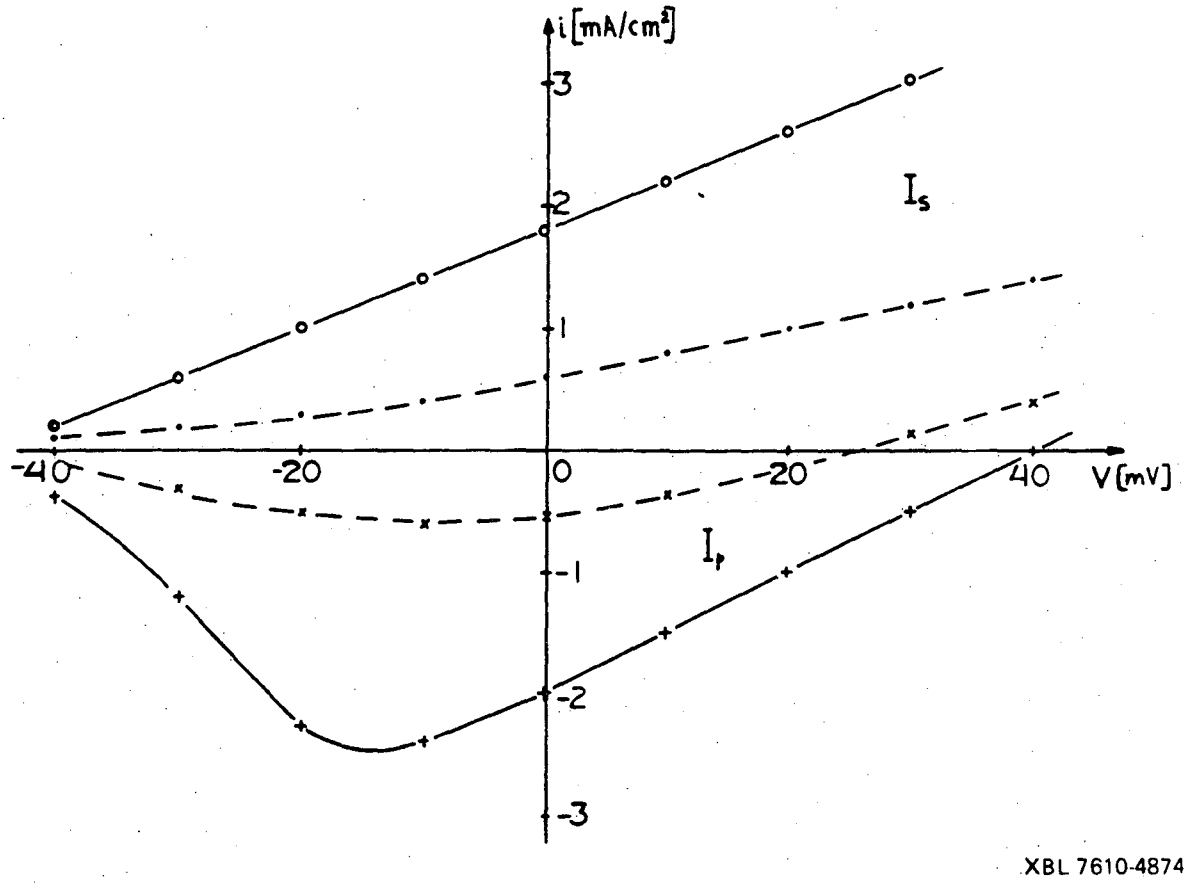

Fig. III.
Current-voltage relation. Peak initial current (I_p) and steady state current (I_s) are read from currents associated with step changes in membrane potential (V). Continuous line is control, broken line is after glutaraldehyde treatment (0.25%) for 10 minutes.

cause a prolongation. This would slow down the turn-off process of the inward current, which, however can not be seen with certainty from this experiment alone. It should be mentioned, that during the glutaraldehyde experiment, the inward currents can still be increased by giving larger hyperpolarizing prepulses, which can remove the existing inactivation .

In general we may assume that glutaraldehyde is able to immobilize a configurational change during excitation by crosslinking normally mobile charged groups in the membrane. The persistence of the color, the increase in membrane resistance and the irreversibility of the current inhibition suggest that the effect of glutaraldehyde involves formation of stable chemical bonds with some membrane constituents. An awareness of the effects of crosslinking agents (fixatives) on the electrophysiological properties of excitable membranes may assist one in the proper interpretation of morphological studies.

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