Effect of Azide and Ca Ion

on the Reversible Changes of Protein

Configuration in Stimulated Nerves

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ABSTRACT   Working with sciatic nerves from the South American frog *Calyptocephalella gayi* we have been able to substantiate the finding of Ungar *et al.*, that nerve proteins undergo a reversible denaturation concomitant with a train of nerve impulses. We also showed that 0.3 mM sodium azide is capable of impairing the reversibility of those changes. Furthermore, 10 meq/liter of calcium ion applied to extracts of resting nerves induce configurational rearrangements in the proteins similar to those produced during stimulation. We also proved that nerves previously kept in low calcium Ringer's solution do not show configurational changes upon stimulation. A tentative interpretation of the results described is that the configurational changes are a consequence of the extra influx of calcium ion due to nerve impulse propagation.

INTRODUCTION

During the last 15 years various lines of research have shown the occurrence of changes in proteins of the nervous tissue during activity (9, 14). Thus such observations have shown that non-protein nitrogen increases in extracts of stimulated brain (10) as well as in extracts of stimulated peripheral nerves (23). Also, the protein turnover is increased in active nerve cells (18), and nerve metabolism seems to shift from glucose utilization to amino acid utilization when nerves are stimulated (20). Furthermore, there is evidence that nerve proteins undergo reversible changes in configuration during impulse conduction (23, 24). These changes have been discussed in terms of Ling's fixed charge hypothesis (15) and it has been suggested that the entry of Na ion into the axon during the action current might be due to a transient configuration of the nerve proteins which in this state would not be able to bind potassium (23).

Another type of information is given by Brink *et al.* (3) who demonstrated that in frog nerve 0.2 mM sodium azide inhibits the extra oxygen consumption

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due to a train of nerve impulses. Hodgkin and Keynes (11) have found that in *Loligo*, 3.0 mM of the same substance is a powerful inhibitor of the sodium pump. The effect of Ca ion upon the excitable properties of nerve cells is also known (2, 7, 8).

We felt that it would be of interest to study the configurational changes described by Ungar *et al.*, particularly with the aim of correlating and understanding these changes in terms of known physiological facts such as those quoted. In the present work we show that sodium azide is an inhibitor of the reversibility of the configurational changes of nerve proteins described by Ungar *et al.* We also demonstrate that Ca ion has a definite effect upon the structural state of nerve proteins. We interpret these results in terms of the movements of Ca ion during nerve impulse propagation. Furthermore, our evidence shows that the protein changes occur after the nerve impulse; that is to say, the changes of protein structure seem to be a consequence of the nerve impulse and not its cause.

METHODS

The criterion for modification of protein structure used in this work is based on the change of absorption spectra of protein solutions produced by an increase in the pH of the solvent. Following Ungar and his coworkers (23), we used the findings of Crammer and Neuberger (4), further substantiated by Tanford and Roberts (22), who demonstrated a shift in the ultraviolet absorption spectrum and an increase in the extinction coefficient of denatured egg albumin. To this end we proceeded as follows: the optical densities of the nerve extracts at pH 7 and 12 were measured at 20°C from 245 to 310 mμ. The ratio optical density at pH 12 over optical density at pH 7, tentatively called "Side Group Ionization Ratio" (SGIR) by Ungar *et al.*, was plotted against the wave length and two peaks were obtained. By means of a variety of methods including titration curves (24), it has been established that the peak at 300 to 305 mμ is a measure of phenolic hydroxyls of tyrosine free to ionize (5, 23) and the peak at 245 to 250 mμ corresponds to $-SH$ groups from cysteine also free to ionize (1, 23, 24).

It should be pointed out that the quantity defined as SGIR is not a simple measure of an ultraviolet absorption spectra. Rather, it shows how a given absorption spectrum increases when the pH of the solution is raised. Accordingly, an increase in SGIR at 245 or 300 mμ implies an augmentation of side groups available for ionization, either through breakdown or uncoiling of proteins.

Sciatic nerves from the South American frog, *Calyptocephalella gayi*, were used in all experiments. The nerves excised from one side of the animals were used as a control group and those from the other side were stimulated. Each group was usually formed by four nerves. Stimulation was supramaximal for Aα fibers and effected with square pulses (0.2 msec. duration), 120/sec. for 30 minutes. The activity of the nerves was continuously displayed on an oscilloscope. Occasionally, the compound action potential diminished in size, due to a decrease in excitability. This was compensated for by increasing the strength of the stimulus until the original size of the

compound action potential was recovered. Sometimes this was not possible to do; in those cases in which we could not reach the end of the experiment with at least 75 per cent of the original size of the action potential the experiments were discarded. After 30 minutes' stimulation and while the nerves were being stimulated and their activity checked, the nerves were frozen by applying to them the surface of a metal cylinder containing dry ice. Then, the nerves were crushed with ground glass in a mortar, and their proteins extracted in 0.1 M KCl plus 0.01 M tris (hydroxymethyl) aminomethane at pH 7 (KCl-tris, from now on). The extraction was made with about 40 ml of KCl-tris for each gram of nerve. The exact proportion is irrelevant since the determinations of SGIR are independent of protein concentration in so far as Beer's law applies. The nerve extracts were centrifuged under refrigeration at 20,000 g for 60 to 90 minutes and the supernatant used. Except when specifically mentioned, the extracts were kept between 0 and 3°C.
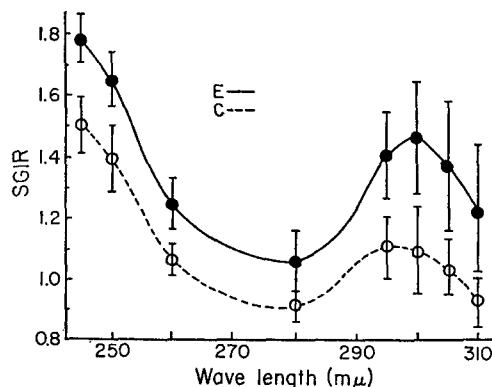

FIGURE 1. SGIR of stimulated and resting nerves. Vertical bars indicate standard deviations. *E*, stimulated; *C*, non-stimulated. Eleven experiments.

To study the participation of Ca ion in the changes of protein configuration various modifications of this general procedure were introduced. These will be mentioned in the section in which such experiments are described.

RESULTS

1. *Effect of Stimulation*

As shown in Fig. 1, stimulated nerves (*E*) revealed higher SGIR than the resting ones (*C*), both at peaks of 245 and 300 mμ. Ungar and coworkers (23, 24) found an increased SGIR associated with stimulation in both peaks (245 and 300 mμ) for rat nerves and only in the 300 mμ peak for frog sciatics. The present work shows that at least in *Calyptocephalella*, stimulation increases both peaks.

In view of the fact that the increased SGIR might be produced by protein

breakdown, the experiments described were repeated introducing dialysis to the procedure. Aliquots of extracts E and C were dialyzed against KCl-tris for 24 hours under refrigeration. As a control of the dialysis, other aliquots were left under refrigeration for 24 hours without being dialyzed. The results of twelve experiments, measured only at 245 and 300 mμ, are shown in Table I. The first and second rows (E and C) show the average SGIR of extracts from stimulated and control nerves. Δ is $E - C$ and P is the probability that E and C belong to different populations calculated according to Student. The columns describe the values (E, C, Δ, and P), (a) immediately after the nerve extracts were made (Init.), (b) after 24 hours of dialysis (Dial.), and (c) after

TABLE I

EFFECT OF DIALYSIS ON THE SGIR OF EXTRACTS OF
STIMULATED AND RESTING NERVES. TWELVE EXPERIMENTS

		Init.	Dial.	24 Hrs.
	E	1.62	1.51	1.56
245 mμ	C	1.54	1.49	1.47
	Δ	0.08	0.02	0.09
	P	0.99	0.80	0.99
	E	1.28	1.16	1.26
300 mμ	C	1.21	1.08	1.20
	Δ	0.07	0.08	0.06
	P	0.98	0.84	0.89

E, extracts of nerves stimulated at 120/sec. for 30 min. C, extracts of non-stimulated nerves. Δ, $E - C$. P, probability, according to Student, that E and C come from different populations. Init., values obtained immediately after the nerve extracts were made. Dial., values obtained after 24 hrs.' dialysis in the cold. 24 hrs., values obtained after 24 hrs.' standing in the cold.

24 hours' standing (24 hrs.). It was found that dialysis reduces the SGIR of E and C, both at 245 and 300 mμ, indicating the presence of small molecular weight substances containing tyrosine and cysteine in the extracts. Only at 245 mμ the loss in SGIR due to dialysis is higher in the stimulated group. In judging these data one has to consider the fact that also from standing 24 hours there is a great deal of loss of SGIR at 245 mμ, showing that the −SH groups are not stable under the experimental conditions. The important point is that the differences in SGIR between E and C are maintained at 300 mμ. At 245 mμ this difference is reduced but still remains a little in favor of E. The probabilities, at both peaks, that E and C belong to different populations, are also reduced with the dialysis. Nevertheless, considering the data obtained at both wave lengths together we feel certain that E and C remain distinct groups.

At this point, evidence should be included which shows that the observed

changes in extracts from stimulated nerves are a specific physiological effect of nerve impulse propagation rather than a secondary action of the stimulating currents. In fact Ungar *et al.* (23) showed that these changes occur in cerebral cortex of cats, dogs, and rats after stimulation of their afferents, that means, after stimulation of the corresponding brachial plexus; the same area (sensory-motor area) of the opposite hemisphere was used as a control. Furthermore, Table II shows the results of eleven experiments made as follows: groups of four sciatic nerves were stimulated as usual for 30 minutes, the distance between stimulating and recording electrodes being about 3 cm. After stimulation the nerves were cut halfway between stimulating and recording electrodes;

TABLE II
SGIR OF EXTRACTS OF STIMULATED NERVES LYING ON THE STIMULATING ELECTRODES (*C*) AND ON THE RECORDING ELECTRODES (*E*)

	SGIR	
	245 mμ	300 mμ
E	1.62	1.23
C	1.62	1.22
Δ	0.00	0.01
P	0.15	0.15

Δ, $E - C$. P, probability, according to Student, that E and C come from different populations.

the portion over the stimulating electrodes was called *C*, and that portion lying on the recording electrodes was called *E*. Extracts of both *C* and *E* groups were made as usual and were studied in the spectrophotometer at pH 7 and 12. Table II shows that the values of SGIR obtained from the part of the nerves lying on the stimulating electrodes (*C*) do not differ at all from those obtained from the distal parts (*E*). Accordingly, the effect described is specific for nerve impulse propagation.

The unmasking of ionizable groups concomitant with stimulation is a reversible phenomenon. This was shown by Ungar *et al.* (23) and we have confirmed it. The results with twenty-eight groups of nerves which were stimulated and then allowed to rest for 1 hour (*E*), and with eleven groups of nerves which were not stimulated (*C*) appear in Table III. It can be seen that the probability *P* is small, indicating that *E* and *C* come from the same sample. In this particular experiment the *C* and *E* nerves did not belong to the same frogs, which explains the scattering of the data.[1]

[1] The reader should not be misled by the fact that two values which should be about the same appear as different in different tables or figures (*e.g.* E_{245} in Fig. 1 is 1.78 and E_{245} in Table I is 1.62 or C_{245} in Table V is 1.72 and C_{245} in Table III is 1.50). This apparent contradiction occurs because every

It should be pointed out that these results and those that follow were obtained without incubating the homogenized nerves with the extracting solution at 37°C, as was done by Ungar *et al.* (23). We thought that this step was undesirable. In fact, the results of thirteen experiments not included in this work, show that incubation increases the SGIR of both extracts, the increase being a little larger for the stimulated group. In other words, incubation increases the difference between the stimulated and control groups, which it seems to us, can only be due to a greater proteolytic activity of the extracts from the stimulated group.

TABLE III

EFFECT OF 1 HOUR OF RECOVERY ON THE STRUCTURAL
CHANGES PRODUCED BY STIMULATION. *E*, TWENTY-EIGHT
EXPERIMENTS; *C*, ELEVEN EXPERIMENTS

	SGIR	
	245 mμ	300 mμ
E	1.53	1.12
C	1.50	1.08
Δ	0.03	0.04
P	0.10	0.20

E, extracts of nerves stimulated at 120/sec. for 30 min. and then allowed to rest in Ringer's solution for 1 hour. *C*, extracts of nerves which were not stimulated. Δ, *E* − *C*. *P*, probability, according to Student, that *E* and *C* come from different populations.

2. *Effect of 0.3 mμ Azide on the Reversibility of the Structural Changes Described*

One group of nerves (*E*) was kept in Ringer's solution plus 0.3 mμ sodium azide for 2 hours under refrigeration (0–5°C), while the other group (*C*) was maintained without azide under the same conditions. Afterwards, both groups were stimulated for 30 minutes and allowed to rest for 1 hour at 20°C, the *E* group in Ringer's solution plus azide and the *C* group in pure Ringer's solution. Thereafter, extracts were made and the SGIRs were determined.

The results are summarized in Table IV. The first and second rows show the average SGIRs of the *E* and *C* groups, at 245 and 300 mμ. It can be observed that the SGIR of stimulated nerves which have been allowed to rest in Ringer's solution without azide is lower than the SGIR of the nerves treated with azide. Since Ungar's results and ours show that the changes under observation are reversible, we conclude that the presence of only 0.3 mμ sodium azide has been sufficient to at least impair the reversibility.

table, unless clearly and otherwise stated, presents the results of a different experiment performed with a different group of frogs and there is a considerable variation in the SGIR of nerve extracts from different frogs and particularly from frogs of different seasons.

3. Effect of 10 Meq/Liter Ca Ion on the SGIR of Resting Nerve Extracts

Sciatic nerves from six frogs were desheathed and then their ability to conduct impulses was checked. Afterwards, the desheathed nerves were immersed for 3 hours in 250 ml of calcium-free Ringer's solution at 0 °C. The solution was changed every hour. Then the nerves were blotted on filter paper and an extract was made in KCl-tris. After centrifugation the supernatant was divided into two aliquots. To one of them (E) enough 1.0 M $CaCl_2$ was added to raise the concentration of Ca ion in the aliquot to 10 meq/liter. To the other ali-

TABLE IV

EFFECT OF 0.3 mM SODIUM AZIDE ON THE REVERSIBILITY
OF PROTEIN CHANGES PRODUCED BY STIMULATION.
TWENTY-EIGHT EXPERIMENTS

		SGIR	
		245 mμ	300 mμ
	E	1.63	1.25
	C	1.53	1.12
	Δ	0.10	0.13
	P	0.98	0.99

E, extracts of nerves which were stimulated at 120/sec. for 30 min. and then allowed to rest for 1 hour, in the presence of 0.3 mM sodium azide in Ringer's solution. C, same as E but in absence of 0.3 mM sodium azide. Δ, $E - C$. P, probability, according to Student, that E and C come from different populations.

quot (C), the same amount of solvent was added. Both E and C aliquots were then incubated at 10 °C for 30 minutes and the SGIR at 245 mμ and 300 mμ was determined.

Table V summarizes the results. One observes that Ca ion is capable of altering the state of resting nerve proteins. This effect is shown in both the 245 and 300 mμ peaks. Apparently the contact with Ca ion produces the liberation of ionizable groups.

4. Effect of Low Calcium Ringer's Solution on the Configurational Changes of Nerve Proteins Produced during Stimulation

In this section, the same experiments reported in section 1 were repeated but the concentration of Ca ion in the bathing medium was reduced from 2.16 meq/liter to 0.2 meq/liter. The nerves (control and stimulated) were previously kept in low Ca^{++}-Ringer's solution with 3.0 mM sodium azide for 2 hours at 5 °C. These nerves were crushed and extracted without freezing since

azide prevents the reversibility of the structural changes under observation.
The use of 3.0 mM sodium azide instead of 0.3 mM as described in section 2 is
justified on the basis that the effects of this drug at these two concentrations on
the ionic changes and oxygen consumption of frog nerve are not qualitatively
different (13). Hurlbut's results (13) show that the effect of azide both on the
resting respiration and on the sodium and potassium shifts is increased as
azide concentration is raised. Accordingly, we have assumed that 3.0 mM
azide has a higher capacity than 0.3 mM to impair the reversibility of the
changes being observed. Still, it should be mentioned that three experiments
were made without 3.0 mM sodium azide in exactly the same manner as de-
scribed in section 1 with the same results. Because of this, these three experi-

TABLE V

EFFECT OF 10 MEQ/LITER [Ca++] ON EXTRACTS FROM
RESTING NERVES. SEVENTEEN EXPERIMENTS

	SGIR	
	245 mμ	300 mμ
E	1.82	1.72
C	1.72	1.56
Δ	0.10	0.16
P	0.99	0.99

E, extracts of resting nerves previously kept in calcium-free Ringer's solution
and then treated with 10 meq/liter of calcium ion. C, same extracts as E but
not treated with calcium. Δ, E − C. P, probability, according to Student,
that E and C come from different populations.

ments were pooled together with the six experiments made under the effect
of azide. Furthermore, the nerves used in section 4 were not desheathed. This
fact introduced an element of uncertainty in the extracellular level of calcium.
Nevertheless, whatever that reduced extracellular level of calcium was, it was
sufficient to produce the results described below. In all the experiments under
section 4 the nerves from the E group were stimulated as usual and their
activity was also continuously displayed on the screen of an oscilloscope. The
ability to conduct impulses at 120/sec. in the azide-treated nerves was not
different from that in the normal nerves. If one assumes that the results of
FitzHugh (6) apply to Calyptocephalella, one would expect a refractory period
of the order of 2 to 2.5 msec. in the presence of 3.0 mM sodium azide. Exclud-
ing all other possible factors except azide, those figures imply the ability to
conduct impulses at frequencies lower than 400/sec. In any case, our azide-
treated nerves only began to have difficulty keeping up with the frequency of
stimulation at 240/sec. and then only after a few minutes of stimulation. This
was noted by a decreased compound action potential which was immediately
recovered by switching to lower frequencies.

Fig. 2 shows the increase in SGIR due to stimulation (ΔSGIR) as obtained from the data used in the elaboration of Fig. 1, that is to say, in the presence of normal calcium-Ringer's solution (hatched, eleven experiments). It also shows the increase in SGIR due to stimulation in the presence of low calcium-Ringer's solution (stippled, nine experiments). In other words, the ordinate is the difference in SGIR between stimulated and non-stimulated nerves. The results are presented as ribbons, the centers of which are the averages of the differences, and the width of which is equal to two standard deviations.
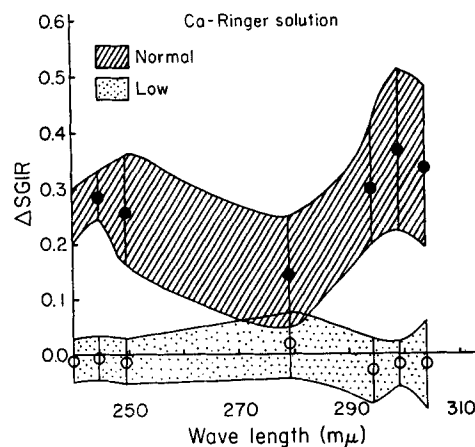

Figure 2. ΔSGIR, SGIR of extracts from stimulated nerves minus the SGIR of extracts from non-stimulated nerves. SGIR due to stimulation of nerves kept in normal Ca-Ringer's solution and low Ca-Ringer's solution. Vertical bars indicate standard deviations of ΔSGIR. Eleven experiments (hatched); nine experiments (stippled).

One sees that in the nerves kept in normal Ringer's solution the differences between stimulated and non-stimulated groups are significantly different from zero. These differences, as expected, are larger in the zones of the 245 and 300 mμ peaks. On the other hand, in the nerves kept in low Ca^{++}-Ringer's solution the differences between stimulated and non-stimulated groups are practically zero; *e.g.*, one sees that the axis of abscissas is contained within the ribbon for low Ca^{++}-Ringer's solution.

DISCUSSION

Ungar and coworkers (23, 24) showed that after intense nerve stimulation nerve proteins undergo some degree of reversible denaturation in which side chains which could not be demonstrated before become detectable. In this respect our findings are in full agreement with theirs. However, by adding N-acetyl-L-tryptophan ethyl ester to the extracts as a substrate it has been

found that potentially the proteolytic activity of stimulated structures is increased (23). In these circumstances it might be hypothesized that the high SGIR of the extracts of stimulated nerves might be due, at least partly, to protein hydrolysis. However, if it is taken into account that the same kind of results are obtained if extracts are dialyzed before studying them in the ultraviolet, it becomes apparent that this cannot be the most important phenomenon involved. The increased SGIR must indicate, then, that the excess of demonstrable side chains has been produced by a different process, namely, protein denaturation but not hydrolysis. Still, keeping in mind that the turnover of nerve proteins seems to be increased during impulse propagation (18), it must be concluded that a small part of the nerve proteins undergo a breakdown due to stimulation but that this is not clearly detectable by the loss in SGIR upon dialysis. Such an increase of nerve protein turnover would be in agreement with the finding that the TCA-soluble fraction of the proteins extracted from cat brain is increased upon stimulation (10). The reader should not conclude, however, that more nerve proteins were rendered soluble in KCl-tris upon stimulation. This was shown by the fact that the optical densities of the extracts at pH 7 from stimulated as well as from resting nerves were the same at all wave lengths studied. This was not so at pH 12. The higher increase in absorbancy shown by the extracts from stimulated nerves at high pH and only in the regions of 245 and 300 mμ means that in those extracts there are more cysteine —SH and tyrosine —OH free to ionize. It can also be concluded from the present work, in agreement with Ungar et al., that the described protein changes are reversible.

The configurational changes described by Ungar et al. have been discussed in terms of Ling's fixed charge hypothesis (23). We felt that if the protein behavior were directly connected with the excitable properties of the nerve cells, other factors affecting nerve excitability could be expected to have an effect on this phenomenon. Since Ca ion is a powerful regulator of the excitation processes (2, 7, 8), we thought that changes in the external Ca^{++} should affect the protein configuration. Thus, it was expected that if low Ca increases nerve excitability, the low calcium[2]-treated extracts should be the ones showing higher SGIRs. We were able to find a clear effect of Ca ion upon resting nerve proteins. Nevertheless, the changes of SGIR were exactly the contrary of those expected. The higher SGIRs were found in the high calcium (10 meq/liter)-treated extracts.

Ca influx increases a little during nerve impulse propagation (12). It is conceivable, then, that the configurational changes described might not occur concomitantly with the action currents, but rather a little later, after calcium

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[2] One should speak of low Ca, since no special precautions were taken to avoid contamination from the glassware used. (See reference 6.)

ion has entered the nerve fiber. According to this assumption, it should be the calcium which enters the fiber which produces the configurational changes. With this hypothesis in mind, we tried the fourth set of experiments in which Ca ion was lowered to 0.2 meq/liter in the external medium. We thought that under this condition, if our interpretation was correct, little or no changes would be detected. The results of our experiments substantiate this hypothesis.

It should be pointed out that the effects of sodium azide could be explained through the effect of calcium ion. Hodgkin and Keynes (12) showed that the flux ratio of calcium across the axon membrane is many orders of magnitude different from what one should expect from the electrochemical gradients. Their data lead to the conclusion that the outflux of calcium is mainly an active flux. If the reversibility of configurational changes in nerve proteins is associated with the active outflux of calcium ions, it would not be surprising to find, as we have indeed observed, that sodium azide, by preventing the elimination of calcium, also impairs the reversibility of the protein changes.

The attention of the reader should be called to a few points which remain obscure. The dose of azide used to impair the reversibility of protein changes in these experiments is insufficient to block the sodium pump in *Loligo* (11) or in *Ommastrephes gigas* (21); it is only a little superior to the dose found necessary by Brink *et al.* (3) to block the extra oxygen consumption associated with nerve impulse propagation in *Rana pipiens*. It might be that either the postulated calcium pump or *Calyptocephalella* is more sensitive to the effect of azide. These are points which deserve further study. Also the possibility that ATP might induce recovery *in vitro* of the azide-treated extracts has been studied in our laboratory. So far, such an effect has not been shown (17), which is in agreement with our interpretation of the mechanism involved.

It should be pointed out that it seems very difficult to associate the interpretation of the structural changes of nerve proteins described here with sodium pumping. In fact, experiments carried out in our laboratory (21) show that it is perfectly possible to have sodium pumping in giant axons without the presence of calcium in the bathing medium.

A few considerations should be taken into account as to the order of magnitude of the quantities involved in the extra influx of Ca ion during stimulation. In order to make calculations regarding the node of Ranvier, several data have been taken from the literature (16, 19). These data permit us to tentatively work with a cylinder 3 μ in diameter and 1 μ in length as an "idealized" node. These figures are assumed on the basis that in frog (16) the great majority of the myelinated fibers have a diameter between 3 and 8 μ; only occasionally a fiber of 8 to 12 μ in diameter is seen. According to our experience this is also the case in *Calyptocephalella*. A node diameter of half the fiber diameter was also assumed. With these data and the extra influx of Ca ion measured in *Loligo* (0.006 pmole/cm^2/impulse) by Hodgkin and Keynes (12), one

can estimate that about 6×10^{-10} pmole of Ca ion enters each node per impulse in our experiments. During 30 minutes of stimulation at 120 per second, this could amount to enough to raise the concentration of Ca ion to 10 meq/liter in a volume of 26 μ^3, that is, a little less than four times the volume of a node. In judging these figures one has to have in mind the fact that labeled calcium microinjected into giant axons shows very little mobility (12). Accordingly, it would seem (a) that the calcium entering the node would not tend to be greatly diluted by diffusion and (b) as calcium ion enters the fiber it would immediately become "bound," so that it would not decrease the original effective concentration gradient. Of course it must be taken into account that the data taken from *Loligo* may be far from the situation in the frog. The only conclusion that can be derived from these figures is that it seems that enough Ca ion might enter the fiber to produce the changes described.

In view of the uncertainties mentioned regarding the calcium hypothesis described, we only ascribe to it the value of a working hypothesis. The main contribution of the present work, as we see it, is that it has been shown that it is possible to obtain nerve impulse propagation without the configurational changes described by Ungar *et al.*

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