

# Discrete Threshold and Repetitive Responses in the Squid Axon Under 'Voltage-Clamp'<sup>1</sup>

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## ABSTRACT

TASAKI, I. AND A. F. BAK. *Discrete threshold and repetitive responses in the squid axon under 'voltage-clamp.'* Am. J. Physiol. 193(2): 301-308. 1958.—By using the so-called voltage-clamp technique, records were obtained indicating the presence of undulatory or oscillatory membrane current under fixed potential difference between the internal and external electrodes. This oscillatory membrane current was observed only when rectangular membrane depolarization was in the range between 15 and about 35 mv. The amplitude of the oscillatory membrane current was of the order of 1 ma/cm<sup>2</sup>. The oscillatory membrane current disappeared suddenly when the depolarizing clamping pulse was reduced below the 'threshold.' Various sources of artefact were examined.

SINCE Hodgkin, Huxley and Katz (1) published the results of their voltage-clamp experiments, it has been widely accepted that, under the voltage-clamp conditions, the membrane current takes smooth, well-defined time courses, showing no sign of discontinuous threshold phenomena. During the course of a series of experiments designed to study the properties of the squid axon membrane treated with tetraethylammonium chloride (TEA), one of us (I. T.) working in collaboration with S. Hagiwara, encountered a strange phenomenon which appeared to conflict with the widely accepted view as to the behavior of the axon under voltage-clamp. The phenomenon observed could be taken as signs of the existence of a discrete threshold and of repetitive firing of responses under the so-called voltage-clamp conditions. Since such a phenomenon was entirely unexpected and appeared to be inconsistent with the basic concept of voltage-clamping, no explicit statements were made of the phenomenon in our previous publications on the squid axon (2).

Recently, we could demonstrate the existence of a similar phenomenon in the nodal

membrane of the toad nerve fiber under voltage-clamp (3). Encouraged by the similarity between the toad nodal membrane and the squid axon membrane under TEA, we expanded our observation on this discontinuous phenomenon to normal squid axons with an improved experimental setup. We found that this phenomenon could be demonstrated in the majority of the fresh squid axons available at Woods Hole.

The phenomenon in question can be stated as follows: when the 'membrane potential' of the squid giant axon is raised above its resting level by 15-25 mv and is kept at this level for a period of about 10 msec. or longer, the membrane current required to maintain the new potential level undergoes rapid undulating or 'oscillatory' changes which resemble the phenomenon of repetitive firing of impulses under ordinary experimental conditions. When the size of the clamping rectangular pulse is reduced by small steps, this phenomenon disappears suddenly at a certain intensity level, indicating the existence of a 'threshold.' When the level of depolarization by the clamping pulse is about 40 mv or higher, the time course of the membrane current observed always resembles that published by the British investigators (1).

In pursuing this phenomenon, we were fully

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<sup>1</sup>The work presented in this article was carried out at the Marine Biological Laboratory, Woods Hole, Mass.

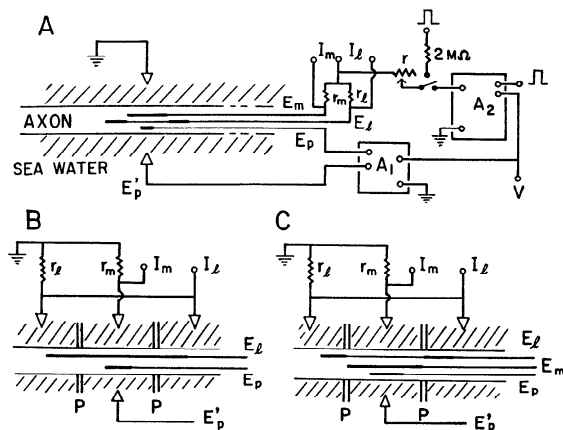


FIG. 1. *Diagram A*: experimental setup used for the experiments of figs. 2, 3 and 4. The drawings of the axon and the internal electrodes are disproportionately short and thick. The rectangular voltage pulse at the input of the high-gain differential amplifier,  $A_2$ , is used to clamp the membrane potential; the other rectangular pulse is used to obtain records of ordinary action potentials. Further details are in the text. *Diagram B*: arrangement used for the experiment of fig. 7. Partitions,  $P$ , are made with glass-plates (1 mm thick) and with a small amount of Vaseline. *Diagram C*: arrangement used for the experiment of fig. 5. Partitions (made with Lucite plate and Vaseline) are watertight.

aware of the possibility that there might have been some kind of artefact in our experiments. We made therefore a number of control experiments designed to explore such a possibility. We try in the present paper to present the results of these experiments as objectively as possible.

A preliminary report of this work has been published elsewhere (4).

#### METHODS

The material, the experimental setup and the procedures employed in the present experiments were similar to those described in a previous paper (2). Giant axons, taken from the squid available at Woods Hole, were used, in many cases without extensive cleaning of the surrounding tissues. In some cases, they were used after extensive cleaning under dark-field illumination. The axon was mounted horizontally on a glass-plate of about 42 mm width, and a set of internal electrodes, consisting of two or three twisted silver wires was inserted along the axis of the axon.

The arrangements of the internal and external electrodes employed in the present experiments are illustrated by three diagrams in figure 1. The arrangement of diagram *A* was

used most frequently. The arrangement of diagram *B* is similar to that used by the British investigators (1) and was used mainly in the early stage of the present investigation. The arrangement of diagram *C* is a combination of the two, *A* and *B*.

In the arrangement of diagram *A*, the internal electrode-set consisted of three silver wires of about  $50\mu$  in diameter. The potential-electrode,  $E_p$ , had an exposed surface of 3–6 mm in the middle of the current-electrodes. The main current-electrode,  $E_m$ , had a 6 mm (sometimes 3 or 8 mm) long uninsulated surface, and the lateral current-electrode,  $E_l$ , had two bare portions of 4 (sometimes 3 or 8) mm length adjacent to the bare portion of the main current-electrode. These internal silver wire electrodes were used both with and without chloriding the surface electrolytically. The external potential-electrode,  $E'_p$ , was a large silver wire covered by a layer of gauze and agar gel. The ground electrode, which was similarly covered with gauze and agar gel, had a resistance of about 10 ohms to current pulses used in the present experiments. The significance and the effect of the lateral current-electrode will be discussed under RESULTS.

The time course of the potential difference between the two potential electrodes,  $E_p$  and  $E'_p$ , was recorded with a unity-gain differential preamplifier,  $A_1$  in figure 1, diagram *A* (cf. fig. 2 in ref. 2); the recording was as a rule d.c.-coupled. The membrane current,  $I_m$ , was determined by recording the IR-drop across resistance  $r_m$  which was generally between 2 and 10 ohms. The high-gain differential amplifier for negative feed-back,  $A_2$  in diagram *A*, was a Tektronix amplifier of type 112 (instead of type 122 in the previous experiments), modified to give an output impedance of about 250 ohms. This was achieved by adding paired cathode-follower stages (two 6CL6 tubes) at the output of the commercially available amplifier. The over-all voltage amplification,  $n$ , by this amplifier was approximately 2,300. The resistance connected between the output of this amplifier and the current-electrodes,  $r$  in the diagram, was varied between 500 and 25,000 ohms. The smallest value of  $r/n$  (cf. p. 864 in ref. 2) available was therefore about 0.3 ohm.

As in the previous experiments (2), negative

feed-back was accomplished by condenser-coupling. This did not give any limitation to our observation, since the duration of rectangular clamping pulses was in most cases shorter than 20 msec. and furthermore the time course of the membrane potential,  $V$ , was always monitored by the second beam of a dual-beam oscillograph (DuMont type 322). Precautions were taken to eliminate all the causes for a shift in the membrane potential resulting from closure of the manually operated key between the feed-back amplifier and the internal current-electrodes. The feed-back circuit was closed immediately before the start of a sweep of the oscillograph beams and was opened immediately after a photograph of the potential and current traces was taken. A period of at least 10 seconds (often 30 sec. or more) was allowed before another clamping pulse was delivered. Most of the axons examined showed no sign of progressive deterioration during the course of experiments which sometimes lasted more than 3 hours.

Most of the experiments described in this paper were carried out at room temperature which was between 20 and 23°C.

#### RESULTS

**Membrane Currents Caused by Clamping Pulses Near Threshold.** When the membrane potential of a giant axon was suddenly raised above its resting level by a rectangular clamping pulse of 50–90 mv, a transient surge of strong current flowing inward through the membrane was observed (see the lower column of fig. 2). The current intensity at the peak of the inward surge decreased linearly with the size of the clamping pulse in this range. When the membrane potential was clamped at the level of the peak potential of the action potential (observed without clamping), the membrane current at the peak of the inward surge was close to zero. For negative clamping pulses, namely when the membrane potential was clamped at levels below its resting level, the membrane behaved like a passive, ohmic (slightly nonlinear) resistor with a parallel condenser. These findings agree perfectly with what has been reported by the British investigators.

In the range of rectangular clamping voltage between 10 and 40 mv, Hodgkin *et al.* reported the appearance of a single small and smooth

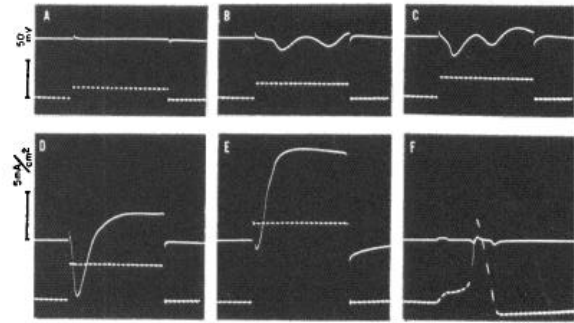


FIG. 2. Records of membrane current associated with rectangular depolarization of a normal axon taken with the arrangement of fig. 1A. Dotted lines in records A to E show the potential difference between the internal and external potential-electrodes controlled by the method of voltage-clamp. Record F was taken by passing a short pulse of outward current through the membrane and later keeping the membrane current at practically zero. Blanking, 0.1 msec. interval. The effective length of the main current-electrode ( $E_m$ ) was 6 mm, that of the lateral current-electrode 3 mm each and the axon diameter 400  $\mu$ . Temperature, 22°C.

inward surge which has slow rising and falling phases. However, we rarely obtained oscillograph records similar to those reported by the British investigators. Two most common examples of our records are reproduced in figure 2 and figure 3, illustrating the difference between our results and those of the previous investigators.

In our records an upward deflection of the potential (lower) trace represents a rise in the axoplasm potential referred to the potential of the surrounding sea water; this recording polarity was chosen to show an ordinary action potential (record F in fig. 2) as a transient upward deflection. The polarity of the current (upper) trace is opposite to what was used by the British investigators; the reason for adopting this new convention is to represent the behavior of an ohmic resistor by a parallelism between the deflections of the two traces.

With a pulse intensity of about 15 mv, or slightly less, we generally obtained a weak inward membrane current (of the order of 50  $\mu\text{a}/\text{cm}^2$  or less) which smoothly changed into an outward membrane current. When the pulse intensity was gradually increased from this level, there was, in almost all the 'normal' axons (i.e. in those producing action potentials larger than 100 mv), a sudden appearance of a large inward membrane current (records B in

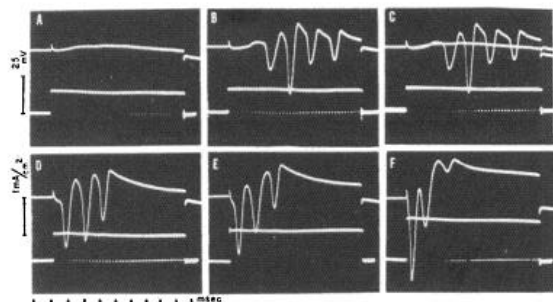


FIG. 3. Records showing the time course of the membrane current (*upper trace*) observed when the potential difference across the axon surface (*lower trace*) was clamped at various levels between 15 and 30 mv above the resting potential. In record C 2 sweeps at slightly different pulse intensities were superposed. Time markers, 1 msec. apart 22°C.

fig. 2 and B and C in fig. 3). In other words, there was a discontinuous, true 'threshold' in our experiments. The magnitude of this discrete inward current varied to a considerable extent with the state of the axon; in many cases it was of the order of 1 ma/cm<sup>2</sup>. The time interval between the beginning of the clamping pulse and the start of the discrete inward surge depended markedly upon the size and the duration of the clamping pulse; at room temperature it was sometimes longer than a few milliseconds.

The first discrete surge of inward membrane current was, as a rule, followed by several similar inward surges. The configurations of these inward surges varied in many cases from one surge to another, but in some cases (e.g. in record D in fig. 7) they appeared to be uniform. It was frequently seen that these individual surges had a more-or-less abrupt beginning and ending and consequently a succession of these surges resembled the pattern of repetitive firing of impulses in an unclamped axon membrane. The interval between individual surges was close to 1 msec. (0.5–1.5 msec.) at room temperature (22°C). In a series of experiments to be published later, it will be shown that this interval is prolonged markedly by a fall in temperature.

When the size of the clamping rectangular pulse was raised from this threshold level gradually, the latency of the first discrete inward surge was decreased gradually, the amplitude of the first discrete surge was increased gradually and the pattern of the undulating or oscillatory membrane current

changed gradually into the type of a smooth damping oscillation. When the clamping pulse reached the level of 40–50 mv above the resting potential, the multiple peaks in the inward membrane current disappeared and the records obtained resembled those published by the earlier investigators except for the fact that the maximum inward current observed was four to nine times as strong as those reported by Hodgkin *et al.*

In order to examine the existence of a threshold and a sign of repetitive responses in our axon under voltage-clamp further, the following control experiments were performed: a) investigation of the effects of imperfection of voltage-clamping, b) the possible effect of the series resistance of the membrane, c) the effect of the guard system and d) the effects of various chemicals.

**Effect of Feed-Back and Compensation.** As has been stated under METHODS, the feed-back amplifier used in the present series of experiments has a gain of 2300 and its output impedance was about 250 ohms. By controlling the resistance between the feed-back amplifier and the internal current electrodes ( $r$  in the figure) between 500 and 25,000 ohms, we examined the effect of imperfection of voltage-clamping. It was our repeated finding that the variation in the amount of feed-back in the range just mentioned had very little or no clear effect upon the rhythm and the amplitude of the repetitive inward surges. An example of such observations is presented in figure 4, top.

When the feed-back was the weakest within the range mentioned above (left upper record), there was a slight elevation in the membrane potential at the time when the inward current reached a peak. Evidently, the feed-back was insufficient to control the membrane potential when the membrane resistance underwent a marked reduction. When resistance  $r$  between the internal current-electrodes and the feed-back amplifier was reduced to about 3000 ohms or less, the trace displaying the potential difference between the internal potential-electrode and the external indifferent electrode took a perfectly rectangular time course. In all (more than 30) axons examined, the repetitiveness in the membrane current remained unaffected when the feed-back was increased to the highest value in the range mentioned above.

It has been postulated (1) that there is a resistance of about  $5 \text{ ohm}\cdot\text{cm}^2$  in series with the excitable membrane which consists of a variable resistance with a parallel condenser. The small resistance of the axoplasm between the internal electrodes and the surface membrane of the axon is undoubtedly connected in series with the membrane capacity. This series resistance should be smaller than about  $10 \text{ ohm}\cdot\text{cm}^2$  in our preparations, since the entire resistance between the internal and the external electrodes is between 10 and 15  $\text{ohm}\cdot\text{cm}^2$  in most of our preparations (cf. p. 873 in ref. 2). We made a series of experiments in which the effect of this series resistance was compensated by an external circuit.

The principle of the method of compensating the series resistance we employed is as follows: In diagram *B* of figure 1, the area of the axon membrane in the middle pool is close to  $0.1 \text{ cm}^2$ . If one assumes that the clamped portion of the axon membrane is uniform, the series resistance of  $5 \text{ ohm}\cdot\text{cm}^2$  should then give rise to a potential drop of 50 ohms times the current passing through the membrane in the middle pool. When resistance  $r_m$  connected to the middle pool is 5 ohms, the IR-drop across the series resistance should be 10 times as large as that across  $r_m$ . We amplified the potential drop across  $r_m$  1000 times with a differential amplifier (Tektronix no. 122) and, using a potential divider which reduced the potential drop by a factor of 100, the signal was superposed upon the rectangular pulse used to clamp the membrane potential. The membrane was thus clamped by the amplified IR-drop across  $r_m$  superposed upon a rectangular pulse. By varying the value of  $r_m$  and also the potential divider, it was possible to compensate for the series resistance of about  $7 \text{ ohm}\cdot\text{cm}^2$  or less. This method of compensation was applied also to the arrangement of diagram *A* in figure 1.

In the right, upper record of figure 4, it is seen that the membrane potential goes down at the moment when there is a strong inward surge in the current trace. (Note that the sign of potential variation is opposite in the left upper record.) By separating the recorded membrane potential into an IR-drop superposed upon the rectangular pulse, it is found that the series resistance of approximately 65

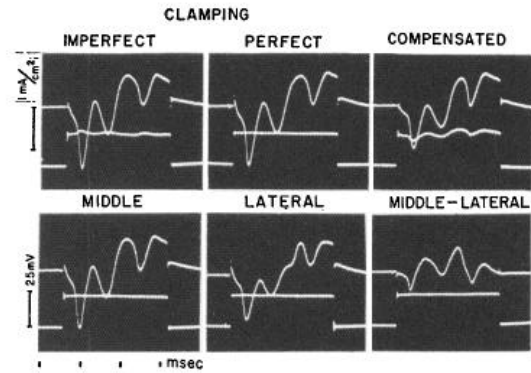


FIG. 4. *Upper column:* records of the current through the main current-electrode ( $r_m$  in fig. 1*A*) taken when the feed-back for clamping the 'membrane potential' was insufficient (*left*), when it was sufficient (*middle*) and when the 'series resistance' of the membrane was compensated (*right*). *Lower column:* Current through the main current-electrode (*left*), that through the lateral current-electrode (*middle*) and the difference between the 2 currents (*right*). The rectangular clamping pulse was approximately 22 mv in all the records in this figure. The experimental arrangement was the same as that for fig. 3.

ohms (about  $5 \text{ ohm}\cdot\text{cm}^2$ ) has actually been compensated. This type of compensation was found to reduce the amplitude of individual inward surges, reflecting the falls in the membrane potential at the peaks of inward surges. However, the pattern of the repetitiveness remained essentially unaffected by the procedure.

**Effect of the Guard System.** When the membrane potential is clamped along a rectangular time course, the internal current-electrode carries a current that flows near the end of the current-electrode (where voltage-clamping is imperfect) as well as the current which is actually used to clamp the voltage. The arrangement of figure 1*B*, was used previously (1, 2) to eliminate the current flowing near the end of the current-electrode. In this arrangement, the fluid medium surrounding the axon is divided into three separate pools by two partitions. If the potentials of the three pools are maintained at the same level during voltage-clamping, the current that flows through the middle electrode should represent the membrane current in the uniformly clamped portion of the axon. However, a strong current required to clamp the membrane potential tends to set up potential differences among the pools which can cause flows of current through the leakage resistance

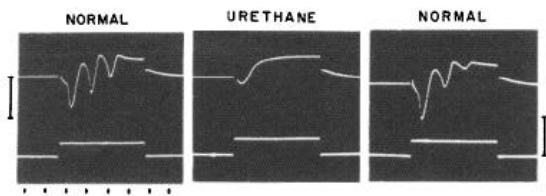


FIG. 5. Effect of urethane upon the repetitiveness of the axon membrane under 'voltage-clamp.' The arrangement of fig. 1C was used, in which the middle pool was 4.5 mm wide, partition about 2.5 mm wide, the effective length of electrode  $E_m$  5 mm, that of  $E_l$  5 mm each. The lateral pools were filled with 3% urethane-sea water. The fluid in the middle pool was first normal sea water (*left*), next urethane-sea water (*middle*) and then normal sea water again (*left*). Calibration: 50 mv for the potential (*lower*) trace and 1 ma/cm<sup>2</sup> for the current (*upper*) trace. Time markers, 1 msec. apart.

across the partitions. This can cause an error in the measurement of the membrane current. Nevertheless, we sometimes employed this method in the present series of investigations.

The second method employed in the present study is illustrated by diagram *A* in figure 1, in which an 'internal guard system' is adopted. If the potential difference between the two internal current-electrodes is maintained at a low level (in practice within a fraction of 1 mv), the potential drops across resistor  $r_m$  (2-10 ohms) should give a faithful index of the currents carried by the main current-electrode to the membrane. The effective length of the main (middle) current-electrode used was between 3 and 6 mm, and that of the lateral one was 3-8 mm on each side of the main electrode.

The third guard system employed is shown by figure 1C, which is a combination of the two methods mentioned above. With this third method, it is possible to compare the current recorded with the internal current-electrode with that observed with the external current-electrode. In combination with these guard systems, we employed the method of narcotizing the portion of the axon in the lateral pools with a 3% urethane-sea water solution. This narcosis of the axon in the lateral pools tends to increase the leakage current across the partitions, but the inward currents observed under these conditions can be regarded as a sign of physiological activity taking place in the axon in the middle pool.

We made a number of experiments by the

use of the methods mentioned above to test whether or not the multiple inward surges described above arise in the portion of the axon near the terminal of the internal current-electrode. In all cases the result of our observation supported the view that these inward surges arise in the 'clamped' regions of the axon rather than from the terminal portion of the clamped axon. An example of the experiments along this line is presented in the lower column of figure 4. In this example, the effective length of the main current-electrode (exposed for 6 mm) was equal to that of the lateral current-electrode (3 mm on both sides). The amplitude of the oscillatory membrane current traversing the resistance ( $r_m$ ) connected to the main current-electrode was slightly larger than that traversing the lateral electrode. Consequently, the difference between the two currents showed a small oscillation which was approximately in phase with the current flowing through the main current-electrode.

**Effect of Narcosis.** The effects of alcohol and urethane upon the current-voltage relationship of the axon membrane under voltage-clamp will be reported fully elsewhere. The effects of these narcotics upon the repetitive inward surges can be summarized as follows: in a deeply narcotized (but not totally inexcitable) axon, the action potential of which was completely graded, there was no sign of discontinuous phenomenon or repetitive membrane currents. Under light narcosis, various degrees of transition between the behavior of the normal axon and that of a deeply narcotized axon were observed.

The records furnished in figure 5 were obtained by using the arrangement of figure 1C. The portions of the axon in the lateral pools were rendered inexcitable with a 3% urethane-sea water solution. Recordings from both the internal and external main current-electrodes indicated that there were repetitive inward surges in the excitable membrane in the middle pool. In the external lateral current-electrode, there was a weak oscillatory current which had an amplitude of about one-fifth of that in the main current-electrodes; we attributed this to a spread of currents across the partitions. When the sea water in the middle pool was replaced with the 3% urethane solution, the membrane became nonrepetitive within 1

minute; in other words, the axon acquired a character to respond to rectangular clamping pulses with single surges of inward current, the amplitude of which varied continuously with the pulse intensity. Approximately 1.5 minutes after introduction of the narcotic into the middle pool, the fluid in the middle pool was replaced with normal sea water. As can be seen in the record, there was a partial recovery in the repetitiveness of the axon membrane under voltage-clamp. The effect of short narcosis of the axon in the middle pool was reversible and the observation could be repeated several times.

The records in figure 6 show the gradual transition of the axon membrane from the normal state to the narcotized. These records were obtained with the arrangement of diagram *A* in figure 1. When the axon was in normal sea water, the action potential of the axon (recorded under current-clamp) was highly all-or-none; that is, the jump in the membrane potential from the subthreshold level to the level of a full-grown response was large and discontinuous. Under deep narcosis, the action potential of the axon became graded; that is, the peak value of the action potential varied continuously with the intensity of the applied current pulse. Under these conditions, little or no discrete inward membrane current was observed when the potential difference between the internal and external electrodes was altered along rectangular time courses. The effect of narcosis upon these properties of the axon was nearly perfectly reversible.

**Effect of TEA.** As has been stated in the introduction, the repetitiveness under voltage-clamp attracted our attention first in axons treated with TEA. Since we did not include the information as to this property of the axon in our previous paper (2), we make here a brief description of these early observations.

The records presented in figure 7 were taken from an axon into which an isotonic solution of TEA had been injected uniformly from one end of the axon to the other. The technique of voltage-clamping in this experiment was described earlier (2). The properties of the prolonged action potential (records *A* and *B*) and the voltage-clamp behavior for large depolarizing pulses (record *F*) were also fully discussed in the earlier paper. Although the

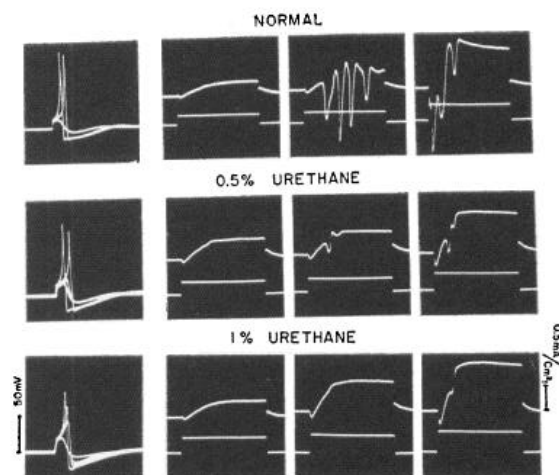


FIG. 6. Loss of the discreteness in the membrane action potential (*left*) and in the membrane current under 'voltage-clamp' (3 records on right) by narcosis.

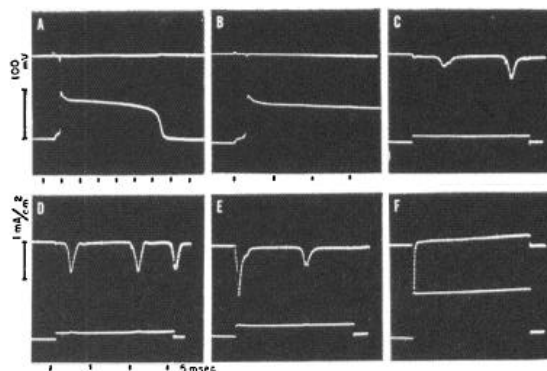


FIG. 7. Action potentials of an axon treated with TEA (records *A* and *B*) and the membrane current under 'voltage-clamp' of the same axon (records *C* to *F*). The arrangement of fig. 1*B* was used. Time markers, 5 msec. apart.

feed-back was not very strong in these experiments, the sign of repetitiveness was clear. It is interesting to note that the repetitive inward surges in these records resemble those observed in the nodal membrane of the toad (3) in its characteristic that the membrane current was almost purely inward-directed during the whole period of voltage-clamping.

#### DISCUSSION

The range of the membrane depolarization in which the repetitive inward currents under voltage-clamp can be demonstrated is not so narrow (roughly between 15 and 35 mv) as to be easily overlooked during a standard voltage-clamp measurement. There are three possible

explanations for the discrepancy between our observations and those made by the British investigators. They are: *a*) There is in our experiments some unknown source of artefact, *b*) squid axons available in England (*Loligo forbesi*) are not as repetitive under voltage-clamp as those we used in Woods Hole (*L. pealii*), or *c*) there is a difference between our technique of voltage-clamping and that of the British investigators.

During the course of preparation of the present paper, we showed our photographic records to Drs. Grundfest, Shanes, Freygang and their associates and asked if they could demonstrate the repetitive phenomenon with their voltage-clamp apparatus. It was our pleasant surprise that their instrument which is similar to that used by the British investigators gave results similar to those presented in figures 2 and 3 of the present paper. Although this fact does not completely exclude the possibility that there might still be some artefact in our experiments, the difference in the feed-back apparatus appears to be completely excluded.

It is not possible to discuss the second possible explanation until we hear more about the squid available in England.

The third possible explanation for the discrepancy between our results and those published by the British investigators is to attribute the discrepancy to a difference in the clamping technique. We took a precaution not to repeat our clamping pulses at a high rate; an interval of at least 10–30 seconds was allowed for the axon to recover from the passage of a strong current during the period of voltage-clamping. It was our impression that clamping pulses repeated at a rate of 1/sec.

tend to alter the axon into a less repetitive (damaged) state. A constant current which arises from the feed-back amplifier when an attempt is made to clamp the membrane potential continuously above or below the resting potential can be a cause of a progressive damage of the axon. The degree of cleaning the axon or of chloriding the surface of the internal silver electrodes did not have a clear effect in our experiments.

We have frequently observed that a local surgical injury (e.g. compression or traction) altered the property of the axon locally and rendered the membrane less repetitive under 'voltage-clamp'; in these experiments, when the clamping electrodes were shifted to the neighboring uninjured part of the axon, normal repetitive inward surges were generally observed. It was our consistent observation that, whenever the discreteness in the membrane current under 'voltage-clamp' was lost, the axon was not capable of developing an action potential in a clean all-or-none manner under current-clamp.

In the following paper, evidence will be presented showing that the discreteness in the membrane current under 'voltage-clamp' arises from patchy excitation of the axon membrane.

We express our thanks to Drs. H. Grundfest, A. M. Shanes and W. H. Freygang for their valuable help and advice. We are indebted to Dr. C. S. Spyropoulos for his help in the experiment.

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