

A STUDY OF THE APPLICATION OF NEUTRON
ACTIVATION ANALYSIS TO THE EFFECT OF
PHARMACOLOGICAL AGENTS ON MEMBRANE
TRANSPORT IN NITELLA CELLS.

by

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ABSTRACT

The analytical technique of neutron activation was applied to living *Nitella* cells in order to measure their sodium and potassium content.

The total flux of 4×10^{11} neutrons per second generated by the Cockcroft-Walton neutron generator was used in the studies. The duration of bombardment depended upon the element in question. The resulting gamma radiation was detected by a NaI (Tl) detector and a 128 channel spectrum analyzer, beta emission by means of a gasflow counter and the .511 MeV. annihilation radiation by means of a coincidence counter. Both types of data from β and γ radiation were analyzed by computer.

From these studies it was apparent that Na and K concentrations could be determined in *Nitella* cells statistically within 5.7% and 3.5% respectively, using β emission data. Gamma radiation, under the same condition, was found to be less diagnostic.

After bombardment the *Nitella* cells were checked for cytoplasmic streaming, membrane potentials and action potentials, all of which were comparable to the values obtained from normal cells.

In order to investigate the possible application of neutron activation to ion fluxes in Nitella cells, the electrical properties of normal cells and cells treated with specific ion inhibitors were investigated. The effect of these agents on the ion transport across the cell membrane during an action potential was observed in the change of shape of the action potential. In order to prove the nature of the current carrier or carriers after treatment with these ion inhibitors, neutron activation can be applied. In the case of potassium, calculations show that, given a flux of 10^{+12} neutrons/cm²/sec, the minimum amount of K detectable is 10^{-12} moles. The amount of K to be detected from an efflux experiment is in the vicinity of 3×10^{-9} moles. Therefore neutron activation is a suitable tool for the analysis of ion fluxes in Nitella cells.

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PURPOSE (PART A)

To investigate the possibility of applying the analytical technique of neutron activation to living Nitella cells, in order to measure the ion fluxes of Na, K and possibly Cl through the cell membrane of cells at rest and of cells at the time of an action potential when changing the external chemical environment by addition of certain drugs.

INTRODUCTION

Ion fluxes in the giant cells of Nitella have been studied by several people (1, 2). In these publications the fluxes were measured by either flame photometry or by radioactive tracers. Flame photometry has the disadvantage that its sensitivity is low, due to the small amount of sample available. Radioactive tracer techniques, as used by these people, are quite sensitive but are also time-consuming when efflux is being measured.

Neutron activation has been used to determine ion content in sepia axons (3). From these studies it was quite apparent

that activation analysis had several advantages over more established analytical methods. Activation analysis does not destroy the cell, making continuous measurements on a single cell possible; it is much faster, depending on the half life of the radioactive isotope studied, and is more sensitive than other conventional methods (4).

THEORY

The essence of neutron activation is to subject a sample to a flux of neutrons from a suitable source. Under these conditions unstable nuclei are formed. These radioactive isotopes have very specific decay characteristics. The mode of decay can be measured and attributed to the element it came from. The amount of activity will be directly proportional to the amount of the parent substance present.

The probability for a specific element to absorb a neutron into its nucleus depends on the kinetic energy of the incident particle and the target area presented by the nucleus. The apparent target area presented by the nucleus is expressed barns (δ) ($1 \text{ barn} = 10^{-24} \text{ cm}^2$) and is referred

to as cross section. The cross sections for most elements have been measured (5).

When a target is subjected to a neutron flux of a specific kinetic energy then the number of processes under consideration in 1 second, is given by equation (I).

$$N = \phi n \delta_{act} \quad (6)$$

(I)

where

N = Interaction of a given type in 1 second.

ϕ = Neutron flux (neutrons/cm²/sec.)

n = Number of nuclei of the given element which are exposed to the neutron flux.

δ_{act} = Activation cross section. (cm²)

The activity of the given nuclide per second in time (t) in the activation process with neutrons is given by equation (II)

$$A_t = \phi \delta_{act} n (1 - e^{-\lambda t}) \quad (6)$$

(II)

where

A_t = The number of disintegrations per second in time t .

λ = Decay constant and is defined as $\frac{\log 2}{t_{\frac{1}{2}}}$ (half life of isotope)

$(1 - e^{-\lambda t})$ = The isotopic saturation factor.

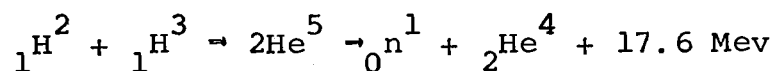
If no accurate value of the neutron flux is available a suitable standard can be bombarded along with the sample. After activation, the two activities can be compared to determine the amount of unknown element present in the sample.

PROCEDURE

I. Source of neutrons

The Cockcroft-Walton neutron generator has been used for these studies.

This neutron generator is capable of producing 4.6×10^{11} fast neutrons per second. The following reaction, using high energy deuterons, produces fast neutrons



The energy of the resulting neutrons is 14.7 Mev. Slow neutrons or thermal neutrons can be produced by using the moderators H_2O or Paraffin wax. The flux in this case is 10^7 - 10^8 neutrons/sec.

II Activation of elements

The elements of interest were Na and K. From Na²³,

Na^{24} ($t_{1/2} = 15.05$ hrs.) was produced by an n, γ reaction i.e. a neutron is absorbed and a γ ray is emitted. The cross-section for this reaction is $.40 + .13$ barns (5) using a thermal neutron flux. Also Ne^{23} ($t_{1/2} = 38$ sec.) is produced by a n, p reaction from Na^{23} using the same thermal flux.

From K^{39} , K^{38} ($t_{1/2} = 7.7$ min.) can be produced by a n, 2n reaction. The cross section for this reaction using fast neutrons is 2.0 barns.

In the case of Na^{24} , the duration of bombardment was reduced to 1 to 1.5 hours. Thermal neutrons were used because the cross section for this reaction ($\text{Na}^{23} \rightarrow \text{Na}^{24}$) is largest in a thermal flux. All samples were contained in polyethylene vials during bombardment.

III. Detection of isotopes

All samples were transferred from the polyethylene vials to a thin plastic envelope which was taped down to a stainless steel counting disc. The mounted samples were placed as close as possible to the scintillating crystal (approximately .5 cm).

a. Gamma ray detection

A NaI (Tl) crystal scintillation counter connected to a 128 channel analyzer was used to detect the γ ray spectrum of

the sample. The 128 channels were calibrated by means of the spectra of Co^{60} , Na^{22} , and Na^{24} . The radioactivity of the activated samples was counted for 2-3 half lives of the required isotope when measuring the concentration of a specific ion. When the half life was being measured, the activity was counted during 1/10 of the half life of the isotope in question at successive intervals after bombardment.

In the case of K^{38} , two 0.511 Mev γ rays are emitted at 180° to each other when an e^+ particle annihilates with an electron. In order to detect this 0.511 radiation a coincidence counter consisting of two NaI (Tl) detectors on opposite sides of the sample and a single channel analyzer set to detect the 0.511 Mev radiation were used. The coincidence counter will only record an event when two .511 Mev γ rays are detected simultaneously, thereby cutting out any background 0.511 Mev radiation present, because the probability that two unrelated 0.511 Mev γ rays are detected by each crystal simultaneously is extremely small. The coincidence counter, therefore, will give a very accurate count of the .511 Mev annihilation radiation.

b. Beta emission detection

β emission was detected by means of a gas flow proportional counter. Total beta activity was measured. Counting

procedures were similar to those described in the previous section. In the case of Ne^{23} ($t_{1/2}$ 38 seconds) the pneumatic transfer system was used in order to cut down on the time to transfer the sample from the neutron generator to the counter. The time for this procedure was about 30-45 seconds. (The pneumatic transfer system is an air pressure operated device which shoots the sample through a pipe into the neutron flux. After bombardment the sample is shot back again into the laboratory for counting purposes).

c. Analysis of data

The γ ray data obtained were analyzed by graphical means. When accurate values were required the data were analyzed by computer using a program capable of plotting the log of the number of counts against energy, and calculating the area under the peaks of the resulting γ ray spectrum. This area is directly proportional to the ion concentration of the element in question.

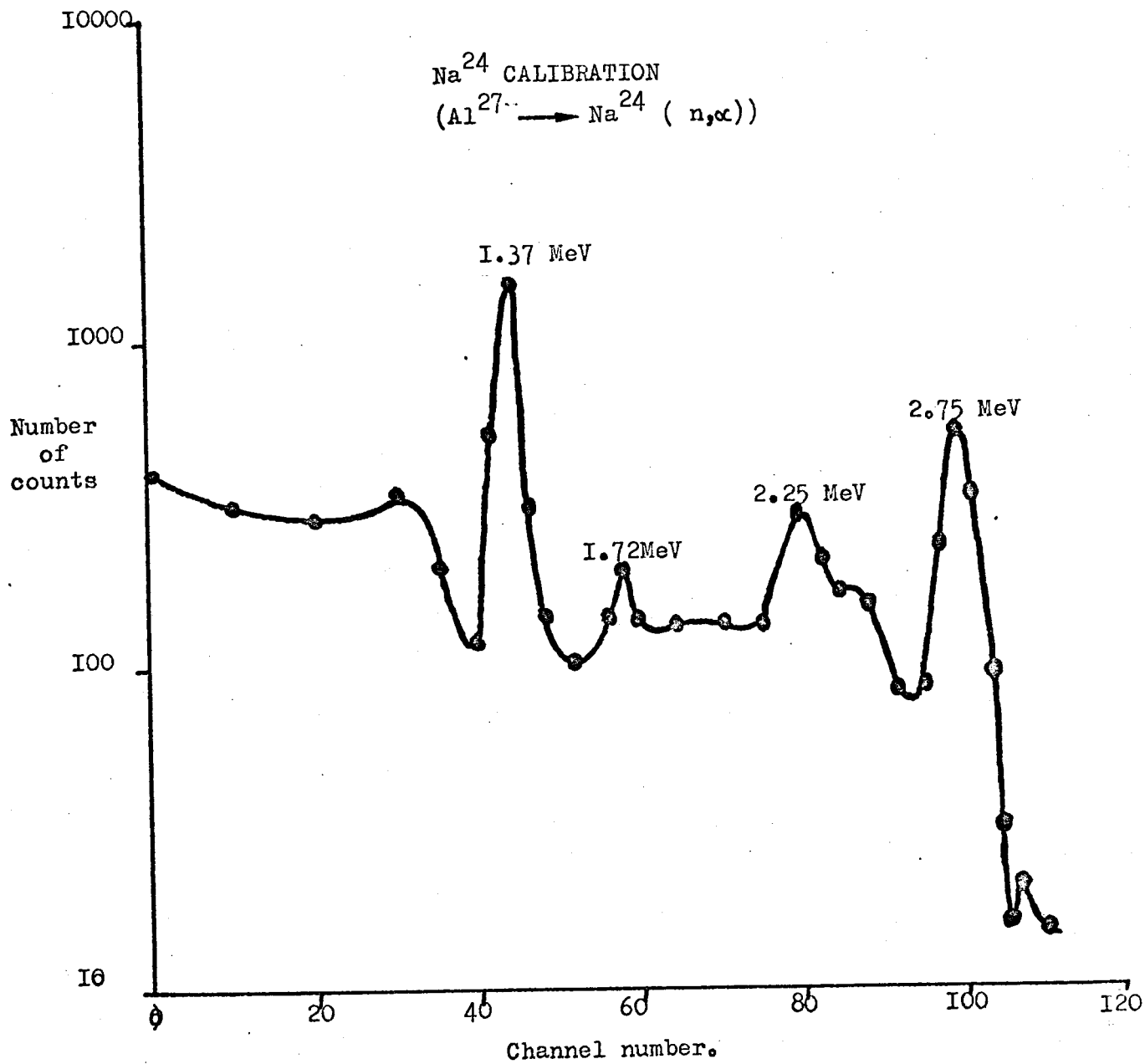
Beta decay data were also analyzed by graphical means. The more complex decay curves were analyzed by computer using Cummings, C.L.S.Q. (7) program. This program will resolve a complex decay curve into its individual components

when given certain approximate half life values obtained from the graphical analysis. The program prints out a FIT value which is a measure of how well a certain half life decay curve fits the original complex curve. The program can also be asked to vary the half life values given, by certain increments in order to obtain the best FIT value possible. (A FIT value of 1.000 is a perfect fit). Besides giving FIT values the program prints out the number of counts per minute (CPM) due to each component at the end of neutron bombardment (EOB) and its standard deviation (SIGMA).

RESULTS AND DISCUSSION

I. Gamma ray Spectra

A typical γ ray spectrum of Na^{24} is shown in graph I. The two peaks at 1.37 Mev and 2.75 Mev are typical for the decay of Na^{24} . The peaks at 1.72 Mev and 2.25 Mev are due to the γ ray interactions with the NaI(Tl) scintillation crystal. When a high energy γ ray hits the crystal, pair production will occur, i.e. the γ ray will form a positive and a negative electron pair. The two short range electrons stop and their kinetic energy is acquired by the crystal.



Graph I.

The positron annihilates, forming two γ rays each with an energy of 0.511 Mev and at 180° to each other. When both the annihilation γ rays are captured by the crystal along with the negative electron of the original pair produced, the peak at 2.75 Mev is detected. When one of the annihilation γ rays is lost, i.e. 0.511 Mev, the peak at 2.75 Mev - 0.511 Mev will be observed. When both the annihilation γ rays are lost the peak at 2.75 Mev - 2 (0.511) Mev is observed (8). A neutron activated Nitella cell gave the spectrum as shown in graph 2. This cell shows Na²⁴ peaks at 1.37 Mev as well as at 2.75 Mev. The latter is less pronounced due to an decrease in counting efficiency with an increase in energy. The total number of counts of the 1.37 Mev peak was 1054 ± 32.4 . This corresponds to a statistical accuracy within 3%. The half life value of the 1.37 Mev peak was difficult to determine due to counting figures which were within the standard deviation of the background. It is therefore difficult from these trials to assign a definite value to the percentage of the number of counts at the 1.37 Mev energy level due to Na²⁴ alone.

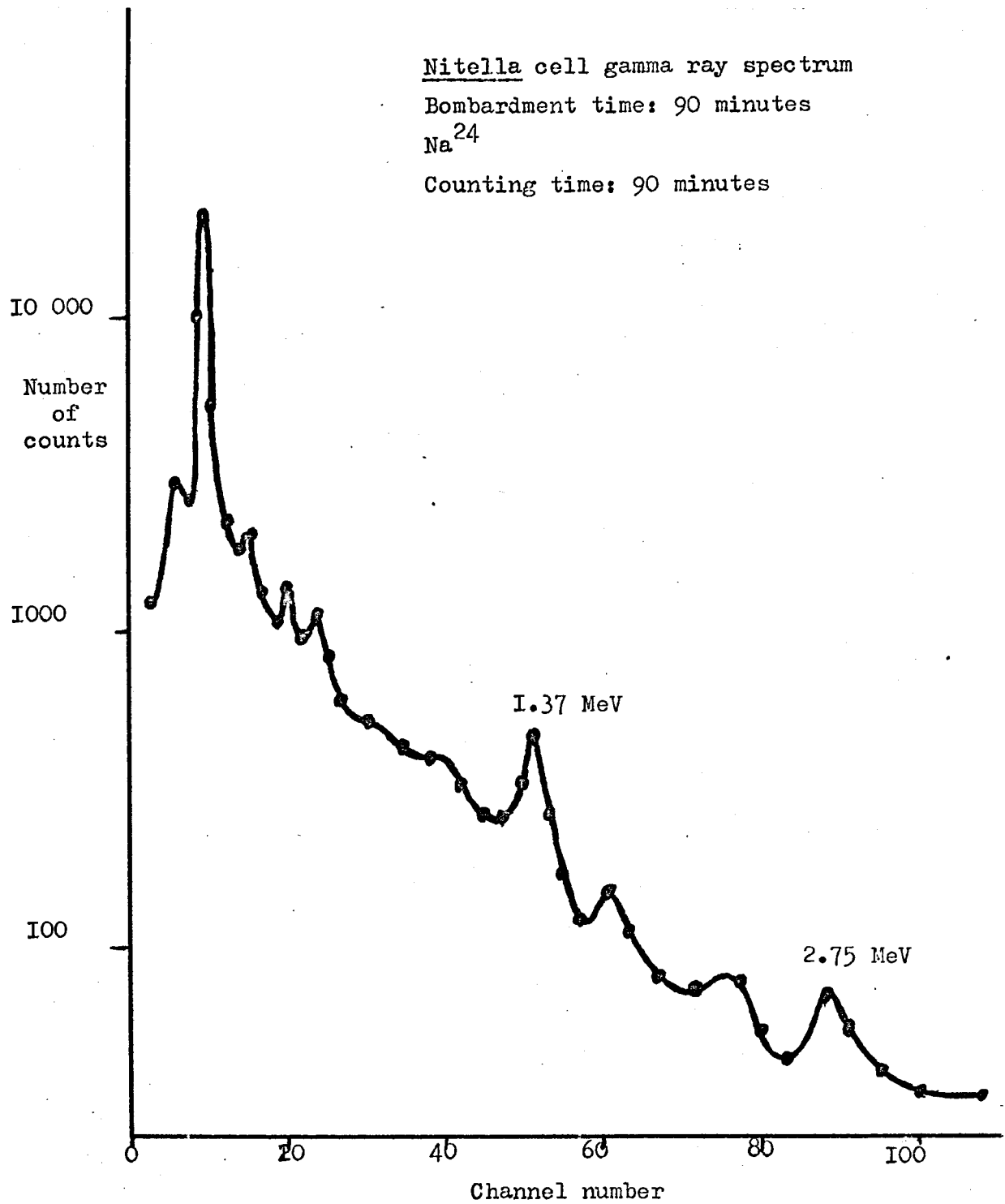
A cell activated for K³⁸ produced a spectrum as shown in graph 3. Upon comparing this spectrum with a typical

Nitella cell gamma ray spectrum

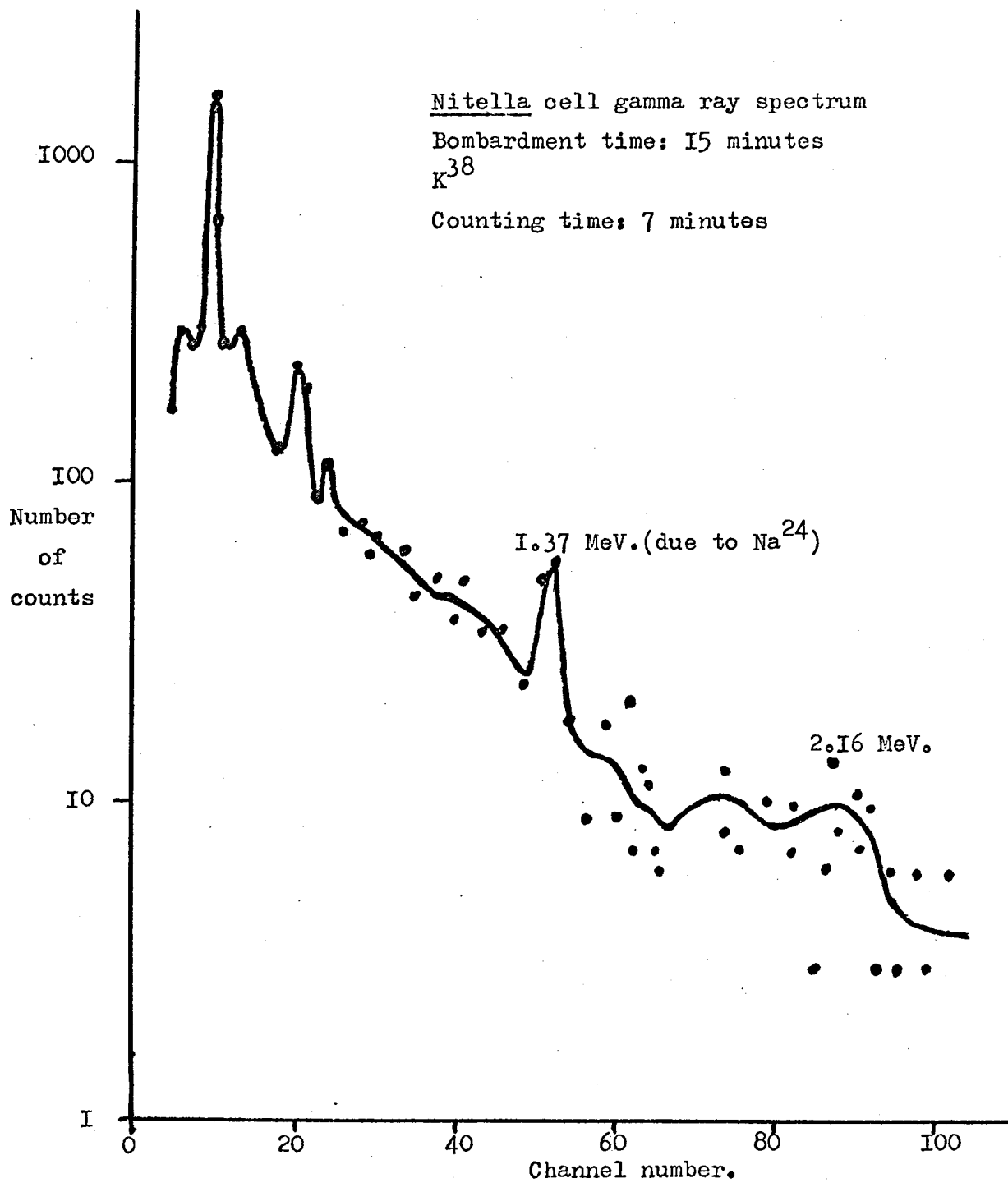
Bombardment time: 90 minutes

Na²⁴

Counting time: 90 minutes



Graph 2.



Graph 3.

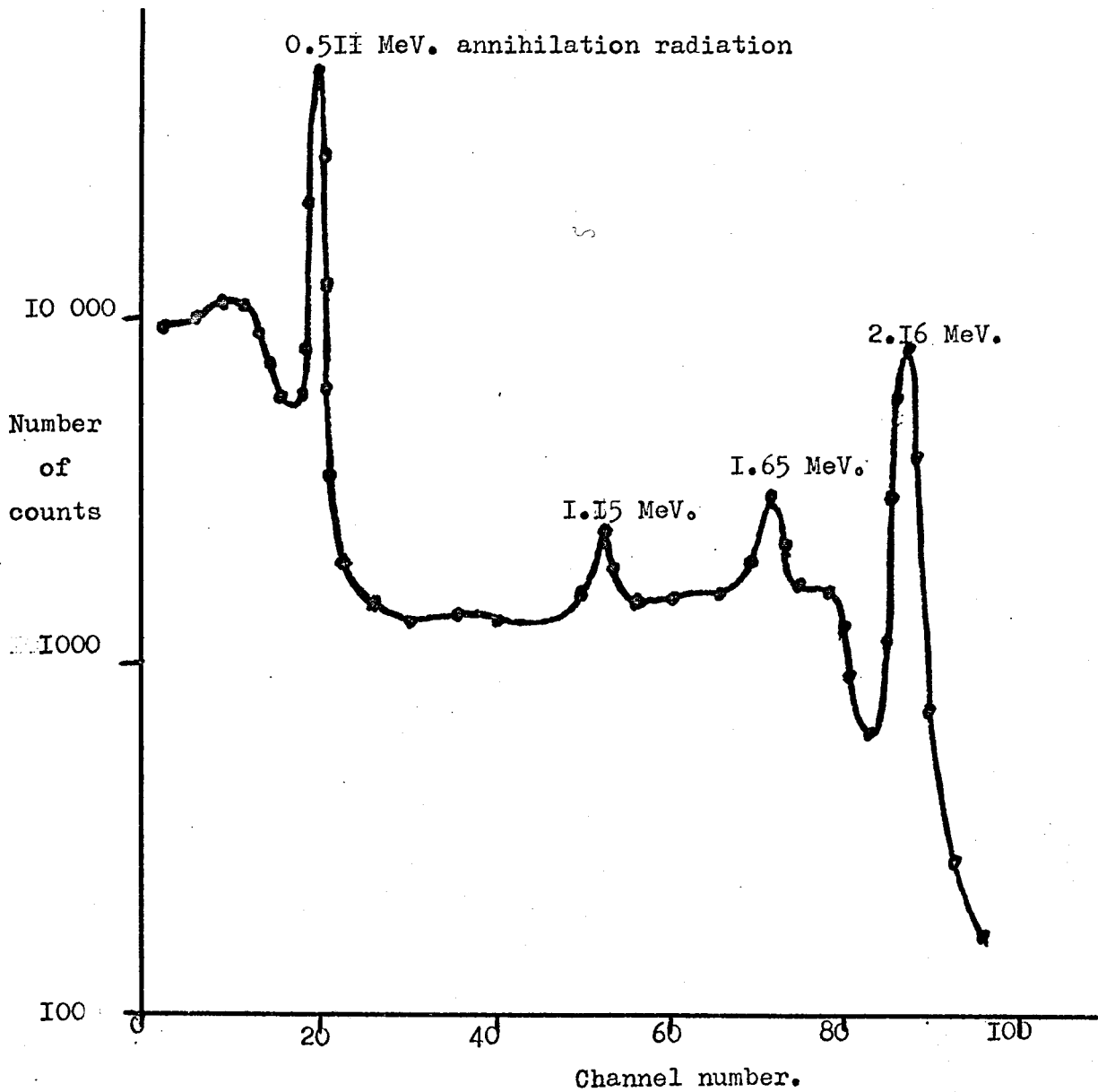
K^{38} spectrum graph 4 it can be seen that the annihilation peak at 0.511 Mev is well established but that the more diagnostic peaks at 2.16 Mev and 2.67 are barely, if at all, above the standard deviation of the background. A half life study of the 0.511 Mev peak, obtained from a 1000 γ KCl sample, showed the presence of two components; (graph 5) a. $t_{\frac{1}{2}} = 7.0$ min and b. $t_{\frac{1}{2}} = 19.9$ min. The lack of points at later times decreases the reliability of these half life figures. A similar study was done on a .1 gr KCl sample using the coincidence counter. A graphical analysis, graph 6, showed again two components, a. $t_{\frac{1}{2}} = 7.3$ min and b. $t_{\frac{1}{2}} = 30.0$ min. The latter activity seems to be due to Cl^{34} $t_{\frac{1}{2}} = 32.0$ min. A repetition of this experiment using a Nitella cell gave data points within the range of the standard deviation of the background making this method of counting unusable for K^{39} analysis of the Nitella cell.

II. Beta emission

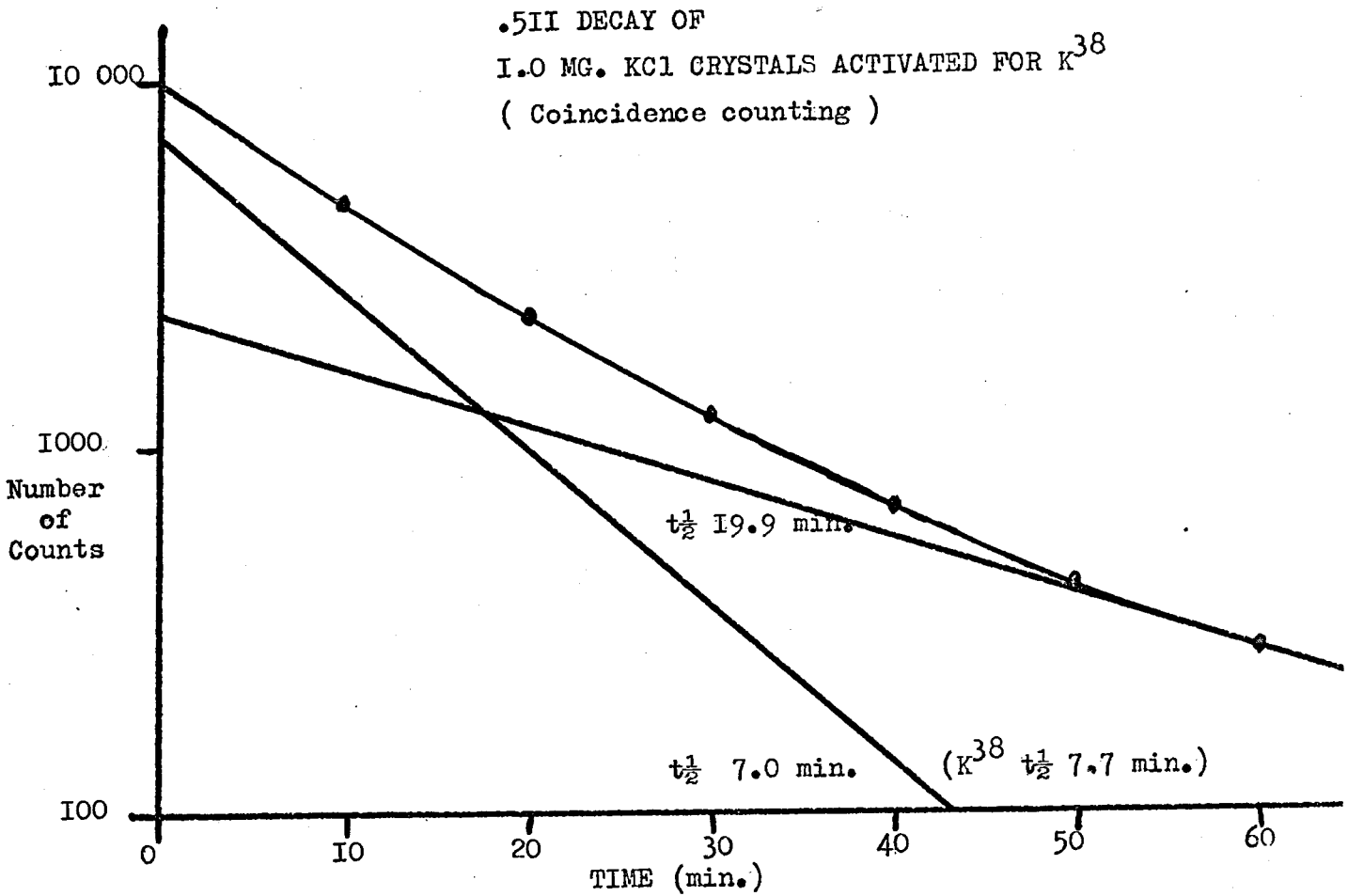
The total beta activity from a Nitella cell, activated for Na^{24} , was recorded at certain intervals to obtain a decay curve (graph 7). Two components seem to be present, one having a $t_{\frac{1}{2}}$ of 1.5 hr the other a $t_{\frac{1}{2}} = 15.5$ hr. The

Potassium gamma ray spectrum

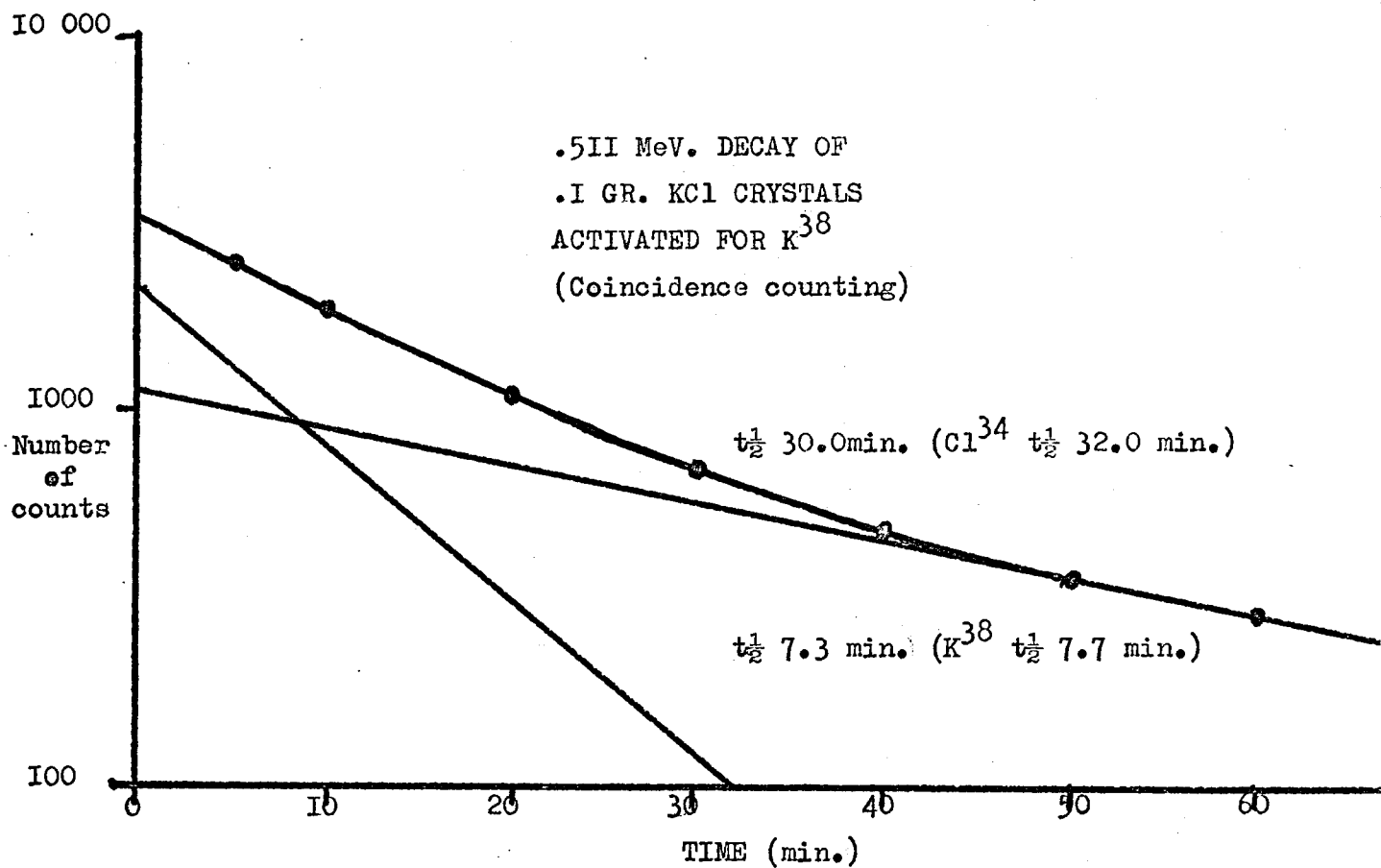
Isotope: K^{38} ($t_{1/2}$ 7.7min.)



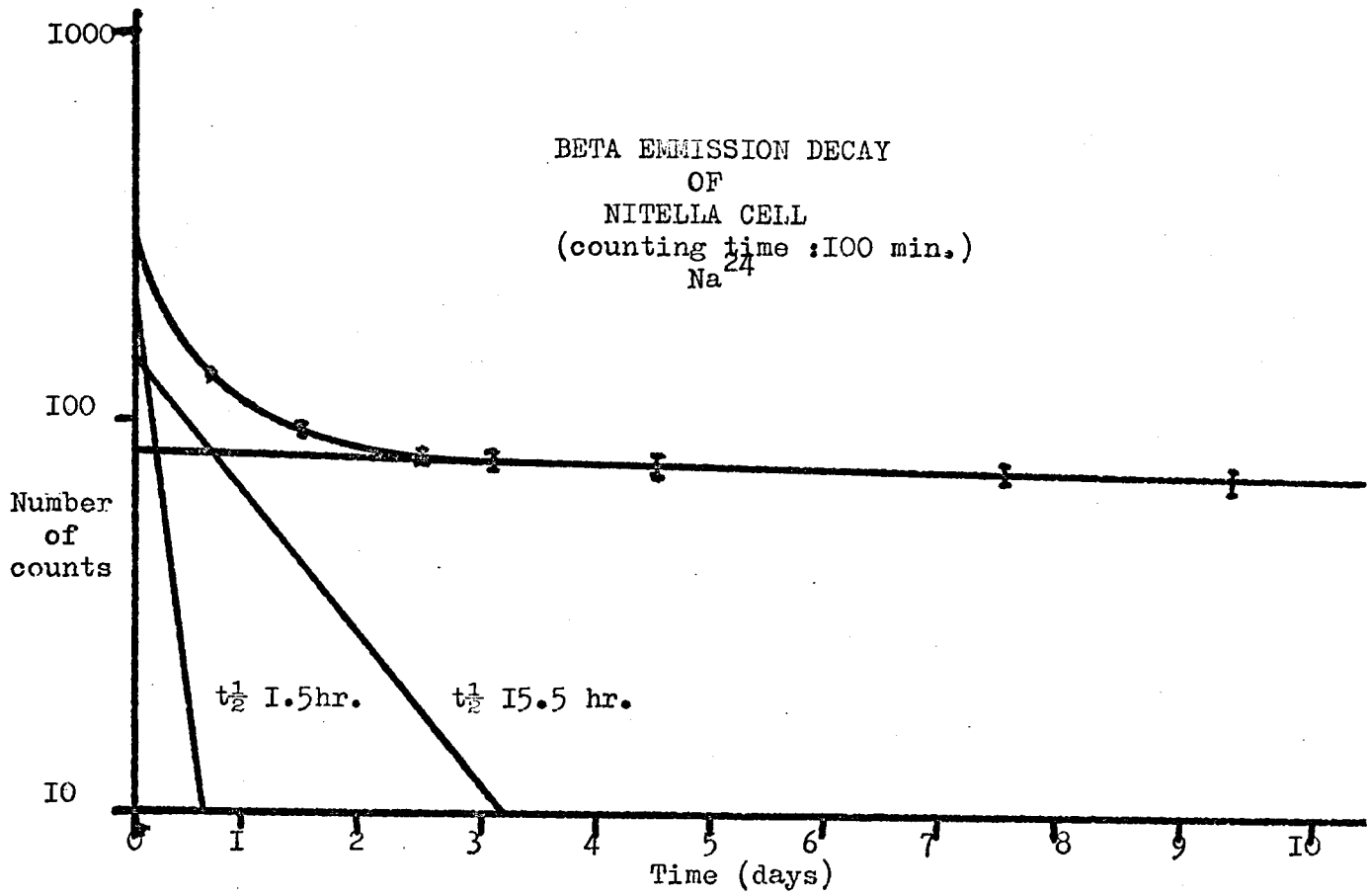
Graph 4.



Graph 5.



Graph 6.



Graph 7.

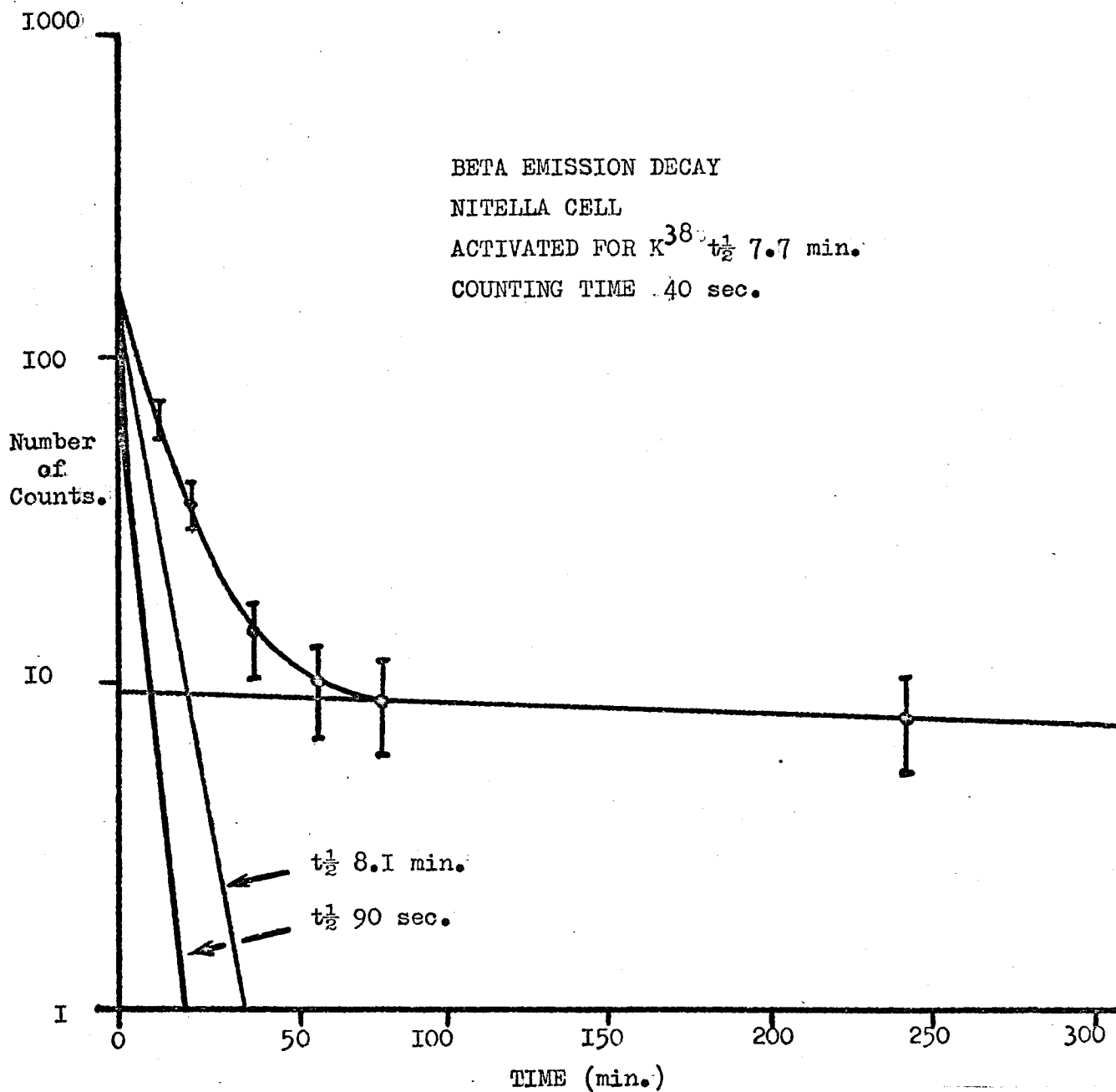
latter seems to be due to Na^{24} activity. A C.L.S.Q. (7) analysis gave a FIT value of 0.925 for these components. The total number of counts at EOB was 143 ± 7.9 i.e. a statistical accuracy within 5.7%.

A similar procedure was followed for a K^{38} activated cell (graph 8). Two components are present, one with a $t_{1/2} = 90$ sec, the other $t_{1/2} = 8.1$ min. A C.L.S.Q. analysis gave a FIT value of 1.134 for a 7.7 min. component (K^{38}) the CPM at EOB value was 386 ± 13 that is, an accuracy within 3.5%.

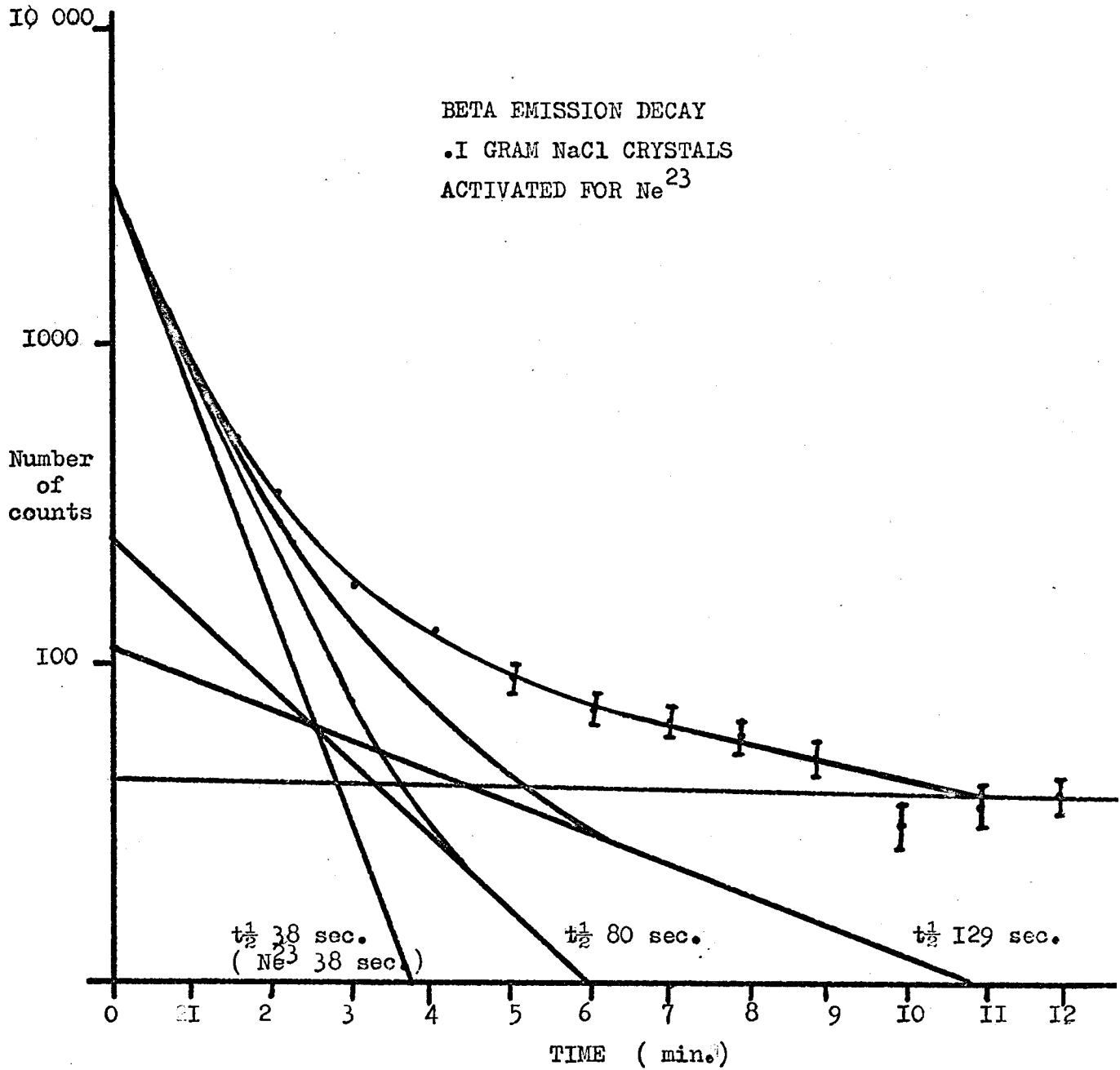
The NaCl sample .1 gr activated for Ne^{23} gave results as shown in graph 9. The three components present have a FIT value of 1.861. When this experiment was repeated for a Nitella cell the results, as shown in graph 10, were obtained. Only one major component seemed to be present. C.L.S.Q. analysis gave a FIT value of 1.140 for a 8.07 ± 0.68 sec isotope. A CPM at EOB of 650 ± 64 i.e. an accuracy within 10% for this isotope, which is probably F^{20} ($t_{1/2} = 11.5$ sec) produced from Na^{23} by a n, α reaction.

III. Effect of radiation on Nitella cells

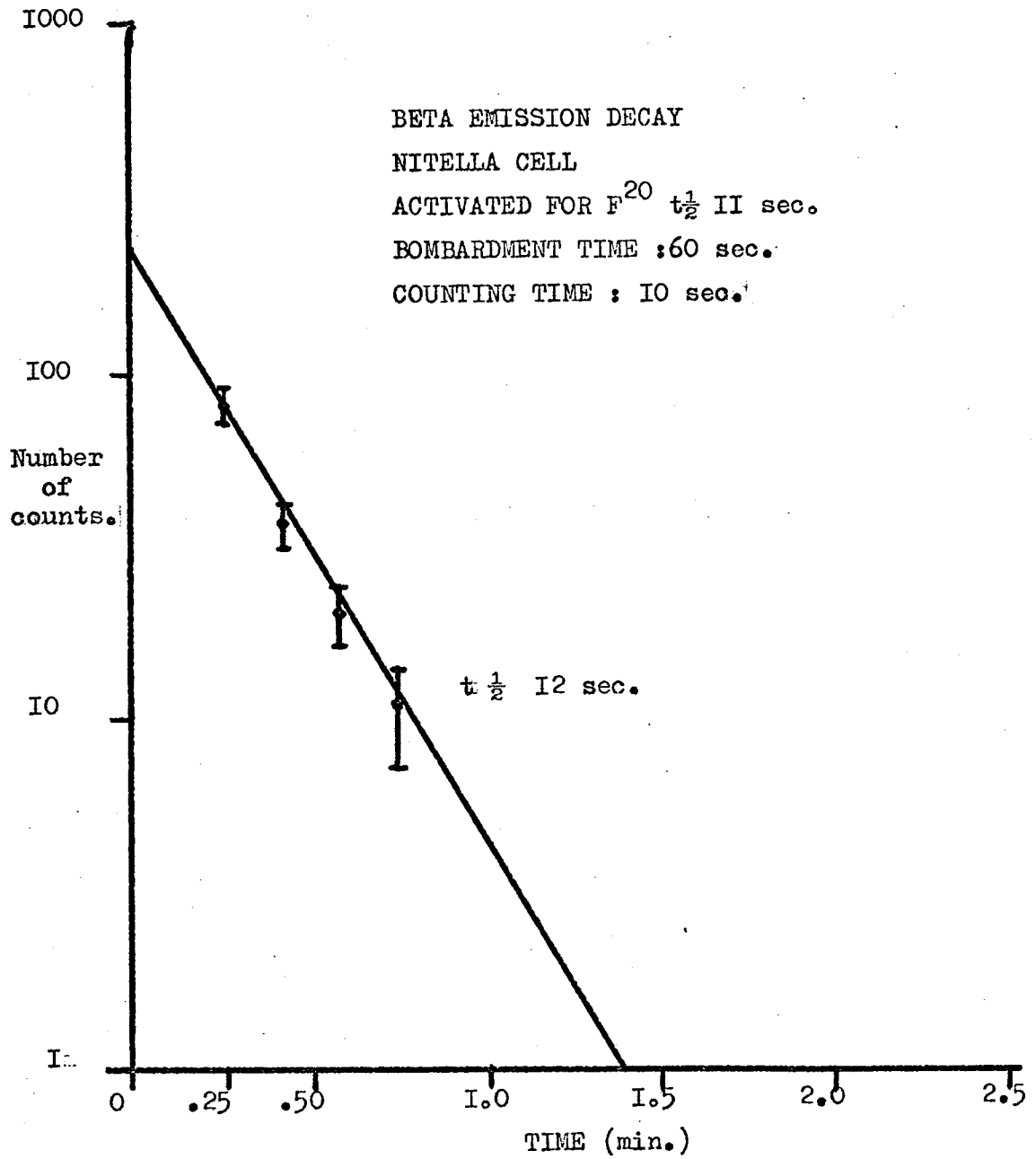
The irradiated Nitella cells were placed in artificial



Graph 8.



Graph 9.



Graph 10.

pond water, (9) under constant light conditions after being checked for radioactivity as described previously. The cells thus treated did not show any signs of degeneration as their values for cytoplasmic streaming, membrane potential and action potential were directly comparable to values obtained from untreated cells. The treated cells seem just as viable as the untreated cells and even in the cells which were irradiated for the longest period of time (1.5 hrs.) growth was observed.

CONCLUSION

-Determination of Na and K can be made respectively within the statistical error of 5.7% and 3.5% using the β emission resulting from exposure to the neutron flux produced by the Cockcroft-Walton neutron generator.

-The low level of γ radiation produced, under these conditions is less diagnostic for a specific ion than the resulting beta activity.

-Neutron activation does not appear to have any effect on cytoplasmic streaming, resting potential, action potential or growth of the *Nitella* cells used.

PURPOSE (PART B)

To investigate ionic and electrical changes during action potentials in Nitella cells in presence of certain drugs and under certain physical conditions.

INTRODUCTION

Nitella cells, like all other cells, have a potential difference between the inside and the outside of the cell, the inside being more negative. This potential difference is called the resting potential. During the passage of an impulse, generated by stimulation, a very characteristic change in the resting potential takes place. This change is called the action potential or spike. A typical action potential in Nitella cells in artificial pond water (A.P.W.) (9) is shown in Fig. 1.

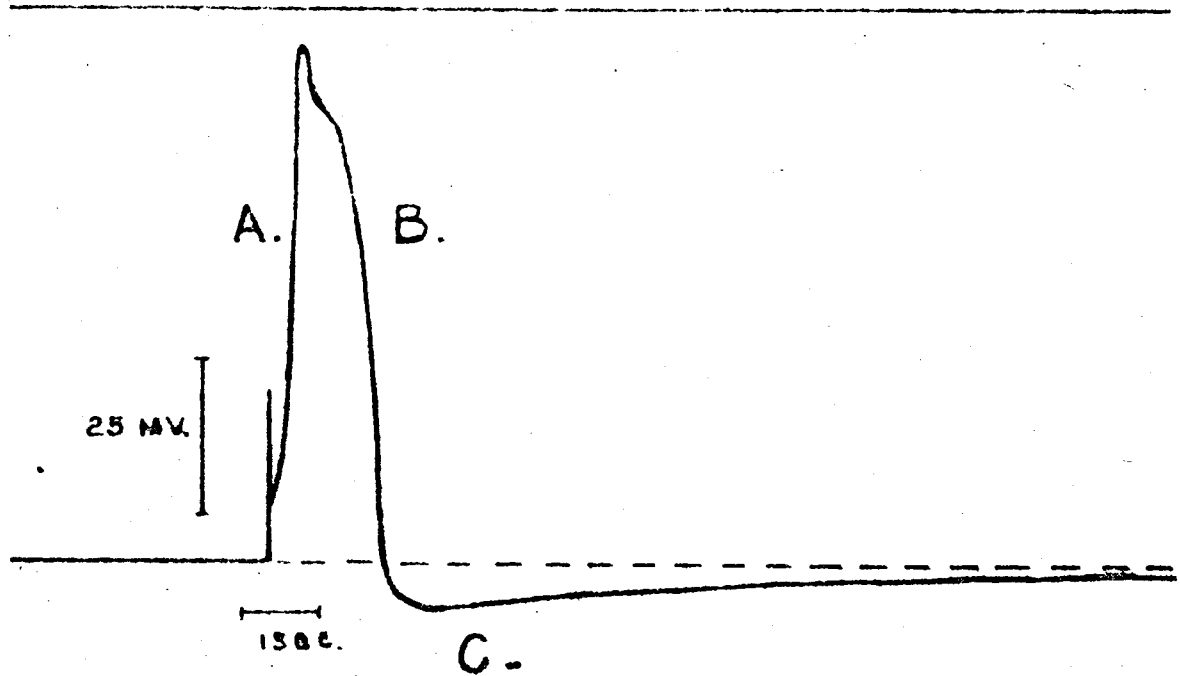


Fig. 1

Recently there has been good evidence (10) that Cl^- ions move out of the cell during depolarization (phase A in Fig. 1), while K^+ ions are thought to move out during repolarization (Phase B Fig. 1). Phase C is an after hyperpolarization, the undershoot, and is in other tissues often associated with a K^+ equilibrium potential more negative than the resting potential.

Hope (11) initially postulated that there was a movement of Ca^{++} ions into the Nitella cell during depolarization rather than an efflux of Cl^- ions. He suggested when the cell was placed in distilled water that an influx of Ca^{++} ions, thought to be bound to the outside of the plasmalemma was responsible for the action potential. Spanswick (12)

measured a Ca^{++} influx in Nitella cell at rest but did not find any appreciable Ca^{++} efflux, as might be expected if Ca^{++} is bound to the membrane.

To clarify the role of various ions in the production of action potentials, certain drugs known to have specific effects on ionic permeability in other cells were tested on Nitella cells.

The drugs and conditions tested are known to have the following actions or effects in the cells specified.

I. Tetra ethyl ammonium (TEA)

Tasaki and Hagiwara (13) have shown that TEA will delay the falling phase of the action potential in squid axons, presumably by slowing down the mechanism which renders the membrane permeable to K^+ ions.

In nerve cell membrane of Onchidium verruculatum Hagiwara and Saito (14) observed that TEA did not change the resting potential, but caused a similar prolongation of the K^+ phase, along with a small undershoot, as in the squid axon. These results were similar to those obtained from cardiac muscle cells (15). At low concentration of TEA in cardiac muscle cells, the plateau was sometimes

preceded by an initial fall from the peak, quite similar to results obtained from Mammalian Purkinje fibres (16).

II. Picrotoxin and gamma amino butyric acid (GABA)

In decapod nerve cells (17) GABA inactivated inhibitory synapses and increased the membrane resistance 50 fold. Picrotoxin was found to reverse the effect of GABA. In cat nerve cells, however, picrotoxin activated excitatory synapses (17). In Crayfish muscle fibres GABA increased and picrotoxin decreased Cl^- conductance in the synaptic membrane (18). They also affected the Cl^- activation mechanisms of inhibitory synapses. The effect on non-synaptic membranes developed slowly and only with high concentrations of the drugs. In general GABA seems to increase Cl^- conductance and picrotoxin appears to reduce it.

III. Urethane

Hagiwara and Saito (14) found that the amplitude of the spike in Onchidium verruculatum decreased when treated with urethane. Eventually the cell was found to become incapable of producing an all-or-none type of spike. The effective conductance of the membrane for the depolar-

ization phase was much smaller than the conductance values obtained from the untreated cell.

The action of urethane seems to be attributed to its lipid solubility (19).

IV. Ethylene diamine tetra acetic acid (EDTA)

Ca^{++} deficiency has been known to produce spontaneous firing in vertebrate cells such as frog muscle and nerve cells (20).

Kishimoto (21) suggested that EDTA chelated the Ca^{++} ions, thought to be bound to the outside of the Nitella cell membrane, producing spontaneous firing due to the lack of available Ca^{++} . The spontaneous firing in the Ca^{++} deficient frog muscle and nerve cells was stopped by addition of ATP and AMP (22). However, in Nitella cells spontaneous activity was induced by an addition of ATP (21).

V. Procaine

Procaine diminished Na^+ and K^+ conductances in the squid axon (23). In arthropod muscle fibres the application of procaine converted the normal graded response into an all or none response. Kuperman (24) suggested that in frog

muscle and nerve fibre, procaine replaced Ca^{++} ions at membrane anionic sites and inhibited the influence of excitable stimuli on processes leading to sodium influx. Procaine seemed specific for the Ca^{++} releasing mechanism in the membranes of these cells. The membrane conductance in these procaine treated frog cells showed an inflection just before the end of the plateau of the action potential.

VI. 2, 4-dinitrophenol (DNP)

DNP is known to uncouple oxidative phosphorylation, the process leading to the synthesis of ATP. DNP inhibits the active transport system at that point where ATP is used (25). A 0.2 M DNP solution completely inhibited the efflux of Na^{++} in Sepia axons. The effect of DNP on the resting potential was not significant for the first two hours. However, after this period the cell adopted the same ionic distribution as the outside medium. In Sepia axons, when placed in Ringers solution, this will lead to an increase in Na^+ ion concentration and a decrease in K^+ ion concentration (26).

VII. Neutron Activation

Damage to biological systems due to neutron activation is caused by chemical changes in the cell. These changes result mainly from ionization, excitation and atom displacement (27). Equal amounts of radiation energy caused different amounts of biological damage depending on the source of the radiation. Secondary ionization due to recoil protons from fast neutrons caused ten times more damage biologically than the same amount of gamma radiation. Thermal neutrons are only twice as effective in producing damage.

MATERIALS AND METHODS

Nitella cells were placed in a lucite chamber. The electrical connections were made as in Fig. 2.

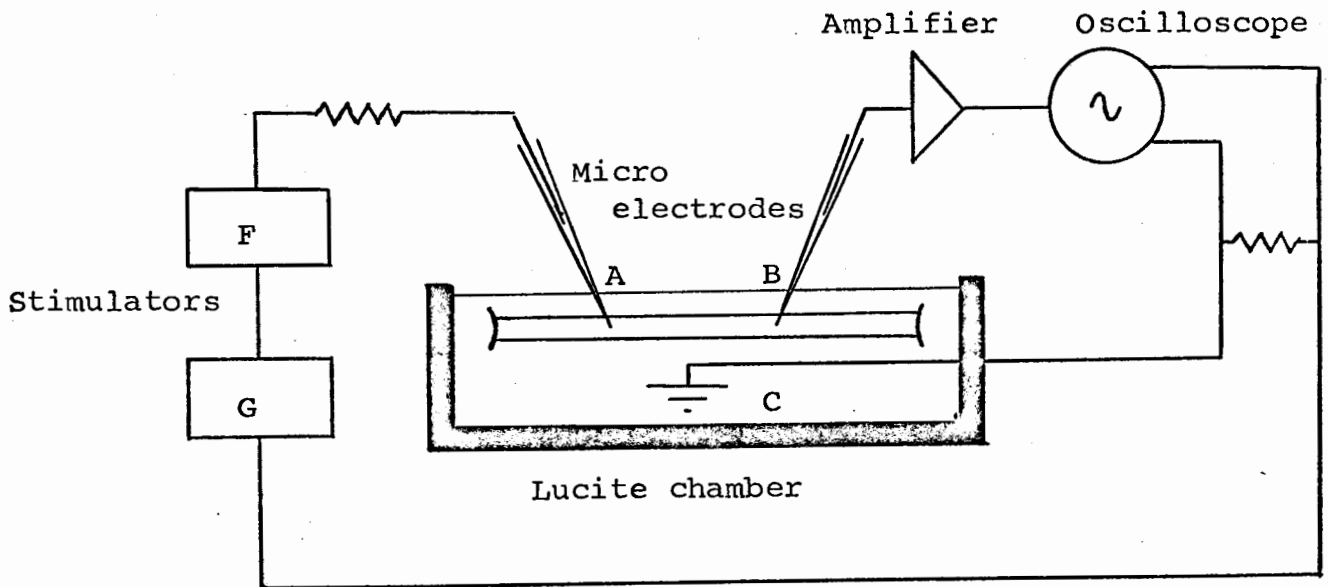


Fig. 2

Glass electrode B was used to record the membrane potential continuously. Upon proper stimulation, such as a voltage pulse from electrode A to the ground electrode, an action potential will be recorded by electrode B. A typical oscilloscope trace of such an action potential is shown by the heavy lines in Fig. 3.

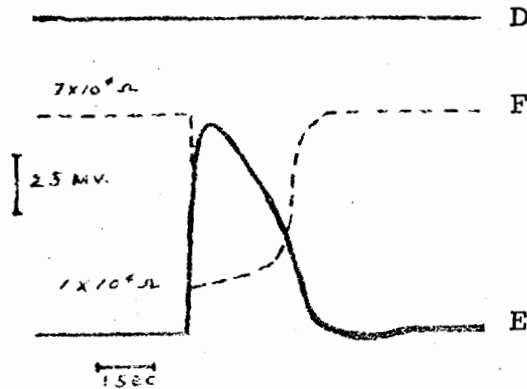


Fig. 3

In this figure, line D is the potential difference between the reference and glass electrodes before the glass electrodes were inserted into the cell and is the zero reference. Line

D is normally stable. Line E is a record of the potential difference with time between cytoplasm and the ground C (fig. 2) after the glass electrodes were inserted. This potential will change during an action potential. When the cell is not stimulated, the distance between lines D and E will represent the resting potential.

To measure membrane resistance, line F fig. 3, electrode A was used to give hyperpolarizing pulses of subthreshold level to C (fig. 2). A typical trace is shown in Fig. 9. Line D and E still perform the same function as in Fig. 3 but the deflections from line D will now represent a record of the current flowing through electrode A and the cell membrane at these times. The current is normally constant.

The deflections from line E are the voltage measurements of the hyperpolarizing pulses. At these deflection points, the current and voltage flowing through the membrane are known and the resistance can be calculated. The resistance changes with time during an action potential can be superimposed onto the voltage trace as shown in Fig. 3, line F. Stimulator F fig. 2 was used to stimulate the cell between electrode A and the ground electrode C.

The glass electrodes used were micro pipettes with the tips drawn out to 5-10 microns, filled with 0.15 M KCl. The ground electrode was silver-silverchloride. Records of the results were made on a two-channel pen recorder and on photographic film from the display on a storage, two-channel, oscilloscope, Tektronix type RM 564.

RESULTS

I. TEA

Nitella cells when subjected to a low concentration of TEA showed a delay of the falling phase, Fig. 4. Higher concentrations of TEA increased this delay of the beginning of K^+ activation Fig. 5.

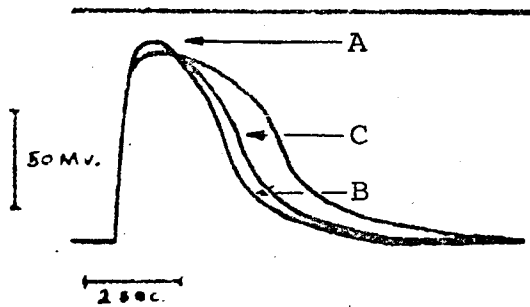


Fig. 4

Nitella cell action potentials. normal, trace A; after addition of TEA, trace B and C.

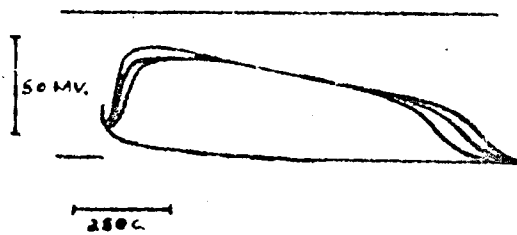


Fig. 5

Nitella cell action potentials, after 5 min. in 0.2M TEA.

The delay of K^+ activation could be reversed by washing the cell with artificial pond water (APW) Fig. 6.

Resistance measurements on a typical TEA response showed a slight increase in the resting membrane resistance as shown in Fig. 7. This figure also shows a decrease in the amplitude of the spike when the action potential is prolonged.

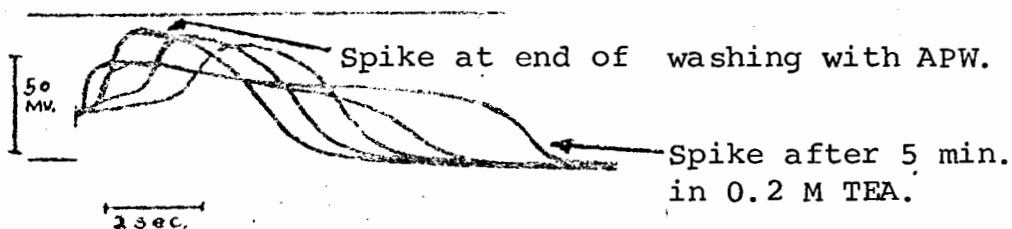


Fig. 6

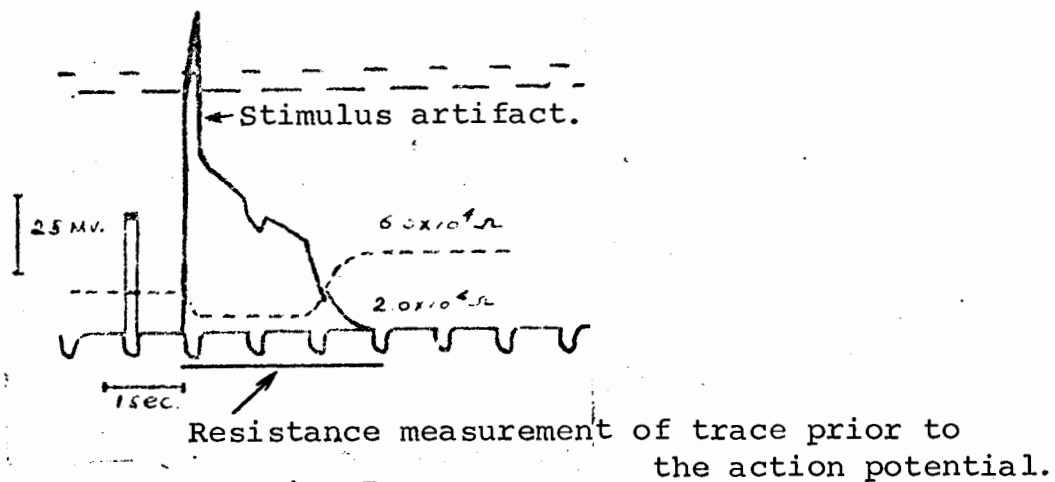


Fig. 7

Thus TEA delayed the falling phase of the spike in the action potential of *Nitella* cells, a result similar to the effect of TEA on other excitable cells.

The TEA effect on action potentials were consistent in the four different cells used.

II. Picrotoxin and GABA

Four cells treated with different concentrations of GABA and picrotoxin (.01 M and .1 M) did not show much variation in their action potentials as compared with normal cells, Figs. 8, 9, 10.

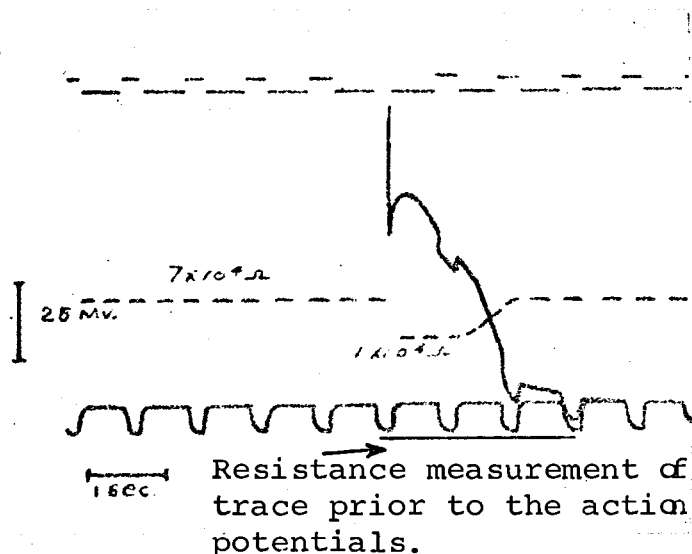


Fig. 8

Untreated cell.

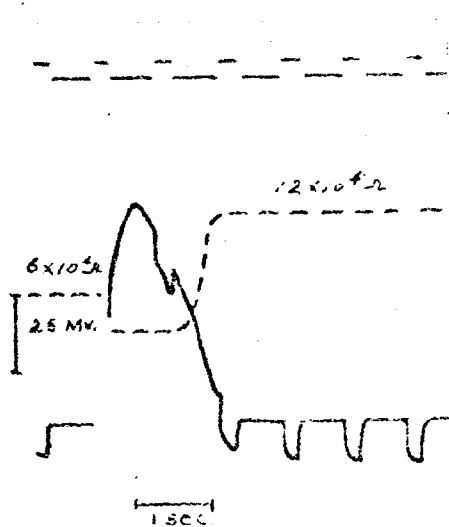


Fig. 9

Same as in Fig. 8 in saturated GABA solution.

A slight difference was noted in the length of time for depolarization of cells in saturated picrotoxin solution as shown in Fig. 11.

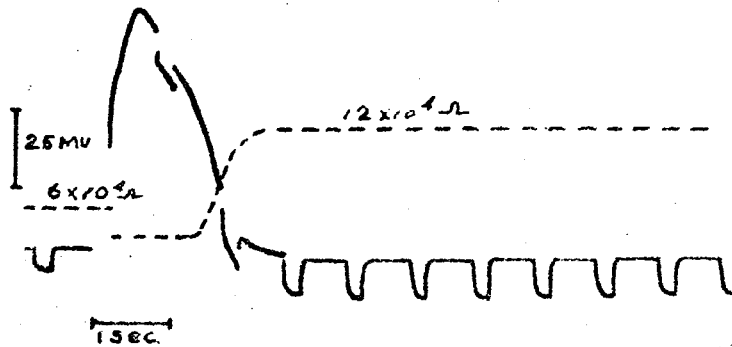


Fig. 10

Cell in saturated picrotoxin for one minute.

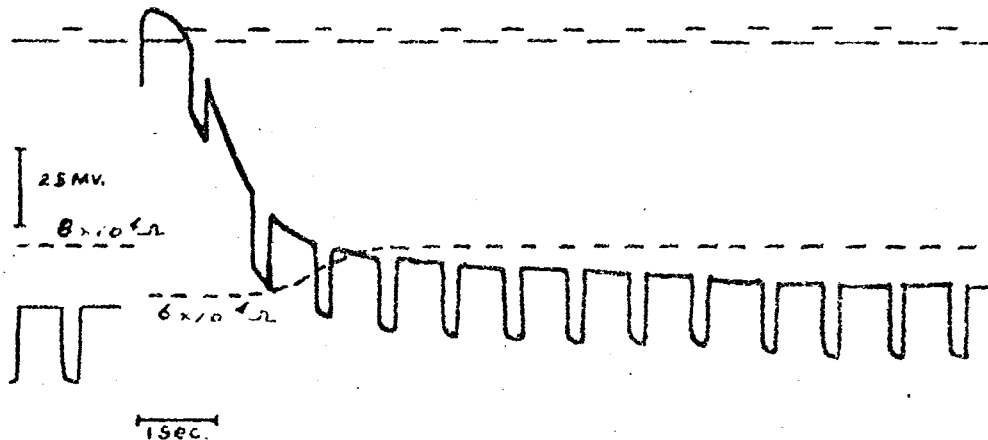


Fig. 11

Same cell as in Fig. 10 in saturated picrotoxin for two hours.

The rising phase in Fig. 11 was more rapid while the falling phase was prolonged. The effect of saturated solution of picrotoxin after 2 hours soaking was similar to the results obtained from nonsynaptic membranes of the Crayfish (17).

Resistance measurements showed with both GABA and picrotoxin, a two-fold increase in membrane resistance after an action potential had passed, Fig. 9, 10.

When these cells were stimulated again the resistance obtained was still approximately twice the previous value. The antagonistic effect of picrotoxin in relation to GABA, as observed by Grundfest (17), was not found in these resistance measurements, indicating that the mechanism at which picrotoxin and GABA act in many animal cells does not seem to be present in *Nitella*.

Since the shape of the action potential was not significantly changed by picrotoxin and GABA, this indicates that the mode of Cl^- flux is entirely different in *Nitella* cell. There is evidence for a different mechanism in *Nitella* since the resting Cl^- flux has been linked directly with the process of photosynthesis (28).

III. Urethane

Cells treated with urethane in both low and high concentrations give relatively normal action potentials. Even after a 12 hour period of soaking in a concentrated urethane solution no major changes were observed, Fig. 12.

Of the four cells tested only one single cell, which had a low resting potential, showed sensitivity to urethane.

The response in Fig. 13 was due to the action of urethane on the cell with a low resting potential. The duration of this action potential was more than twice that obtained before the urethane treatment.

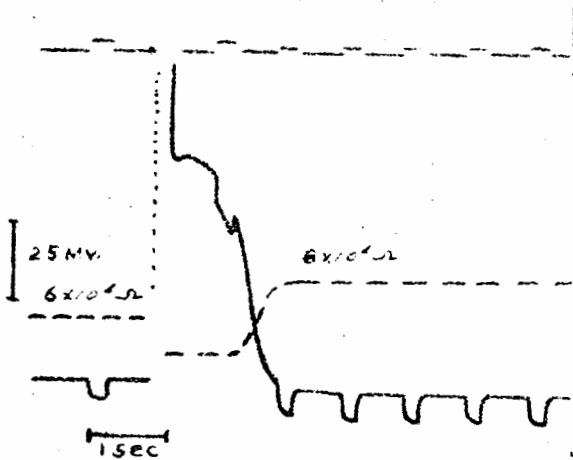


Fig. 12

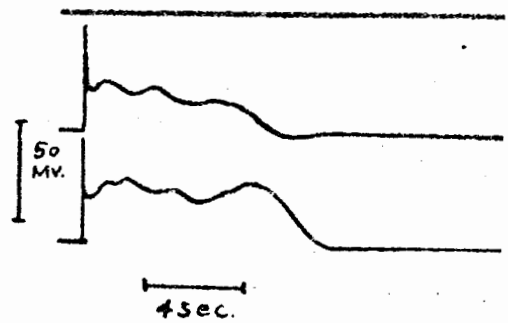


Fig. 13

Resistance measurements during the action potentials in Fig. 12 showed a similar effect as in animal cells, a decrease in resistance during the depolarization after which the resistance returned to a higher value than before stimulation. After several stimulations the resistance doubled. After the 12 hour treatment with urethane, the same cell had a membrane resistance close to that of the normal cell before the urethane was added. The cell, therefore, was eventually capable of counteracting the effect of urethane on the membrane resistance. A small change in the shape of the action potential accompanied the change in resistance.

IV. EDTA

Three Nitella cells treated with a few drops of an aqueous solution of EDTA gave an action potential shown in Fig. 14. The amount of EDTA used in this experiment was about 10 picomoles, a larger concentration stopped all effective response to stimulation. Instead, spontaneous firing, as previously recorded by Kishimoto was observed, Fig. 15.

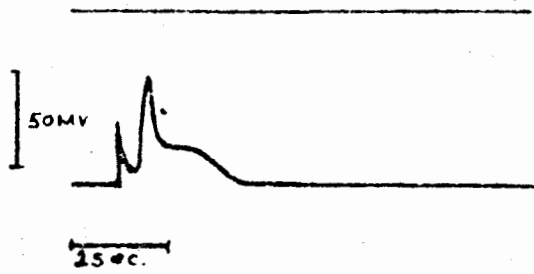


Fig. 14

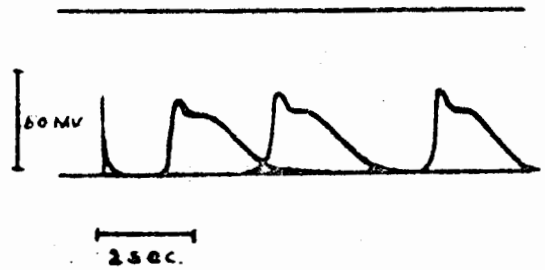


Fig. 15

If the cells were not washed, $Pb Cl_2$ restored a normal response, Fig. 16.

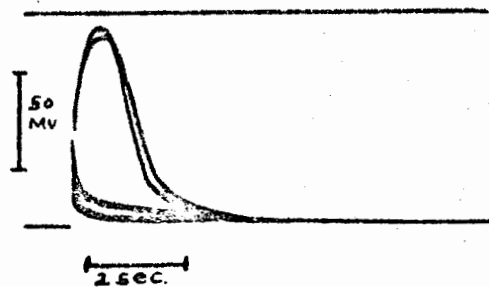


Fig. 16

Lead ions will displace calcium ions from Ca EDTA complex irreversibly (29). Since $Pb Cl_2$ has no effect on the action potential in normal *Nitella* cells but caused the return of the normal action potentials in cells treated with EDTA, it appears that the action of EDTA is caused primarily by its chelating properties with Ca^{++} and not by some secondary effect.

It was observed that the EDTA treated cells needed several stimuli before the action of the chelator took place. This suggests that Ca^{++} ions may indeed be bound to the membrane, as proposed by Kishimoto (30), and so be unavailable to EDTA until stimulation releases them from their binding sites.

V. Procaine

Procaine delayed the depolarizing phase and resolved the rising phase into three components, Fig. 17. Higher concentrations of procaine prolonged the falling phase, i.e. K^+ efflux Fig. 18.

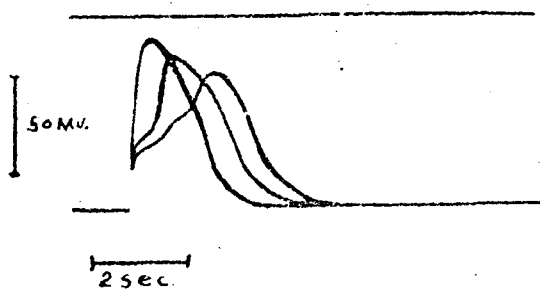


Fig. 17

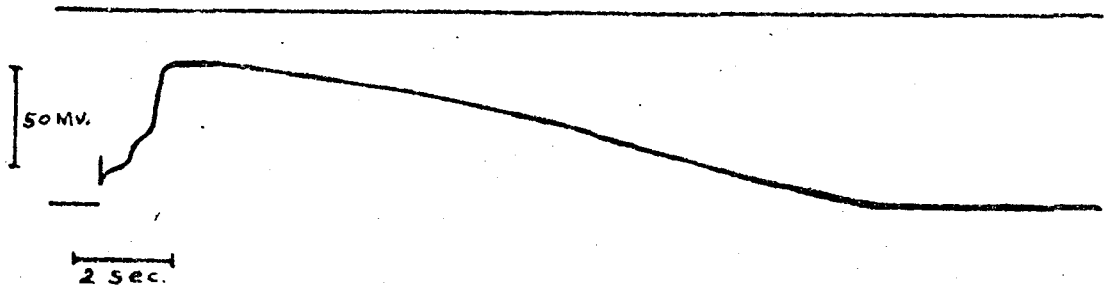


Fig. 18

Resistance measurements showed a response, Fig. 19 similar to those obtained by Kuperman on Crayfish. These results were consistent in the three cells treated with a .01 M procaine solution.

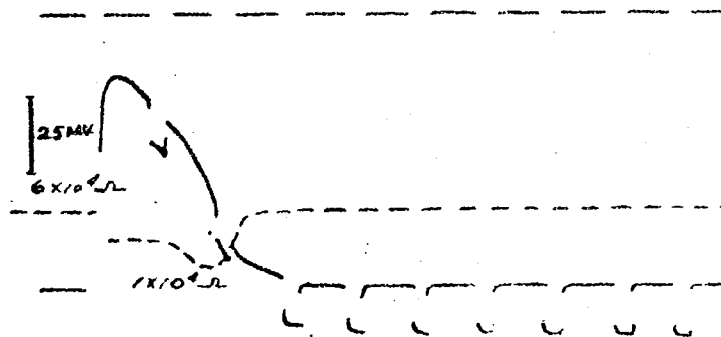


Fig. 19

VI. DNP

A Nitella cell treated with a .01M DNP solution showed a tremendous undershoot upon stimulation Fig. 20. This indicates that change from permeability of K^+ to non-permeability in the hyperpolarization phase, is an active process requiring ATP.

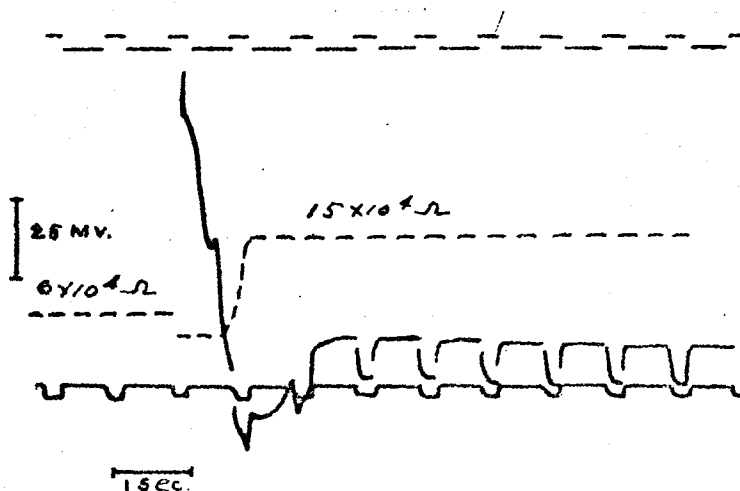


Fig. 20

Resistance measurements showed a two- to -three-fold increase in membrane resistance, Figs. 20, 21, after an action potential had taken place. This indicates that the permeability of the cell may be influenced by oxidative phosphorylation. Different concentrations of DNP seem to have different effects as shown in Fig. 21 where a saturated solution of DNP in A. P. W. was added.

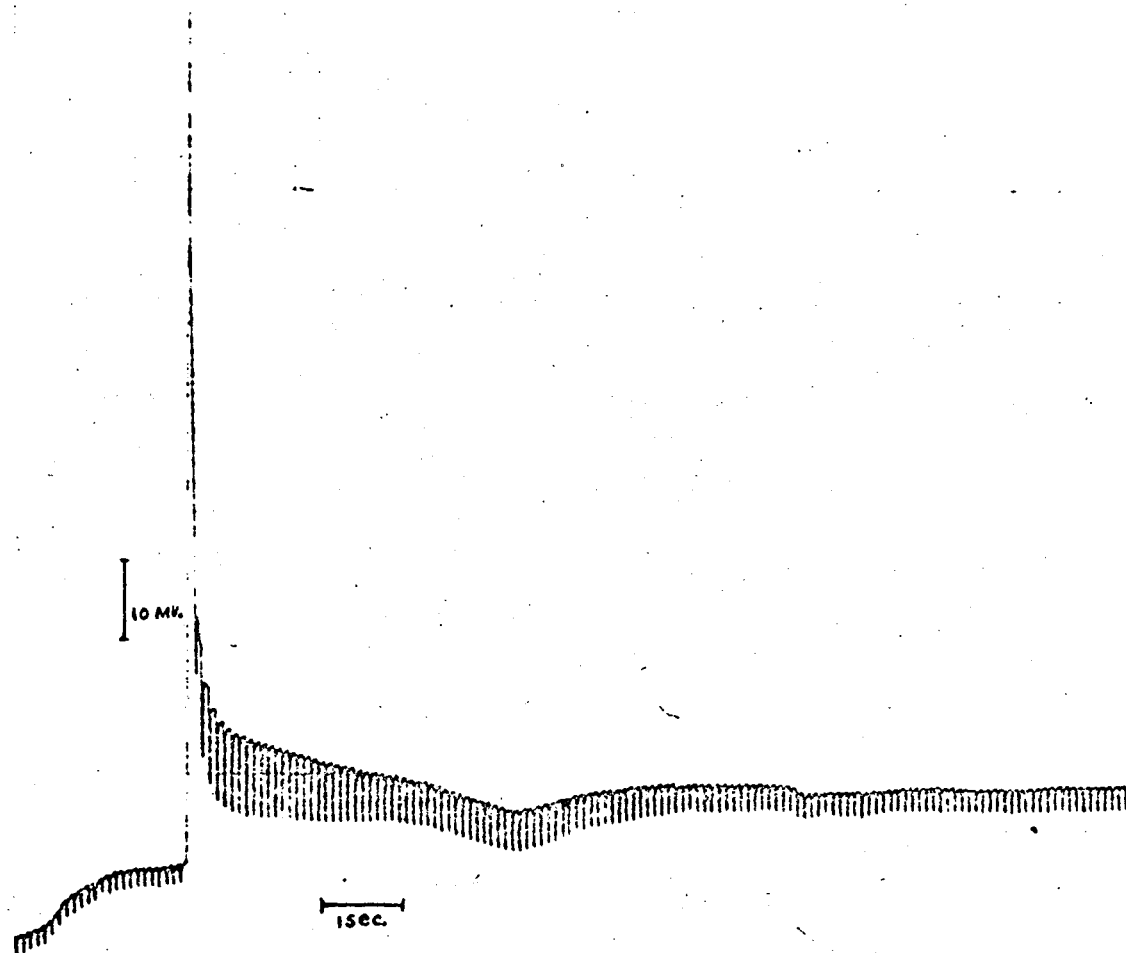


Fig. 21

VII. Neutron Activation

Nitella cells subjected to a fast neutron flux of 10^8 neutrons/cm²/sec for one and a half hours, showed normal cytoplasmic streaming. As mentioned before, no apparent damage from this treatment could be observed. The cells

were kept alive for weeks after neutron activation and new cells were found to be growing from the irradiated cells.

Cells checked for electrical properties after neutron activation showed normal action potentials. One cell investigated directly after neutron activation showed normal streaming and spontaneous firing, Fig. 22, just as those found previously with EDTA treated cells. The shape of the action potential was however quite different showing 3 distinct components.

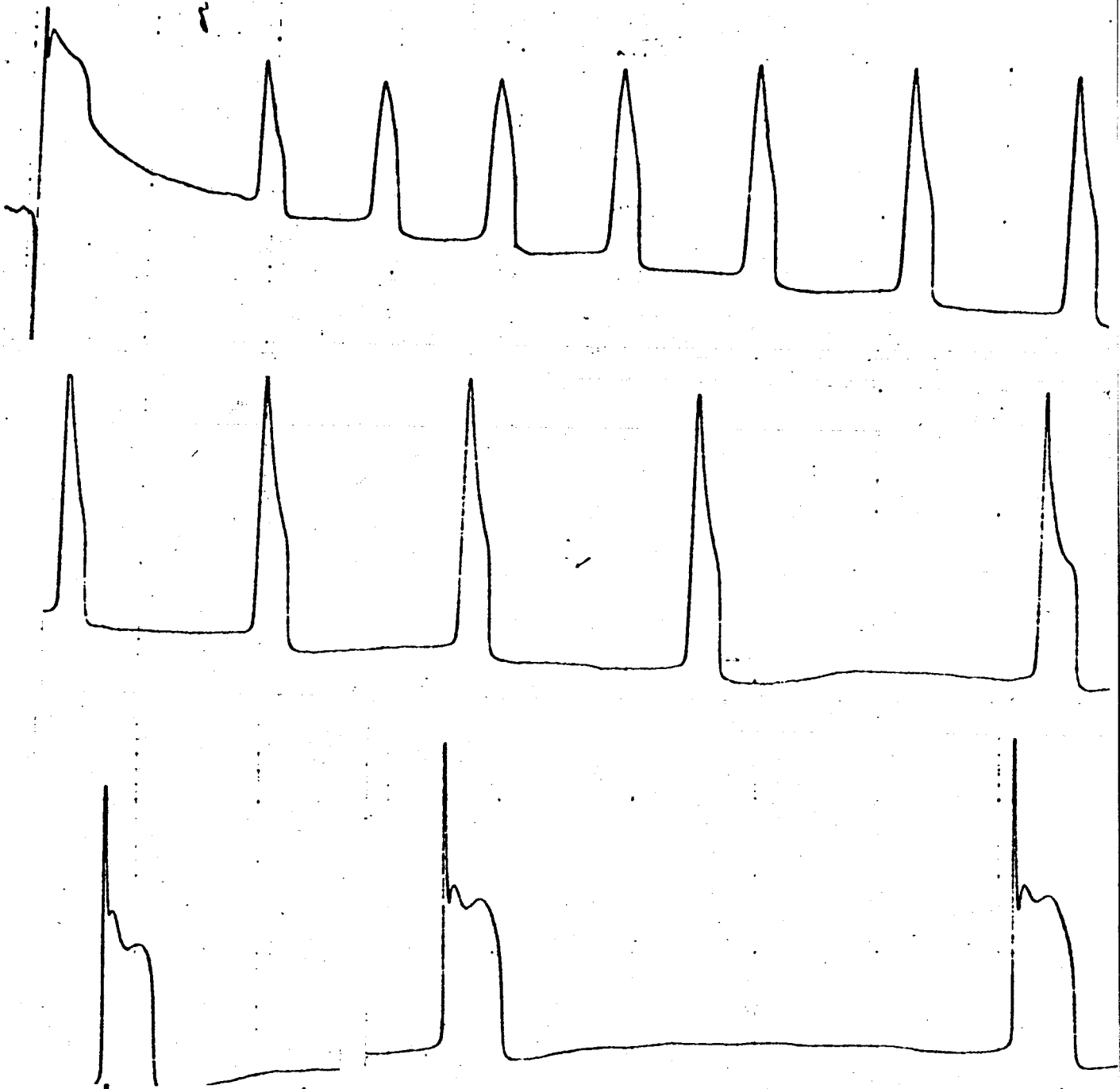


Fig. 22

VIII. Distilled water

The effect of distilled water on the spike of a normal cell can be seen in Fig. 23. The spike recorded in APW did not show any undershoot like those generally observed in the spikes recorded in distilled water. The action potential was also considerably delayed in distilled water as seen in Fig. 24. This effect was readily reversible.

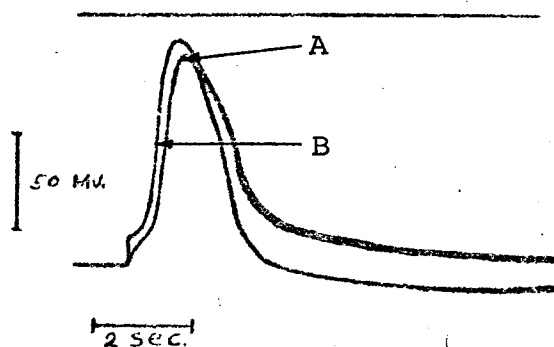


Fig. 23

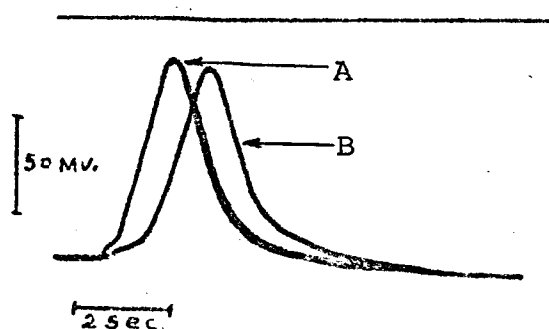


Fig. 24

Traces A, action potentials of Nitella cells in A. P. W.

Traces B, action potentials of Nitella cells in distilled water.

Three cells washed with distilled water and stimulated repeatedly became insensitive to stimulation and no action potential could be generated. The addition of .05 ml APW to the 2 ml bathing solution would restore the normal spike. Again this was reversible. Addition of any of the following .05 ml .2 M $Mg Cl_2$ (Fig. 25), .2 M $Ca Cl_2$ or .2 M $Na Cl$ (Fig. 26) to an insensitive cell caused a slight restoration of the spike.

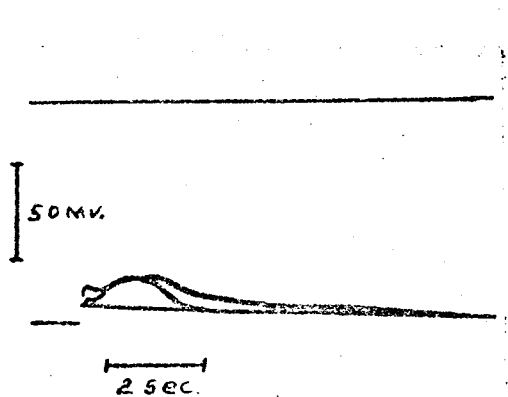


Fig. 25

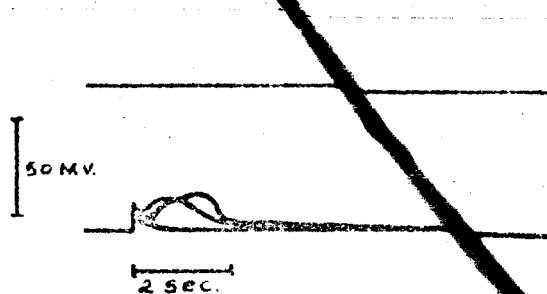


Fig. 26

Higher concentrations of these ions did not improve the response. Addition of .05 ml of .2 M $(\text{NH}_4)_2\text{CO}_3$ gave the response as shown in Fig. 27. The action potential was clearly divided into two components. The last component, presumably the K^+ efflux was delayed by 1.5 seconds after initial depolarization had taken place.

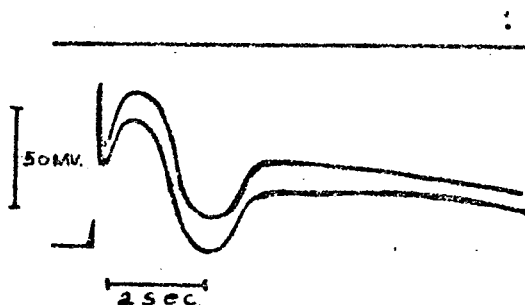


Fig. 27

IX Stimulation

Repetitive stimulation of Nitella cells resolved the normal action potential into two major components, Fig. 28. Similar results were observed with procaine treated cells, Fig. 22. However, this trace showed one extra component.

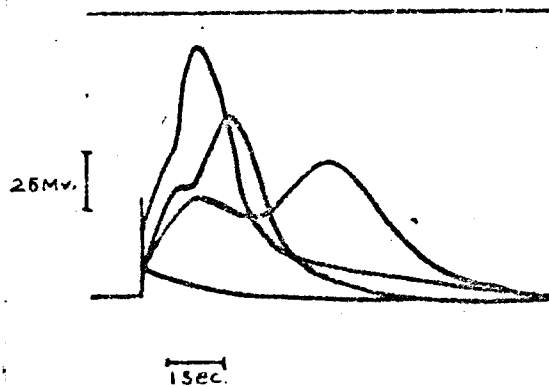


Fig. 28

Some *Nitella* cells contracted when stimulated by damaging the membrane with a glass electrode. A particular cell was observed to contract semi-rhythmically for about five minutes. Each contraction was associated with a slight depolarization of the membrane Fig. 29.

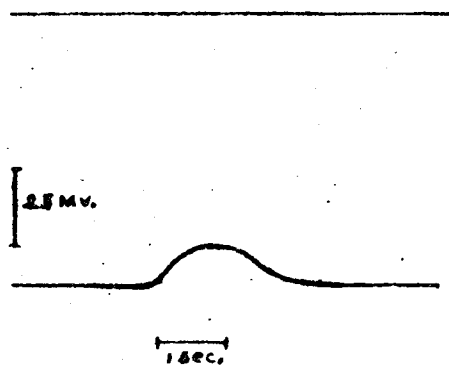


Fig. 29

This is direct evidence for the contractile protein in *Nitella* cells as referred to by Kishimoto to explain cytoplasmic streaming (31, 32).

DISCUSSION

From the results obtained it is apparent that the action potential in *Nitella* cells contain at least three components, Figs. 13, 17, 18, 22. Usually, only two components were observed, Figs. 14, 15, 22, 27. Under special conditions a third component was found to be present in the first one, indicating a close interdependence. The last two components have been attributed to Cl^- and K^+ efflux respectively. The first component could be caused by a Ca^{++} influx, as some of the EDTA experiments indicate.

If this first component is Ca^{++} then an interesting similarity with muscle and nerve cells can be observed. In these vertebrate cells Ca^{++} is involved in the action potential depolarization process (33). The small Ca^{++} influx found by Spanswick was not large enough to account for complete depolarization of the membrane, but it could

explain a triggering mechanism for Cl^- efflux. The EDTA experiments also suggest that the presence of Ca^{++} on the membrane will inhibit spontaneous activity. In heart muscle fibre Ca^{++} controls the pace maker activity, an increase in Ca^{++} concentration will raise the critical depolarization for excitation therefore decreasing the rate of beating. Regular firing has been observed in Nitella cells Fig. 22. The frequency was slowly decreasing and is probably due to an increase in the Ca^{++} ion concentration at the active site which controls the triggering of Cl^- efflux.

In muscle fibre Ca^{++} ion release is the initiating factor for contraction. The contractions observed in Nitella cells were associated with a slight depolarization. It would be interesting to show that this depolarization is caused by a Ca^{++} influx. The mechanism by which the submicroscopic contractions are synchronized to make a major contraction is unknown. Somehow, damage to the cell will initiate this synchronization process.

There is evidence in muscle and nerve cells that Ca^{++} is non-ionically bound to the membrane (34). A similar

binding system for the Ca^{++} in Nitella cells would be more likely than the postulated ionic binding mechanism, because in the latter case negative charges will be left behind when Ca^{++} is released during an action potential repelling any Cl^- ion moving out during depolarization. Ionic repulsion, in such a system, would be economically unfavourable for the cell as more energy is needed to transport Cl^- out of the cell.

It is apparent from these studies that the mechanisms controlling action potentials in Nitella cells have many aspects in common with muscle and nerve cells. It is interesting to postulate that Nitella cells have the same basic biochemical and physiological mechanisms as these cells. Under special conditions and treatments, these similarities can be brought to the surface and observed. During action potentials in the muscle fibres of many crustacea, Ca^{++} moves in while K^+ moves out of the cell. In mammalian muscle and nerve cells Na^+ and K^+ are causing the action potential. The same system operates in squid axon. In Nitella cell Cl^- and K^+ systems are operating. In all cases Ca^{++} has an important role during the initial stages of the action potential. In these cells only the

membrane depolarization mechanism is caused by different ions while the hyperpolarization process is in all cases, caused by K^+ ions. It seems unlikely that only the depolarization ion phase would be entirely different in these cells. It is more probable that during depolarization in these cells all mechanisms are operating i.e. Ca^{++} , Cl^- , Na^+ but that one system has been more developed in one cell than another. According to this theory one should be able to find Ca^{++} , Cl^- , and Na^+ transport systems during action potential in all these cells.

The above speculations are all based on changes in ion concentrations in the cells. Unless these ionic changes are measured they would be difficult to prove or disprove. So far no analytical method has been applied to these cells which is sensitive enough to detect changes in ionic concentrations during an action potential accurately. Neutron activation analysis as discussed in the first section of this thesis was shown to be sensitive within 3-5% for Na and K, using the available neutron flux of 8×10^7 neutrons/cm²/sec. This flux is rather low compared to other fluxes available some of which are in the range of

$10^{12}/\text{cm}^2/\text{sec}$. According to equation no. II the sensitivity should theoretically, increase enormously. However, when working with these fluxes other factors have to be taken into account causing the practical increase in sensitivity to be less than the theoretical calculated value. However, the increase in sensitivity would put the changes in ion concentration during action potentials in *Nitella* cells well within the range of detection. As an illustration, potassium efflux could be measured in the following manner. A Nitella cell is placed in a small chamber filled with artificial pond water, which can easily be replaced with new artificial pond water. The *Nitella* cell is stimulated to initiate an action potential. After several action potentials the bathing solution can be replaced. This procedure can be repeated several times and the washings assayed for its potassium content by means of neutron activation according to the procedure explained in part A of this thesis.

The approximate amount of potassium efflux, if present, could be estimated to be in the range of 1 picomole (10^{12} moles) / cm^2 (35). A *Nitella* cell of 10 cm length and 1 mm diameter would have surface area of 3.17 cm^2 . Therefore

3.17×10^{-12} moles of K are released during one action potential. If ten cells are stimulated for 100 times simultaneously then the total amount of K efflux is increased by a factor of 1,000. The amount of K to be detected in such an experiment would be approximately 3×10^{-9} moles.

Assuming that a neutron flux of 10^{12} neutrons/cm²/sec. is available and that the lowest activity which will give statistical results within a few percent error is 100 counts per minute then the minimum number of target atoms can be calculated using equation II shown below.

$$A_t = \phi \delta_{act} n (1 - e^{-\lambda t})$$

(II) (see page 3)

If the sample is bombarded with a neutron flux for an infinite length of time as compared to the half life of the required isotope and if the activity of the sample is counted directly after bombardment then the expression on $(1 - e^{-\lambda t})$ reduces to 1 and equation II can be rewritten as:

$$A = \phi \delta_{act} n \quad (III)$$

where A = activity/sec.

Equation III can be solved for n.

$$n = \frac{A}{\phi \delta_{act}} \quad (IV)$$

$$A = 100 \text{ counts/min. or } \frac{100}{60} \text{ cnts./sec.}$$

$$\phi = 10^{12} \text{ neutrons/cm}^2/\text{sec.}$$

$$\delta_{act} = 2.2 \times 10^{-24} \text{ cm}^2$$

Substituting these values into equation IV value of

n is obtained.

$$n = \frac{100}{60 \times 10^{12} \times 2.2 \times 10^{-24}} = 7.56 \times 10^{+11} \text{ molecules.}$$

$$\text{or } \frac{7.56 \times 10^{+11}}{6.02 \times 10^{23}} = 1.25 \times 10^{-12} \text{ moles.}$$

The minimum amount of K that can be detected in this experiment is 1.25×10^{-12} moles. The amount that needs to be detected in order to show potassium efflux during the action potential in Nitella cells is 3.17×10^{-9} moles and is well within the range of detection using a flux of 10^{12} neutrons/cm²/sec.

It can, therefore, be concluded that neutron activation analysis is a suitable method to analyze ion fluxes in Nitella cells.

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