Electrophysiological Analysis of the Electrogenic H^+-pump in *Chara corallina*

by

Yuko Takeuchi

Department of Biology, College of
General Education, Osaka University

Contents

Introduction

All cells keep their life with biomembrane, which separate the cell from external circumstances. One important function in the membrane is the transport of ions and other substances to maintain their almost constant internal condition. Generally K^+ is rich and Na^+ is poor inside the cell. Uneven distribution of Na^+ cannot be explained by the simple electro-diffusion mechanism alone. Some active mechanisms to transport ions against the electrochemical potential gradient are acting in the cell membrane.

Ussing and Zerahn (1951) proposed the short-circuit method to determine the active transport system quantitatively. In their report , the same solution was prepared on both side of the membrane, then the electric current , which is necessary to short circuit both side, was measured. At the same time, the flux of ions were measured to know which ion was actively transported. Skou (1957) isolated from periferal nerve the ATPase which possesed the ability of Na^+-K^+ anti-transport.

Before 1960 , active transport system had been considered as electrical neutral one, but in the 1960's it was proved that many active transport systems worked electrogenically. Na^+-K^+ ATPase, Ca^{++} ATPase in the animal cells and H^+ ATPase in the mitochodria and chloroplasts have been investigated very actively (Kerkut & York 1971, Thomas 1972, Kostyuk et al. 1972, Harold 1977).

In plant cells the active pump was also found electrogenic, i.e, it hyperpolarizes the resting potential. Slayman (1965) investigated electrical properties of Neurospora crassa, and found that internal potential depolarized from -200mV to -30mv by various respiratory inhibitors. Besides, he showed a plot of membrane potential versus the intracellular ATP concentration. This plot yielded a saturation curve, which was readily fitted by the Michaelis -Menten equation (Slayman et al. 1973).

Subsequently H^+ extrusion pump was discovered in many plants (Spanswick 1981). Scarborough investigated biochemically the difference between ATPase isolated from Neurospora crassa and H^+-ATPase of mitochondria (1980). On the other hand, Cl^- electrogenic pump was also reported in some plant cells. Gradmann and Bentrup showed in marine alga, Acetabularia, that the resting potential was about -160 mV in the light and depolarized to -80 mV in the dark (1970). Saddler found in Acetabularia the necessity of external Cl^- for the electrogenic pump (1970).

Recently , many data have accumulated to show that the electrogenic pump contributes not only to the transport of other ions or amino acides through the hyperpolarization of resting potential but also to the cotransport or antiport of non electrolytes and neutral electrolytes (Bentrup 1980). The electrogenic H^+ pump will also work as the regulator of the internal pH. (Sanders, Hansen and Slayman 1981). Besides, many studies show that the non-uniformity of membrane potential play an important role in the growth and morphogenesis of plant cell (Jaffe and Nuccitelli 1977). If the electrogenic pump does not distribute equally over the surface of cell membrane, non-uniformity of the membrane potential will be performed.

In this paper I analyzed the mechanism of electrogenic H^+ pump in the Chara corallina. The Chara corallina is one of fresh water algae. Cylindrical giant internodal cells grow as large as 1 mm in diameter and 10 cm in length. So this material is very suitable for electrophysiolocal study. Moreover, Chara corallina has been precisely investigated for a long time on its excitability and its relationship with cytoplasmic streaming. The internal concentrations of various ions are also measured(Tazawa, Kishimoto and Kikuyama 1974).

In this paper, the method of exact measurement and calculation of electrophysiological parameters such as conductance and electromotive force of Chara corallina will be described. An ideal condition to separate the

electrogenic pump from the passive electrodiffusion channel, the primary ion which drive the electrogeic pump and the driving force of electrogenic pump are discussed in this chapter.

Chapter 1

A method for a quantitative expression of the electrogenic pump

Introduction

Electrophysiologically there are two reasons to suppose that the electrogenic pump contributes to the membrane potential. First, the measured resting potential is generally more negative than the equilibrium potential of any of the ions present. Second, the resting potential depolarizes with decline of energy metabolism during treatment with anoxia, dark, low temperature or metabolic inhibitors.

It is very important problem how to express the activity of electrogenic pump. Kitasato showed first the active H^+ extrusion mechanism of Nitella in 1968. He measured K^+ and Cl^- effuluxes by external pH changes. And sum of the slope ion conductances , g_k and g_{Cl} was found to be negligible compared with membrane conductance. In the range of external pH between 5 and 6, the chord conductance of H^+ was almost equal to the membrane conductance. He assumed that the H^+ conductance was so high that the cell membrane behaved like a H^+ electrode. But the observed membrane potential was always about 70 to 80 mV more negative than H^+ equilibrium potential at various pH . The extent of this hyperpolarization was considered to be the pump activity . But the depolarized state by metabolic inhibitor (Dinitrophenol(DNP)) was not equal to the H^+ equilibrium potential. Kitasato explained this DNP effects with the increase of passive conductance. His idea stands on the assumption that H^+-pump behaves as an ideal current source, and all conductances are equal to passive conductance. This model is the same as the current source model of Neurospora by Slayman et al.(1973). (Fig. 1-b)

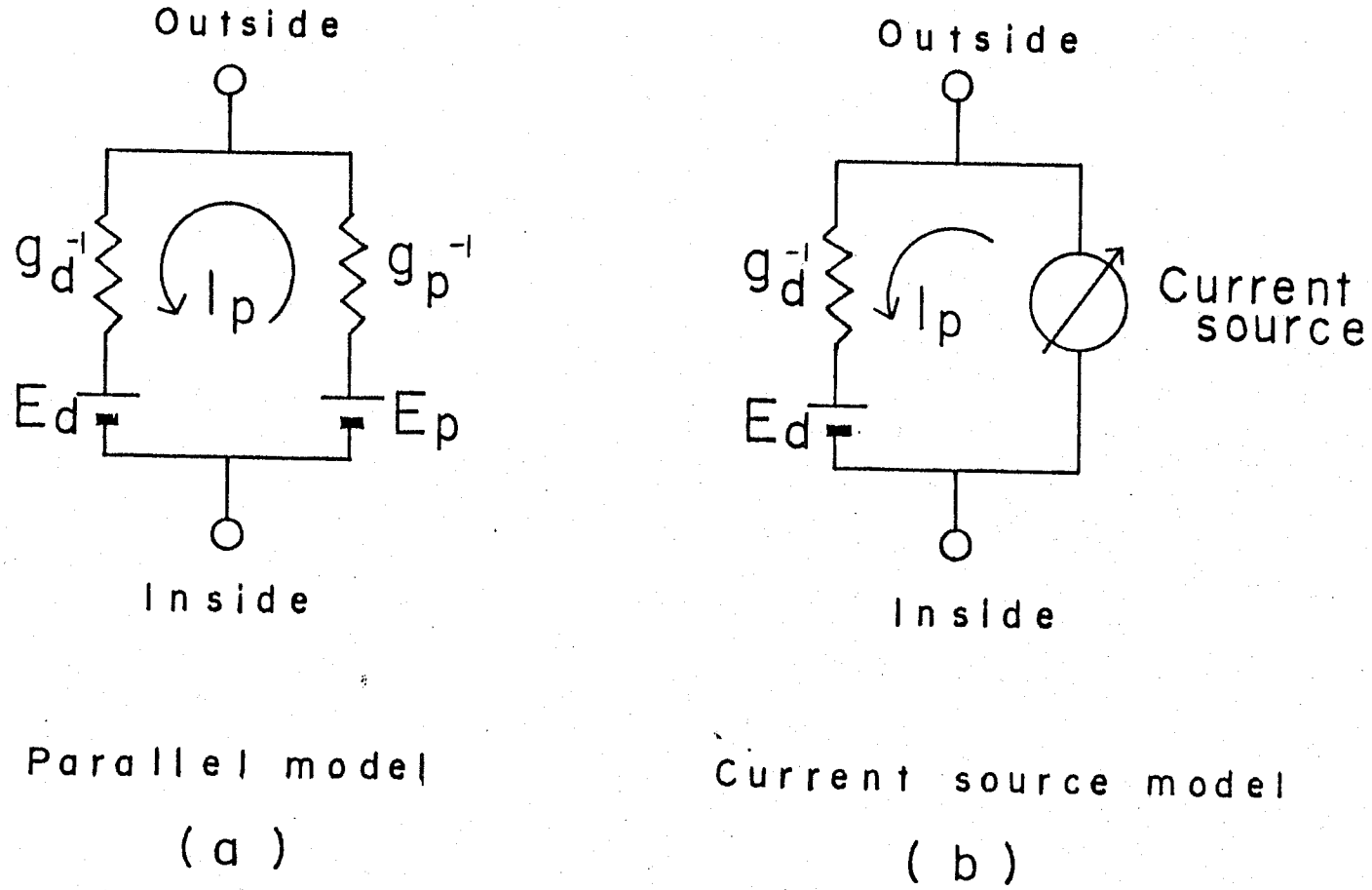


Fig. 1 Equivalent circuit model for the membrane having the electrogenic ion pumping mechanism.
g_d and E_d are the conductance and the electromotive force of the diffusion channel.
g_p and E_p are the conductance and the electromotive force of the pump channel.
I_p is the pump current.
In the paralell model the pump conductance is voltage dependent.

In the current source model (Fig.1-b), pump activity is explained as follows

$$E_m = E_d + E_p$$
$$E_p = E_m - E_d = - I_p / g_d$$
$$I_p = - E_p \times g_d = (E_d - E_m) \times g_d$$

E_m, E_d, E_p is the electromotive forces of the total membrane, the diffusion channel and the pump channel respectively. g_d is the conductance of the diffusion channel and I_p is the pump current.

In Neurospora this model could explain their experimental results, because the resistance of membrane does not seem change appreciably, while the membrane potential depolarized with pump inhibition.

On the other hand, Keifer & Spanswick (1978) proposed another model (Fig. 1-a). In this model the membrane is expressed with a pararell circuit, one is passive path and the other is active path, each of which has its own resistance. The pump current is explained as follows.

$$g_m = g_d + g_p$$
$$E_m = g_d \times E_d / g_m + g_p \times E_p / g_m$$
$$I_p = g_p (V_m - E_p)$$

where g_m, g_d, g_p is the conductances of the total membrane, the diffusion channel and the pump channel respectively. They found that application of 5 μM CCCP, 50 μM DCCD, 50 μM DES or 0.1 mM DNP depolarized the membrane potential and increased the membrane resistance. They concluded that these inhibitors had influence not only on the membrane potential but also on the pump conductance.

The crucial point is whether the electrogenic pump has a conductance or not. In other words, it is important to find out whether the pump current may change with membrane potential or not. Simmen and Tazawa (1977) chose the current source model in their experiments, in which the resistance did not change appreciably, while the membrane potential depolarized by about

100 mV by the internal perfusion with 0 mM ATP medium. On the other hand, recently Smith and Walker (1981) obtained the I-V curve by the open vacuole method in the tonoplast-less perfused cell. According to them 2 mM ADP inhibited the 2 mM ATP effect on the current by about 50% and decreased g_m from 33 to 24 $\mu S/cm^2$. Their results seem to indicate an inhibitory effect of 2.0 mM ADP on the pump conductance.

There are several points to be taken care of in the estimation of the membrane conductance. In plant cells following problems needs to be solved.

1. There is a possibility of change in the electromotive force (emf) when we applied the test current pulse to measure the membrane resistance.
2. Even if current electrode and the membrane potential electrode are in the same cylindrical cell, the spacial uniformity of the current flow may be sometimes be uncertain.
3. The plant cell has a thick cell wall and their outside medium is low ion strength, so the contribution of their series resistance to the total resistance is fairly large.

In this report I solved these problems by following methods. To solve the first problem, I analyzed the voltage response which is caused by the test current pulse as small and as short as possible under the current clamp condition(Ohkawa & Kishimoto 1975).

About the second problem, I took advantage of the Chara corallina, because the internodal cells of this alga are so big that both of the current metal wire electrode and the voltage electrode could be inserted in a same cell. Actually the platinum-iridium wire coated with platinum black was used as an internal current electrode to keep the electrical impedance as low as possible. This wire was introduced into the central vacuole of the internodal cell from the nodal end to maintain the uniformity of the applied current flow.

I could solve the third problem by curve fitting of voltage response

from which series resistance could be subtracted by calculation. The responses were analyzed after A/D conversion with a microcomputer. This method is applied also in the analysis of the membrane excitation. The change of membrane conductance could be followed from time to time during the several seconds of the Chara excitation.

If there is an ideal inhibitor against the pump, it must inhibit only the electrogenic pump, without affecting the passive channel. In this chapter, I compared two drugs and determined which was the more ideal inhibitor of the pump. They are Triphenyltin chloride (TPC), which is known as the inhibitor of CF_1 and F_1 (Stockdale et al. 1970, Gould 1976, Papa et al. 1982) and 2,4-dinitrophenol (DNP), which is known as uncoupler.

Material

The giant internodal cells of Chara corallina were used throughout this work. Chara corallina was cultured in our laboratory with the tap water in which extracts of soil were added. A fluorescent lighting (12 hr light and 12 hr dark) about 2000 lx was employed. Before the experiments Chara internodal cells of which the average diameter was 0.7 mm and the length was 6 cm were cut from the adjacent cells and were put into the artificial pond water (APW) under the natural sun light condition beside the window for at least one day. The APW contained 0.05 mM KCl, 0.2 mM NaCl, 0.1 mM $Ca(NO_3)_2$ and 0.1 mM $Mg(NO_3)_2$. The pH was adjusted to about 7 with 2 mM MES (2-N morpholinoethane sulfonic acid). During experiments the external solution was perfused at a constant rate of about 1 liter per hour. Temperature and pH of test pool were monitored with a thermister and a glass pH electrode respectively.

Result

The measurements of electromotive force and conductance

I calculated the membrane conductance and electromotive force (emf) from the voltage responses which were caused by application of the square current pulse. Current clamp was performed following the method of Cole and Moore (1960). As shown in Fig. 2 an axial wire which was used as a current electrode was introduced into the internal cell from the nodal end. The axial wire coated with platinum black was either a tungsten or a platinum-irridium alloy. The diameter of it was about 0.1 mm. A pair of chlorinated silver plate coated with platinum black was used as an external current electrode. Such a coating are useful for the stability of electrode potential and for the decrease of the electrode impedance.(Cole and Kishimoto 1962)

The membrane potential was measured between the internal Ag/AgCl glass electrode filled with 3 M KCl contained with 5 mM EGTA and the external reference electrode. The tip of the latter was about 30 μm diameter and was filled with agared 100 mM KCl. The tip of internal glass capillary electrode was frequently covered with cytoplasm during several hours. To avoid this 5 mM EGTA was added into the microelectrode. The membrane potential was amplified with a differential preamplifier of unity gain, which was consisted of three integrated circuits, IC (1009, Teledyne Philbrick) having $10^{11}\ \Omega$ input impedance. OP-07 (PMI) was used in the current clamp circuit. The speed of current clamp was from 50 to 100 μsec. The space constant of the internode was about 3 cm at rest and 6 mm at the peak of the excitation. To keep the spacial uniformity of the measuring region, the length of which was chosen as 6 mm long.

Analog data of the current pulse and the voltage response were converted into digital data with Data acquisition system (Datel, MPAS 8D), and

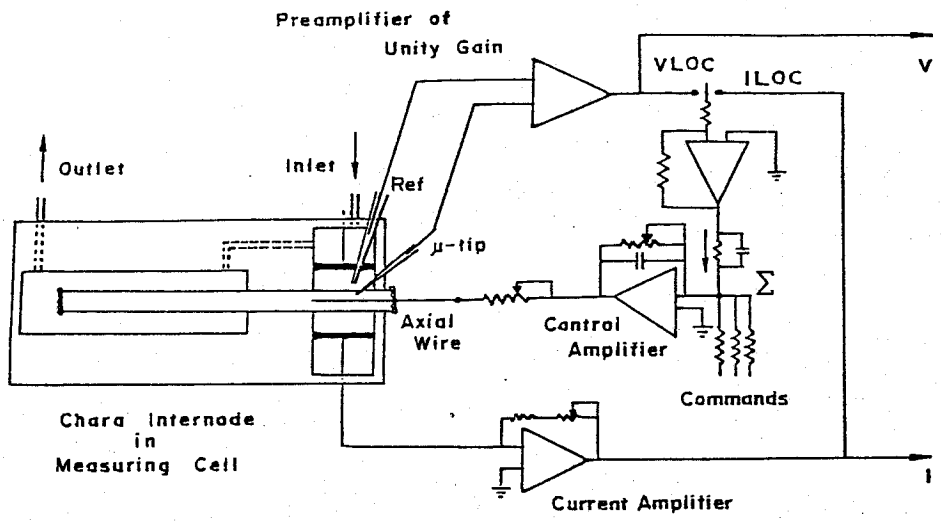


Fig. 2 Current clamp system for Chara internodes

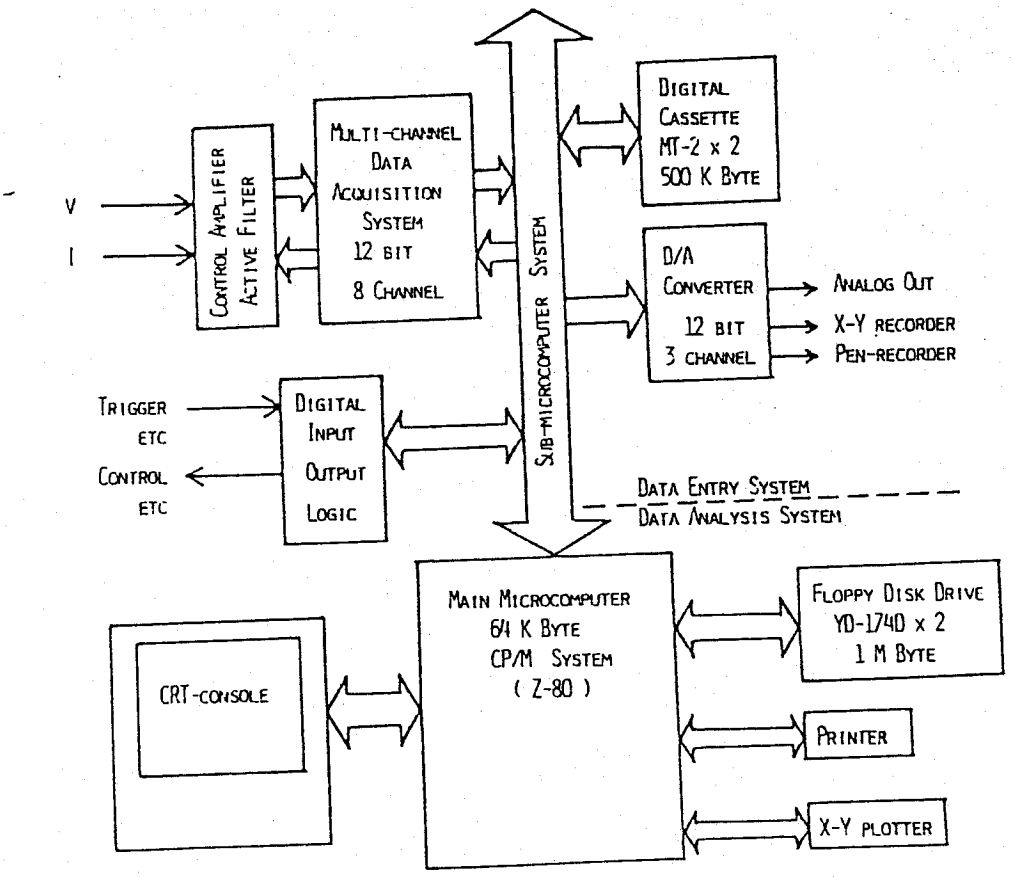


Fig. 3 A block diagram of the method for data entry and analysis

recorded with a digital cassette tape recorder (MT-2, TEAC) and with a floppy disk drive (Y-E DATA) (Fig. 3). The data of voltage responses were simulated with a nonlinear least square program or with a modified Powell's program using microcomputer. This was performed with a CP/M system (DIGITAL RESEARCH) which uses Z-80 as a central processor.

The calculation of membrane conductance

The biomembrane has its own emf when the current was not applied externally. So the relation between current(I) and voltge(V) is expressed with a following equation according to the law of Kirchhoff.

$$V = E + ZI$$

where E is the emf and Z is the impedance of the Chara membrane.

When a small perturbation is given to the current, the relation of such a perturbed current (i) and the resulting voltage response (ΔV) is as follows.

$$\Delta V = iZ + I \times \Delta Z + \Delta E$$

Under the current clamp condition (I is kept 0)

$$\Delta V = iZ + \Delta E$$

Generally, the series resistance between the internal electrode and the external electrode can not be ignored, because Chara internode has a thick cell wall and external solution of low ion strength. When the series resistance (r_s) exists in circuit as shown in Fig.4(a), the above equation is written as follows.

$$V(t) = r_s \times i + r_m \times i \, [1 - \exp(-t / c_m \times r_m)] \qquad (1)$$

where r_m and c_m are resistance and capasitance of the Chara membrane respectively. Each parameter was calculated by computer simulation. But simulation with single time constant was not fully successful (Fig.5(a),(b)). When we simulate the data with two time constant equation such as shown in the following (Eq.2), the standard error of curve fitting

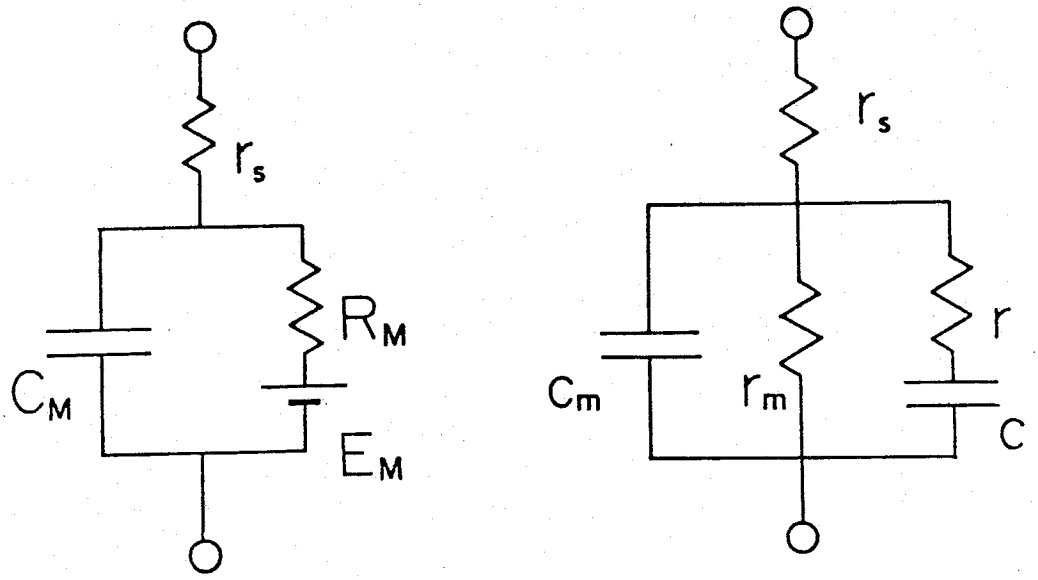


Fig. 4 Circuit models for the Chara membrane

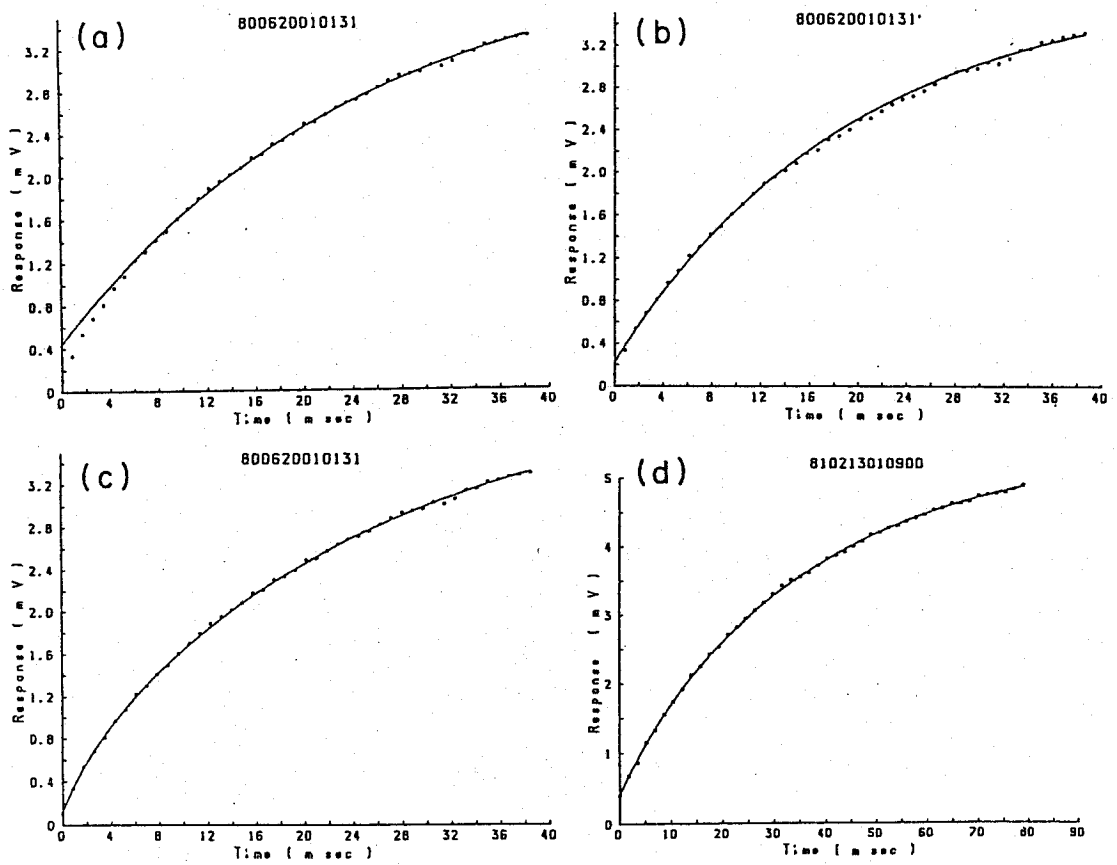


Fig. 5 The voltage responses of the unperfused *Chara* membrane (●) caused by a small square current pulse (0.182 $\mu A \cdot cm^{-2} \times 38.6$ msec). (a) Date fitting with the single τ model. $v(t) = 4.334 - 3.830 \exp(-0.035t)$ mv; (t in msec). In this example the fitting was performed for only the later part of the voltage response. Thus, a deviation from the data is evident for the earlier part of the response. (b) Another example of fitting the same data with the single τ model. $v(t) = 3.848 - 3.616 \exp(-0.0477t)$ mv; (t in msec). In this example the fitting was performed to minimize the standard deviation using the entire set of data of the same voltage response. (c) Data fitting of the same data with the two τ model. $v(t) = 4.334 - 3.830 \exp(-0.035t) - 0.388 \exp(-0.360t)$ mv; (t in msec). The standard deviation is reduced to a fifth. (d) Data fitting of a voltage response of the plasmalemma alone, with the two τ model. In this example, the voltage response was caused by a square current pulse (-0.232 $\mu A \cdot cm^{-2} \times 78.93$ msec). $v(t) = 5.416 - 4.853\exp(-0.02739t) - 0.1642\exp(-0.1339)t$ mv; (t in msec). These results of fittings were plotted with a X-Y Plotter (Watanabe, WX4671).

decreases to 10% of the one obtained with the single time constant equation (Fig.4 (b), Fig.5 (c))

$$V(t)= r_s \times i + (r_m \times i\ [1-\exp(-t/c_m\ r_m)] + r_2 \times i\ [1-\exp(-t/c\times r)]) \quad (2)$$

There are two possibilities why the third term in Eq.(2) was needed. First, in plant cells tonoplast exist in series with plasmalemma. The resistance of the tonoplast is about 10% of plasmalemma (Walker 1957, Findlay & Hope 1964, Tazawa, Kishimoto and Kikuyama 1974). Second, there is a change in emf under the subthrethold current pulse.

To investigate the first cause, I used tonoplast free cell obtained by EGTA perfusion. The detail of perfusion technique will be described in the next chapter. As shown in Fig. 5(d) even in the case of plasmalemma alone, the standard error calculated with two time constant model is much smaller than with the single time constant model. Then we must consider the second reason. As demonstrated clearly by Hodgkin and Huxley (1952) ionic conductances of the squid giant axon are voltage dependent. Similar results were reported in Chara and Nitella by Beilby and Coster (1980), Hirono and Mitsui (1980).

I applied a test current pulse as small and short as possible (smaller than 3 mV and shorter than 38.6 msec). However, I found the change of emf in the voltage response. Therefore, we need to subtract this term from the total voltage response to know the exact value of membrane conductance and capacitance. I calculated each parameter accurately by computer simulation using two time constant equation (Fig.4(b) and Eq.2). By the results of accurate simulation we confirm that the value of membrane conductance at resting state calculated by single time constant is overestimated by about 4% compared with the one by the two time constant. All results under current clamp condition in this reports were calculated by this method.

The determination of pump activity

If an ideal inhibitor attacks the electrogenic pump selectively, following equations will hold under the current source model (Fig. 1-b).

$$G = g_d \; ; \qquad E_p = E_m - E_d$$

where g_d is the conductance of the diffusion channel, E_d is the emf of the diffusion channel which are estimated after inhibition and E_p is the pump emf. In general membrane conductance decreases by pump inhibition. Therefore, we need to conclude that the passive conductance is affected every time by the pump inhibitor in the current source model, even if it is an ideal inhibitor. This is paradoxial indeed.

On the other hand, if we assume that the pump as well as passive channel has its own conductance, then the membrane emf and the conductance which are measured are expressed as follows (Fig. 1-a).

$$G_m = g_d + g_p \tag{3}$$

$$E_m = (g_d \times E_d + g_p \times E_p) / (g_d + g_p) \tag{4}$$

When an inhibitor blocks only the electrogenic pump, then the pump conductance (g_p) will decrease to zero and the membrane potential will decay to the passive diffusion potential (E_d). In other words, the final values of conductance and emf correspond to the passive conductance (g_d) and the passive emf (E_d) respectively. Therefore, the pump conductance (g_p) and the pump emf (E_p) can be calculated as follows:

$$g_p = G - g_d \tag{5}$$

$$E_p = (g_m \times E_m - g_d \times E_d) / g_p \tag{6}$$

Change of pump parameters, i.e, g_p and E_p during pump inhibition can be traced with these equations.

The effects of pump inhibitors

DNP is known as an uncoupler of H^+ or electron flow and is an inhibitor of ATP synthesis. After treatment with 0.2 mM DNP in the dark for about 40 min the resting potential of the Chara membrane depolarized from -190 mV to -110 mV and the conductance decreased from 200 to 85 μScm^{-2} (Fig. 6). When I chose these final values as E_d and g_d respectively, the pump activity can be calculated according to the Eqs. (5) and (6). In this case the pump conductance before inhibition was 115 μScm^{-2} which is greater than the passive conductance. The pump emf is -230 mV and the pump current was 7 $\mu A\ cm^{-2}$ before inhibition. During pump inhibition with DNP, both of the pump conductance (g_p) and the pump current (I_p) gradually decreased to zero, but the pump emf (E_p) showed a transient hyperpolarization. This transient hyperpolarization is so unexpected that the reason of its will be discussed later.

Fig. 7 shows the change of pump and passive parameters at the peak of action potential during DNP treatment. The peak conductance which is 10 times as large as the conductance at the resting state was caused mainly by the marked increase of g_d. With the progress of DNP inhibition the peak of g_d decreased and the peak of E_d moved to more negative level. Actually no action potential took place at the end of the DNP treatment. These results show that DNP affected not only the electrogenic pump but also the passive channel as well. So DNP can not be regarded as an ideal pump inhibitor.

Next we used 2 μM Triphenyltin chloride (TPC) which is known as a inhibitor of CF_1 in the chloroplast and F_1 in the mitochondria. The TPC effects were investigated in much the same way as DNP. APW containing 2 μM TPC was added when membrane potential was stable in the dark condition. The membrane potential gradually depolarized from -200 mV to -100 mV, while g_m decreased from 150 $\mu S\ cm^{-2}$ to 60 $\mu S\ cm^{-2}$ after 60 min (Fig. 8). Using this data g_p, E_p and I_p were calculated in the same way as in the case of DNP.

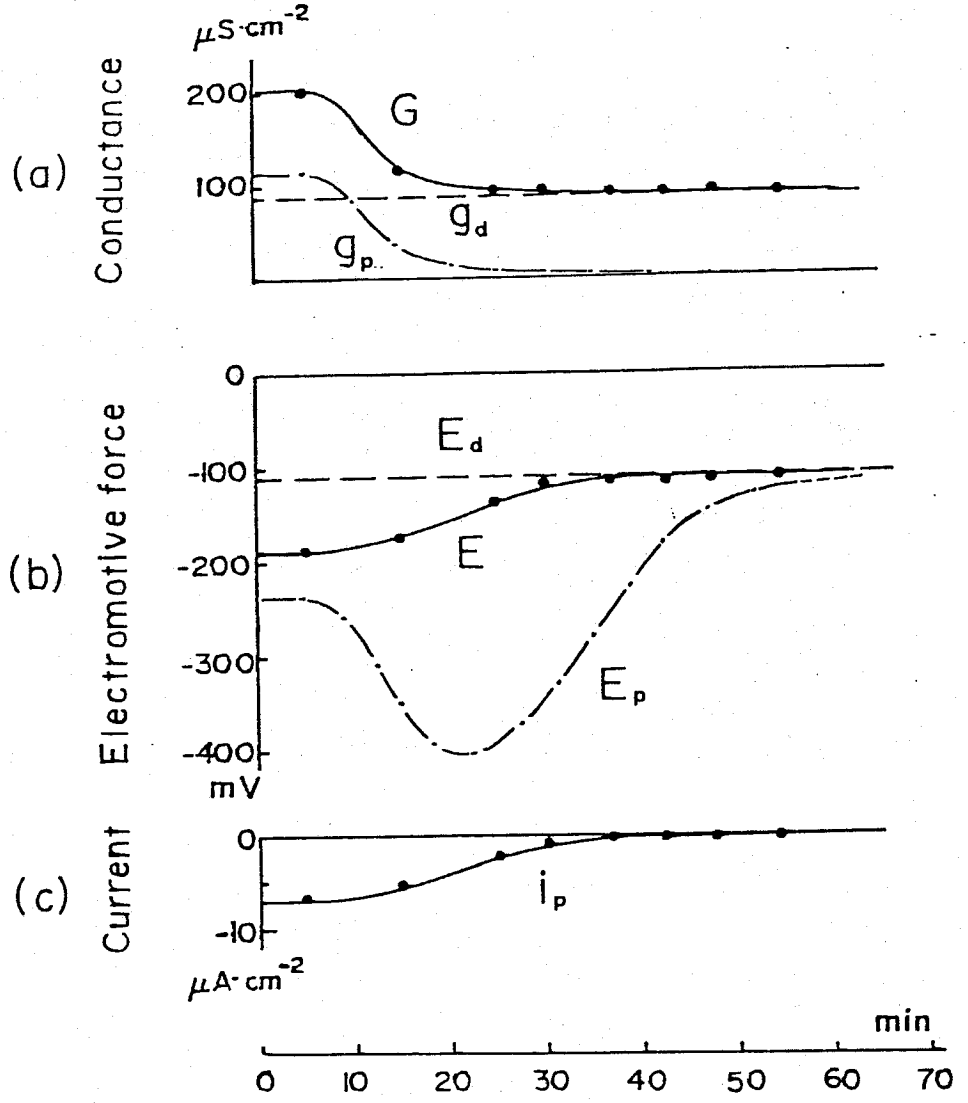


Fig.6 . Changes of conductances, (a), electromotive forces (b) and pump current (c) of Chara internode during progress of DNP (0.2 mM) poisoning. Temperature 25°C The conductance, g_d, and electromotive force, E_d, of the passive ion transport pathway are oftained from the final values of measured conductance, G, and electromotive force, G. The conductance, g_p, of the electrogenic pathway decreased monotonically to zero, while the electromotive force, E_p, decreased asymptotically to E_d after a transient hyperpolarization. The pump current, i_p decreased monotonically to zero.

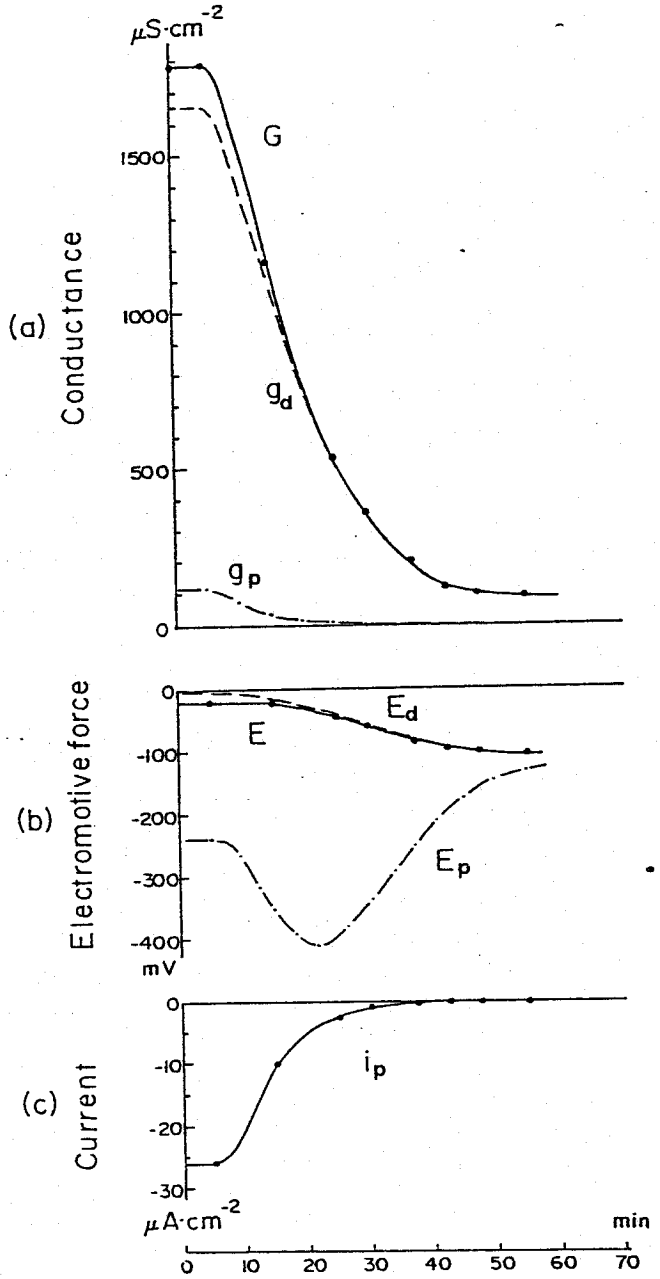


Fig.7 . Conductances (a), electromotive force (b) and pump current (c) at the peak of excitation of Chara membrane during DNP (0.2 mM) poisoning. Temperature 25°C. The changes of conductance, g_p, and electromotive force, E_p, of the electrogenic ion transport pathway are assumed to be the same as those in Fig.6 . The rapid decrease of conductance is mainly due to the decrease of conductance, g_d, of the passive ion transport pathway. The electromotive force, E_d, at the of action potential shifted toward a more negative level. The large pump current, i_p at the peak of excitation decreased rapidly to zero.

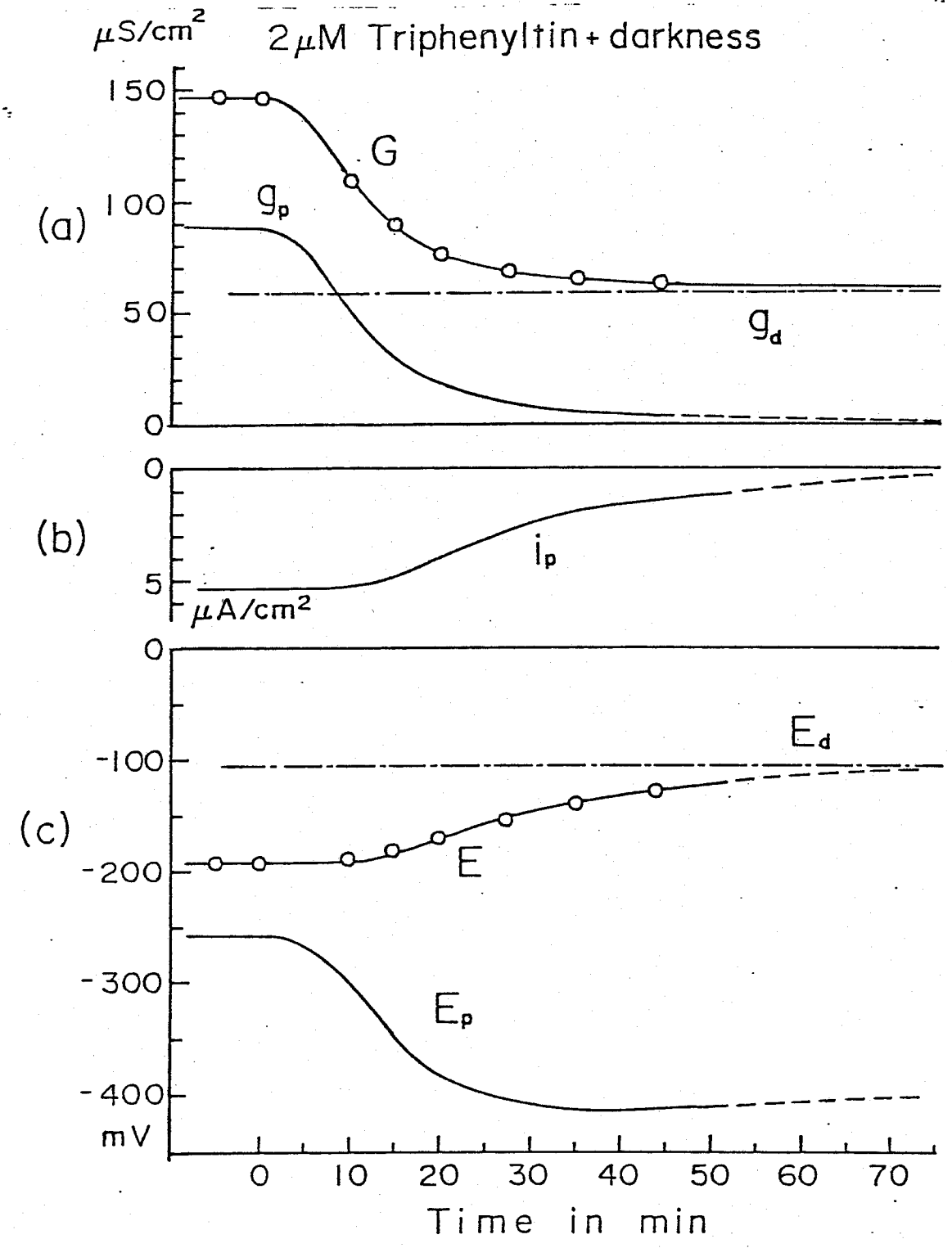


Fig. 8 Changes of conductances (a), pump current (b) and electromotive forces (c) of Chara internode during progress of TPC (2 μM) poisoning. Temperature, 25°C. The conductance, g_d (60 μS cm^{-2}), and electromotive force, E_d (-100mV), of the passive ion transport pathway are obtained from the final values of measured conductance, G, and electromotive force, E. The conductance, g_p of the electrogenic pump pathway decreased monotonically to zero, while the electromotive force, Ep showed a transient hyperpolarization. The pump current decreased monotonically to zero.

As shown in Fig. 8 before inhibition g_p was 90 μS cm^{-2} and I_p was 5.3 μA cm^{-2}. I_p and g_p decreased to almost zero with TPC treatment. On the other hand, the pump emf showed a transient hyperpolarization.

Then I investigated the effect of TPC on the membrane excitation. Fig. 9 showed the change of g_d, E_d, g_p and E_p at the peak of the action potential by TPC. The peak value of g_d was a little decreased by TPC treatment, while that of E_d did not change appreciably. This reduction of peak g_d may be caused by the direct effect of TPC on the passive channel. For example the duration of action potential was prolonged by TPC treatment. But the emf of passive channel did change little and action potential took place even after 60 min. This facts show that TPC is a much better inhibitor of the electrogenic pump than DNP.

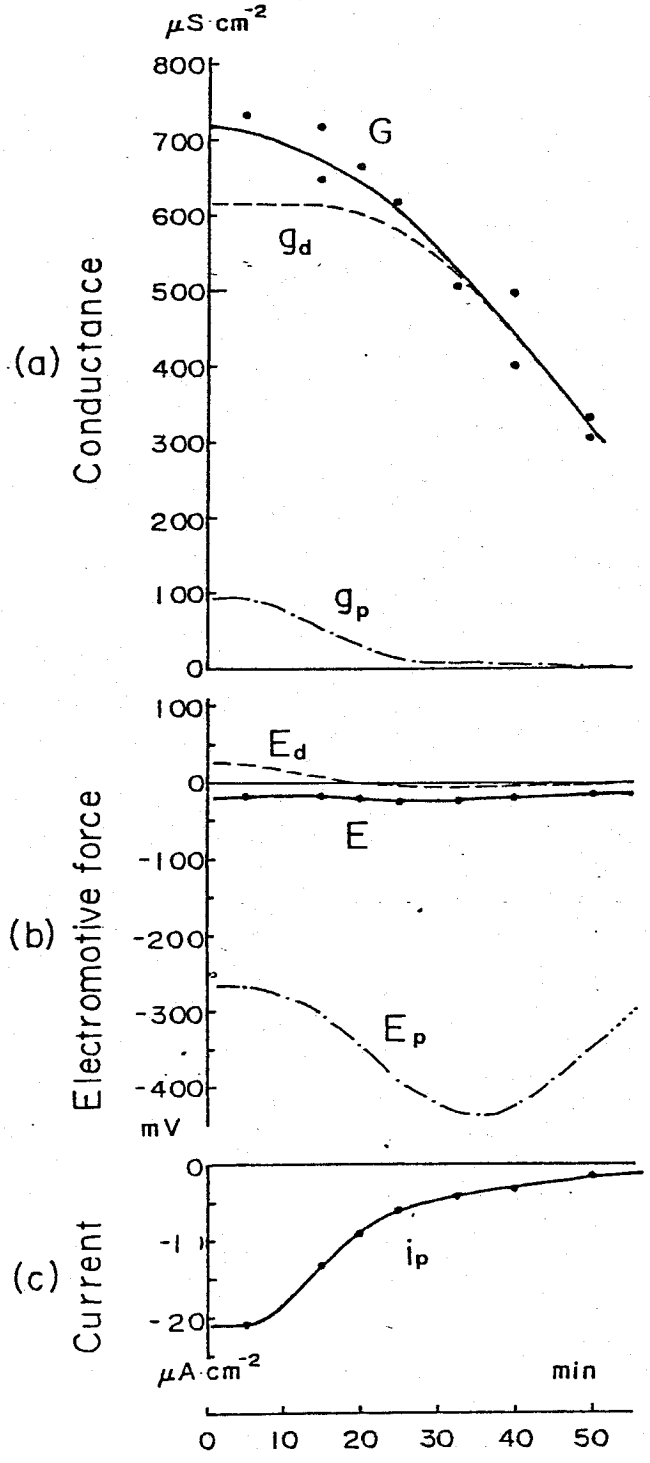


Fig. 9 . Conductances (a), electromotive force (b) and pump current (c) at the peak of excitation of Chara membrane during TPC (2 μM) poisoning. Temperature 25°C. The changes of conductance, g_p, and electrogenic ion transport pathway are assumed to be the same as those in Fig. 8 . Comparatively slow decrease of conductance, G, is mainly due to the decrease of conductance, g_d, of the passive ion transport pathway. The electromotive force, E_d, at the peak of action potential did not change appreciably by TPC poisoning. The large pump current, i_p at the peak of excitation decreased to zero by TPC.

Discussion

The membrane conductance was measured with high degree of accuracy by applying short and small test current pulse under the current clamp condition and later by the curve fitting with computer simulation. The decrease of conductance as well as the depolarization of membrane potential occurred by the pump inhibitor which is known as the blocker of ATP synthesis. These results agree with the report by Spanswick (1972).

Generally pump current is affected by the membrane voltage and the ATP concentration. Finkelstein (1964) and Rapoport (1970) published theoretical analyses of voltage dependency of the pump channel.

Gradmann (1975) obtained the I-V relation of Cl^- active pump in Acetabularia. Recently Gradman et al. (1978) also measured the I-V curve of H^+ pump of Neurospora and showed the potential dependency of pump. These experiments were based on an idea that pump have the conductance.

For example the total conductance calculated by the passive ion fluxes in Chara corallina is not equal to the actual measured cunductance (Williams, Johnston and Dainty 1964). Keifer & Spanswick (1978) showed the remarkable change of g_m during pump inhibition, and he concluded that pump conductance occupied most of the g_m. In his report, g_d is equal to 3-5 $\mu S\ cm^{-2}$ and g_p is 67 $\mu S\ cm^{-2}$. But in my experiment the ratio of pump conductance and passive conductance ranged from 1 : 1 to 10 : 1.

At the resting state E_p is about -250 mV and after inhibitor treatment in the dark, the pump emf showed a transient hyperpolarization. This hyperpolariza-tion of E_p will be discussed in the following Chapter. I used two pump inhibitors and concluded that TPC is a much better inbibitor than DNP. DNP damaged the generation of action potential, while TPC caused only a slight effect on the action potential.

Finally I shall discuss my assumption under which the pump activity was

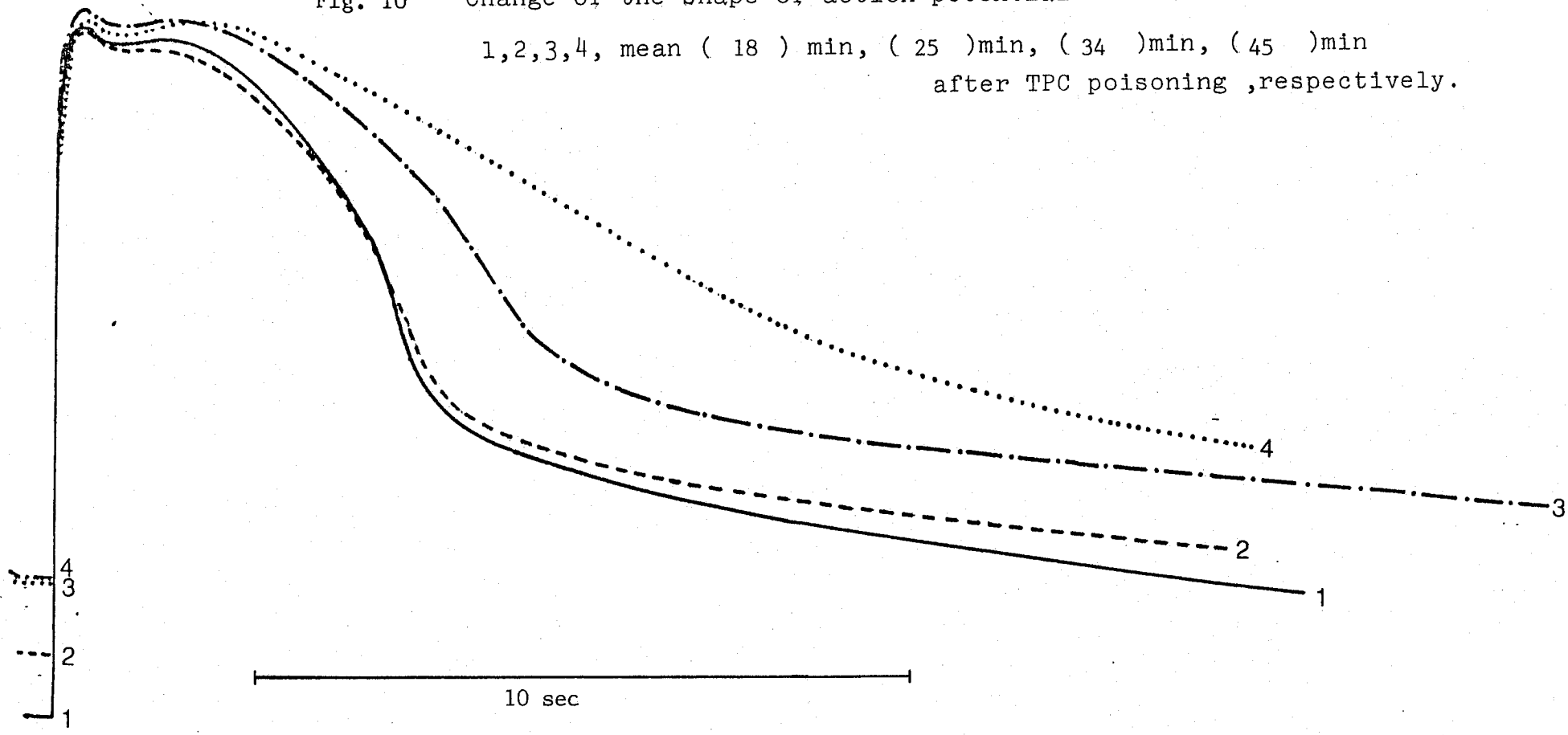
calculated. The first assumption is that passive factor does not change by pump inhibitor. Second is that at the late stage of pump inhibition, the remaining factor is only passive channel. Third is that pump parameters (g_p, E_p) may change only a little during action potential.

It is reported that TPC acts as Cl^-/OH^- exchanger in mitochondrial membranes (Aldridge et al. 1977). But even if TPC may form a Cl^-/OH^- exchange path in Chara corallina, TPC may have no effect in passive conductance, if Cl^- and OH^- are exchanged under electroneutral condition. There is a possibility that the passive channel will change according to the cytoplasmic pH change, if TPC works as Cl^-/OH^- exchanger. As will be shown in the next chapter, the conductance of the passive channel is almost unchanged with external pH change. So the change of the conductance by TPC can be caused by the change of the pump conductance. Next I consider about the second assumption that the pump factor is completely lost by TPC. As will be described in the next chapter, the changes of the conductance and membrane potential after TPC treatment were only a little by the change of pH and temperature, while marked change occurred before inhibition. These facts show the validity of second assumption.

Whether the pump parameters (g_p, E_p) remained unchanged or not in the process of action potential is an unsolved question. It is interesting to note that the duration of action potential prolonged by TPC treatment.(Fig. 10). As shown in Fig.11, the recovery to resting state from excited state seemes to be accelerated by the pump current. The current which flow through passive channel from active pump may accelerate the inactivation process of action potential. Recently Kishimoto analysed the change of pump parameters during the process of action potential using i_p-V curve of the pump channel (1984). From his data g_p decreased to some extent, but it is worth noting that the pump conductance is much smaller than that of the passive channel at the peak of action potential.

Fig. 10 Change of the shape of action potential in chara corallina

1,2,3,4, mean (18) min, (25)min, (34)min, (45)min
after TPC poisoning ,respectively.



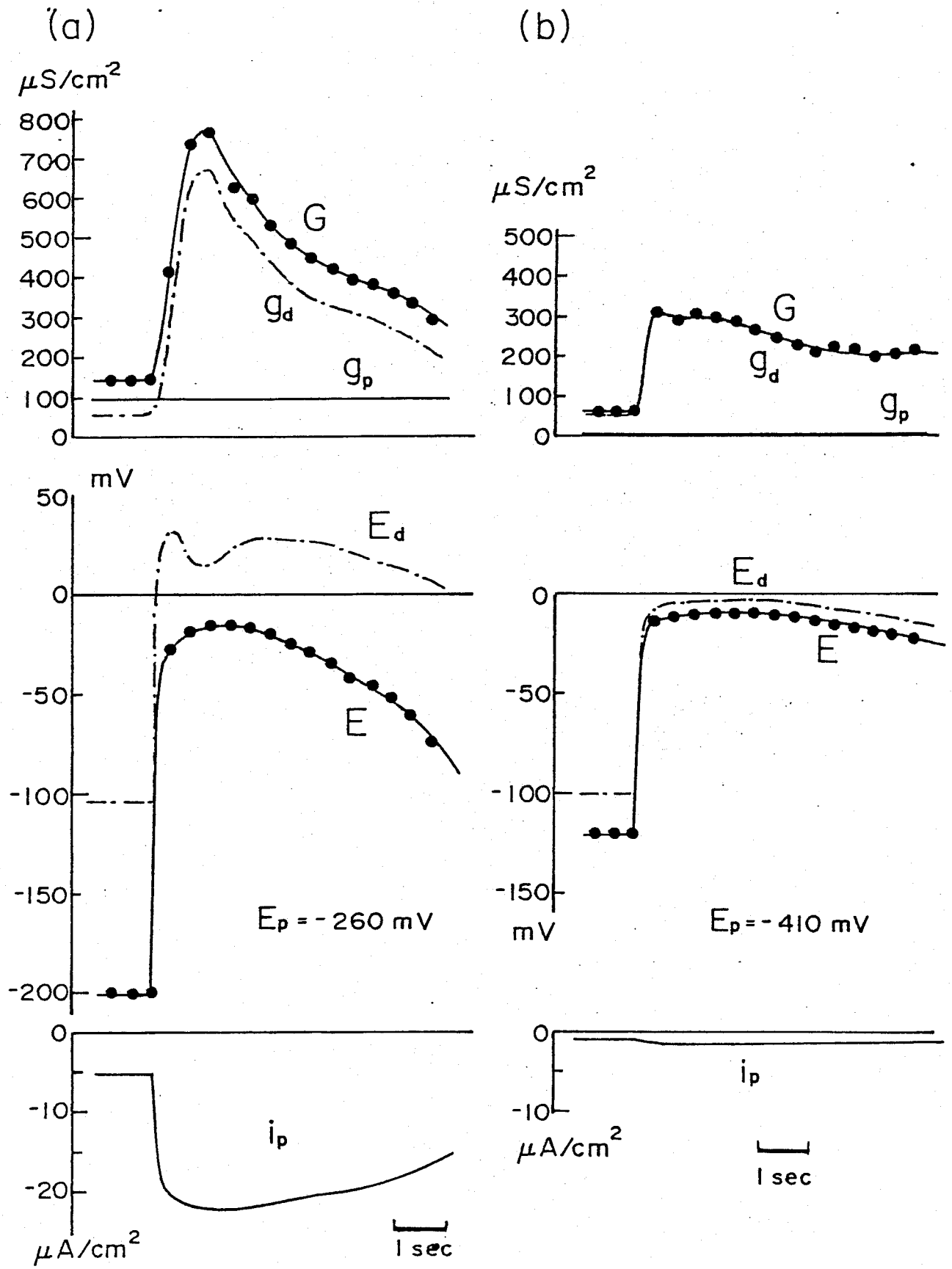


Fig. 11 Change of conductance, electromotiveforce and pump current during action potential of Chara corallina before (a) and after (b) TPC (2 μM) poisoning.
Temperature is 25°C, g_d and E_d were calculated using the g_p, E_p value in fig. 9. It is assumed that g_p, E_p were unchanged during action potential.

Chapter2

The pH dependency of the electrogenic pump

Introduction

Many experiments have been performed on the pH dependence of the membrane potential in Chara and Nitella. First, Kishimoto (1959) investigated the pH dependency on the membrane potential and reported that the membrane potential is most hyperpolarized at pH 7 and depolarized below and above pH 7. Kitasato (1968) reported that membrane in Nitella seemed to behave as a pH electrode and claimed that passive H^+ conductance was very large.

In order to explain the pH dependency of membrane potential, the following possibilities should be taken into account.

1) The change of permeabilities of other ions than H^+ may occur by the change of the external pH.
2) The change of activity of the electrogenic pump of H^+.
3) The change of other active transport system beside H^+.
4) The change of permeability of H^+ of the passive diffusion channel.
5) The change of other transport system coupled with H^+.

If I choose the fourth possibility, the membrane potential must retain the pH dependency even after the pump inhibition. But Saito and Senda (1974) showed that the dependency of membrane potential on the external pH in Nitella was decreased by various metabolic inhibitors and cooling. They proposed the existence of the electrogenic H^+-pump. Richard and Hope (1974), Keifer and Spanswick (1978) reported also the existence of the electrogenic pump. Besides, there is no report indicating that fluxes of other ion than H^+ are much reduced by the metabolic inhibition. Therefore, the second possibility that H^+ pump activity changes with the external pH

change is most likely. However, possibilities of cotransport and anti-transport with H^+ still remains.

The facts which were common in all of these reports were that the resting potential was most hyperpolarized between pH 7 and pH 8, and that the membrane potential changed with a slope ranging 20 - 50 mV/pH below pH 7 and depolarized again above pH 9. However, the data so far published on the conductance change against external pH are not equivocal. Saito and Senda (1974) reported that the conductance at pH 8 was larger than at pH 5, while Richard and Hope (1974) reported the opposite results. Keifer and Spanswick (1978) showed that in the light the membare conductance was larger at pH 8 than at pH 6, while in the dark the conductance was smaller in alkaline medium. The reason for such a qualitative difference among conductance data has not been elucidated yet. Anyway, accurate measurements both of the pump emf and the pump conductance are very important to know the pump mechanism.

In Chapter 1 the method of accurate measurement of the pump emf and the conductance by using TPC, an ideal inhibitor of H^+ pump, was described. In this chapter, pH dependency of the electrogenic H^+ pump will be analysed. The H^+ pump of plasmalemma was more clarified in tonoplast-less cell. The effect of EGTA perfusion was also described.

Result

The effects of perfusion

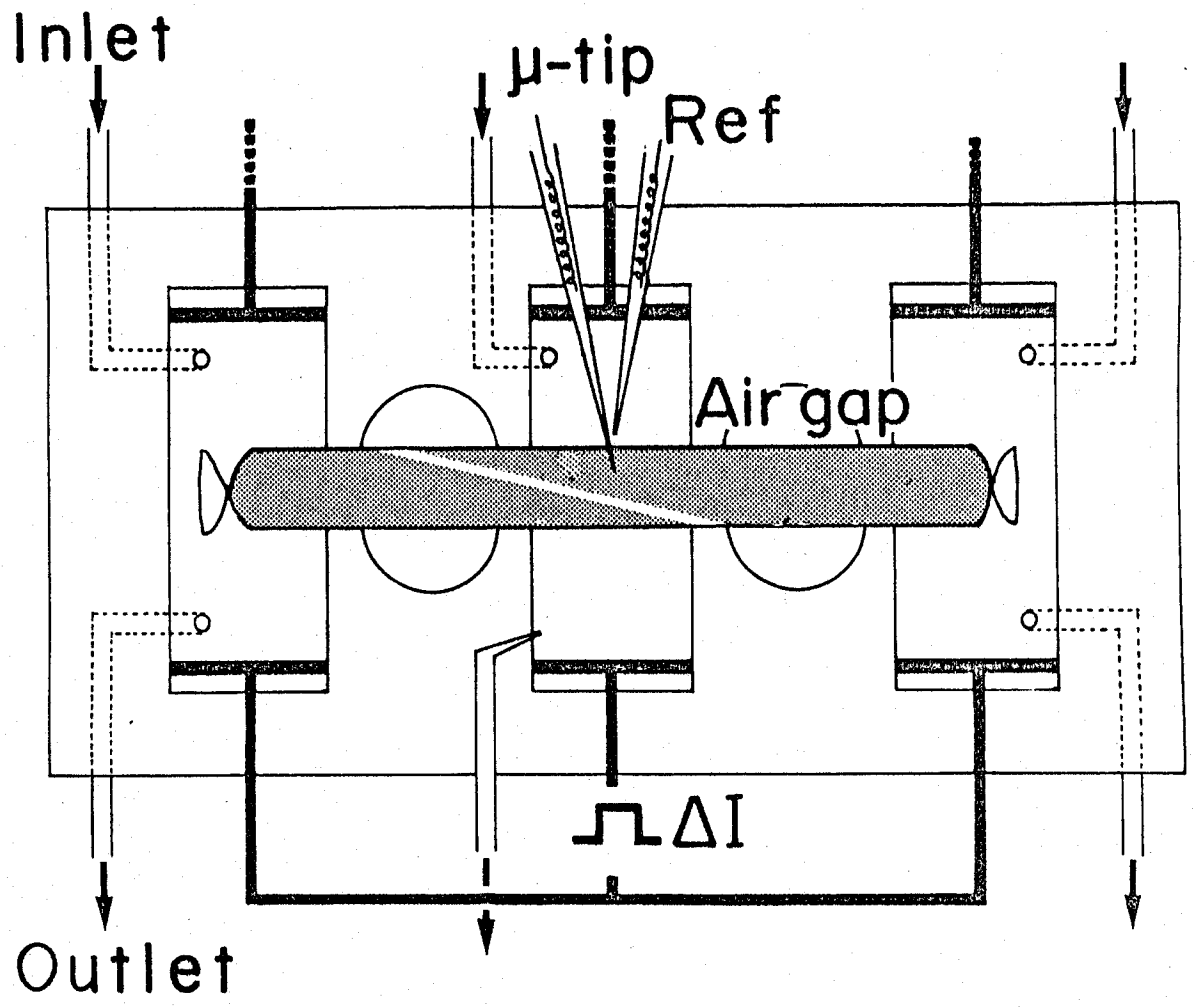
In the case of perfused cell, test current pulse was supplied from external current electrode as shown in Fig. 12. The internodal cell of Chara corallina was cooled before perfusion, then the surface water of the cell was wiped away to reduce the turgor pressure. The cell was cut at both ends and then internal medium was exchanged with the perfusion medium contains 5 mM EGTA (contain 20 mM K^+), 2 mM ATP, 66 mM KCH_3SO_3, 6 mM $MgCl_2$, 300 mM sorbitol and pH was adjusted at pH 7.02 with 5 mM TES (n-Tris (hydroxymethyl) methyl-2-aminoethanesulfonic acid) buffer.

During the internal perfusion I observed under the microscope the border of cytoplasm and vacuole was destroyed. After 5 min, both ends of the cell were tied off with a piece of polyester thread (Tetron #60). The cell was transferred first into the 150 mM sorbitol APW and then placed in the normal APW to recover its osmotic pressure.

When I perfused the Chara cell with a medium containing 2 mM ATP, I observed that normal resting potential (about -200 mV) was maintained more than 1 hour. But conductance of the perfused cell membrane had a smaller value than that of the unperfused cell. The cause of this conductance change may be in the modification of the intramembrane structure which supports the channel protein because of the low ionic strength of the perfusion medium.

The action potential in the perfused cell became to be a plateau type. The duration of the plateau ranged from 30 sec to 1 min. The duration of the action potential of the unperfused cell was several sec generally. This suggests that the low concentration of internal Ca^{++} by internal application of EGTA modified gating mechanisms of K^+ and Cl^- channels (which contribute to the action potential). However, the duration of action poten-

Fig. 12
The measuring vessel used for current clamping of internal perfused cell.



tial could be shortened by various treatments such as lowering the external pH (Fig. 13), lowing temperature and lowing osmotic pressure.

When 1 μM TPC was added to the perfused cell externally, the membrane potential depolarized from -200 mV to -130 mV and the membrane conductance decreased from 60 μS to 40 $\mu S/cm^2$ during 60 min at pH 8.0 (Fig.14). The process of inhibition was roughly the same as the unperfused cell. The change of E_p and g_p are calculated from the Eq. (5),(6) in Chapter 1. It is worth noting that in the perfused cell E_p did not show a transient hyperpolarization during the process of inhibition.

The pH dependency of the electrogenic pump

The pH dependency in the light of unperfused cell which has both plasmalemma and tonoplast is shwon in Fig. 15 (E, G). External pH was varied by using following buffers.

pH 5.5 - 7 :MES 2-(N-Morpholino)ethanesulfonic acid, monohydrate

pH 7 - 8 :TES N-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid

pH 7.5 - 9 :TRICINE N-Tris(hydroxymethyl)-methylglycine

Each buffer was used as 2 mM solved in APW. This cell had been cultured with the tap water, the pH of which was about 8.5. The resting potential was the most hyperpolarized near this pH and that the membrane conductance became gradually large as the pH became lower than 8. Under alkaline condition like pH 9, the membrane potential was depolarized and the conductance increased again. When TPC was applied to this cell in the dark at pH 8.0, the membrane potential depolarized to about -100 mV. The conductance decreased by almost 40 %. The pump activity was almost perfectly inhibited at this state. So the pH dependency of the passive channel could be obtained by changing the external pH after TPC treatment in the dark. The pH effect on the electrogenic pump can be analysed according to the method destribed in Chapter 1.

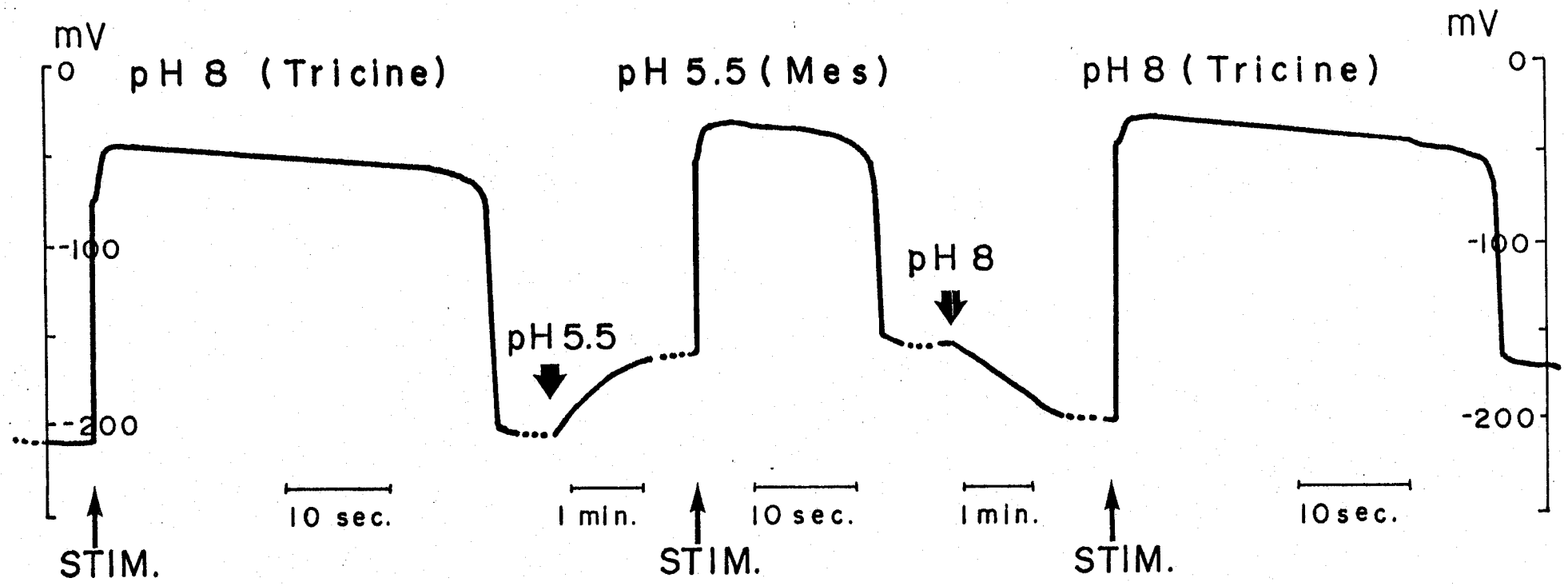


Fig. 13 The action potential of perfused cell at 10°C.
The duration of its was shortened by acid treatment.

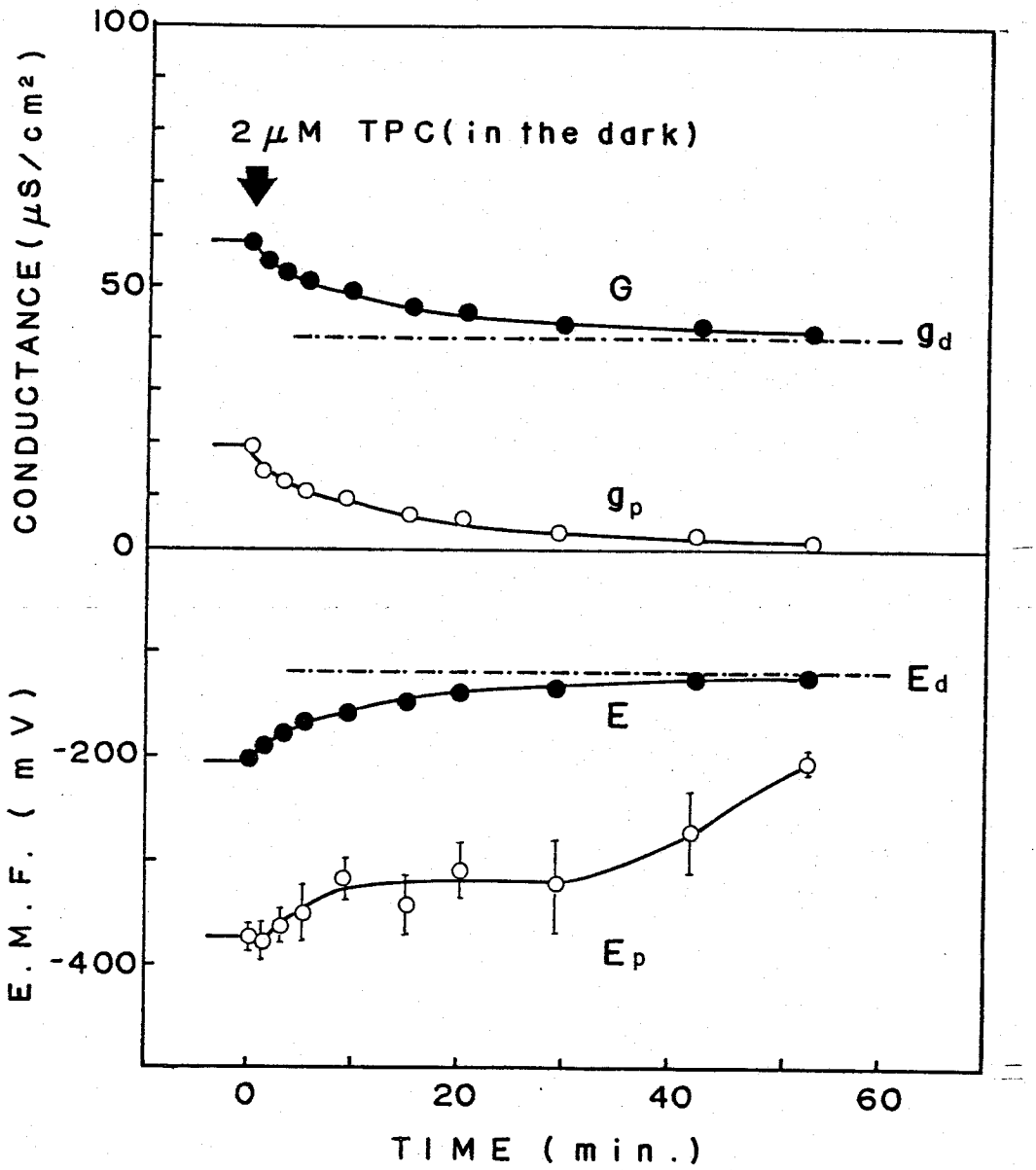


Fig. 14 Changes of conductance and emf of the plasmalemma during the process of 1 μM TPC poisoning. This cell was perfused with 5 mM EGTA. Temperature was 10°C and pH of the APW was adjusted to 8.0 with 2mM TRICINE buffer. g_d, E_d are parameters of passive channel and g_p, E_p are parameters of pump channel.

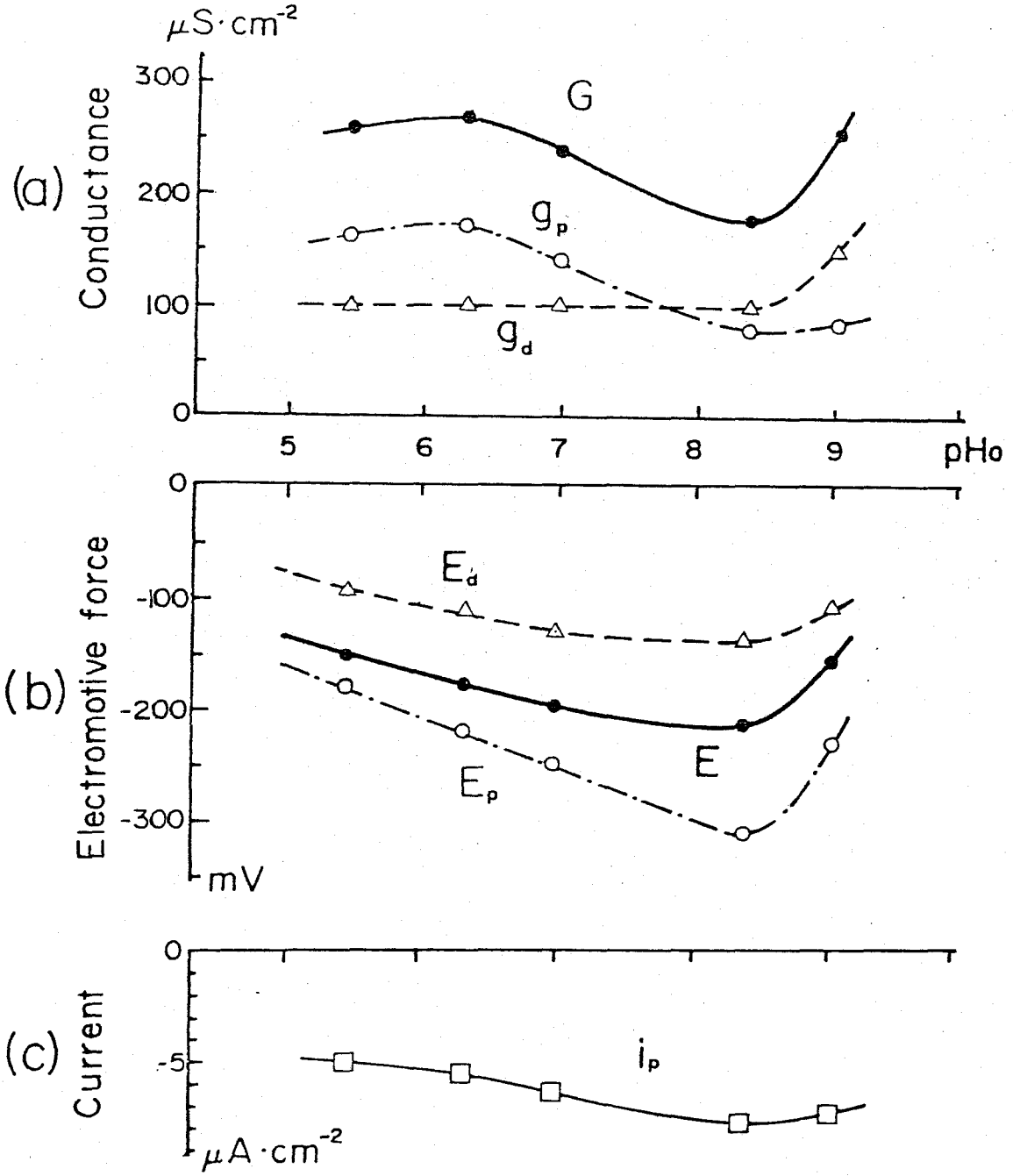


Fig. 15 Changes of conductances (a), electromotive forces (b), and pump current (c) of Chara membrane with external pH change. The pH of the external APW was adjusted with 2 mM TRICINE or 2 mM MES. The data are on a single Chara internode. That is, the first series of measurement at different pH solution was carried out without 2 μM TPC in the light. Then, the second series of measurement was carried out on the same cell after poisoning with 2 μM TPC in the dark. The values of g_d and E_d were taken from those in the second series of measurements. From these two series of measurement g_p and E_p of the electrogenic pump channel can be calculated with equations (5), (6), in chapter 1.

After TPC treatment the passive emf changed with a slope of 20 mV/pH, while the conductance changed little (Fig. 15 (g_d, E_d)). On the other hand the pump emf (E_p) changed with a slope of 52 mV/pH and the pump conductance (g_p) decreased with the external pH. (Fig. 15 (g_p, E_p)).

Fig. 16 show the pH dependency of perfused tonoplast-less cell. The membrane emf(E) changed with a slope of about 20 mV/pH and the membrane conductance(G) gradually increased in the acid medium. On the oter hand, at the late stage of TPC poisoning the membrane potential depolarized from -200 mV to -130 mV and the conductance decreased from 60 μS to 40 $\mu S/cm^2$ at pH 8.0 (Fig.14). Changes of the passive parameters (g_d, E_d) and pump parameters (g_p, E_p) of the plasmalemma against external pH are shown in Fig. 16. This result is similar to that of the unperfused cell. The parameter which changed markedly with pH was E_p. The slope of change in E_p was 60 mV/pH. E_p of the tonoplast-less cell was more negative than that of the unperfused cell. The pH effects on the conductances of passive channel and active pump were very similar to those of unperfused cell. The marked difference is that the slope of passive emf is 20 mV/pH in the unperfused cell and 6 mV/pH in the perfused cell. These results indicate that the passive channel of plasmalemma was affected by perfusion to some extent.

Another merit of perfusion method is to regulate the internal concentration of ions and ATP. Kikuyama et al. (1979) reported that the cellular ATP concentration decreased to below 1 μM by perfusing with 1 mg/ml hexokinase and 5 mM glucose. I reinvestigated the pH sensitivity of such a cell. As shown in Fig. 17 the emf and the conductance of hexokinase treated cell, which had no ATP supply to the pump, is similar to that of TPC treated cell in Fig. 16 (E_d, g_d). This result also supports our assumption that TPC is an ideal inhibitor of the electrogenic pump of the Chara plasmalemma.

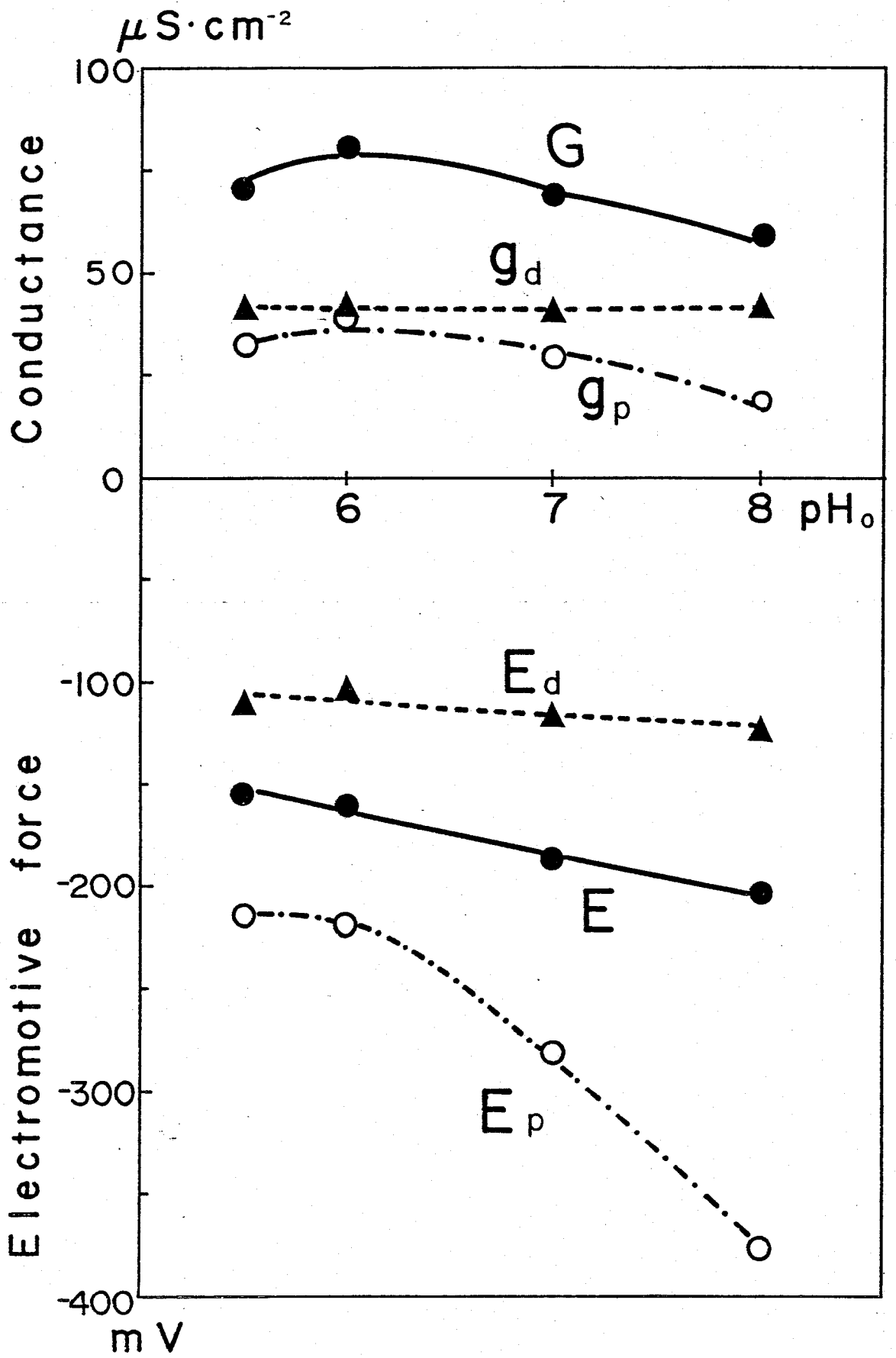


Fig. 16 pH dependency of plasmalemma.
g_d, E_d are the conductance and emf of passive channel, and
g_p, E_p are the pump factor. Other situations are same as Fig.12

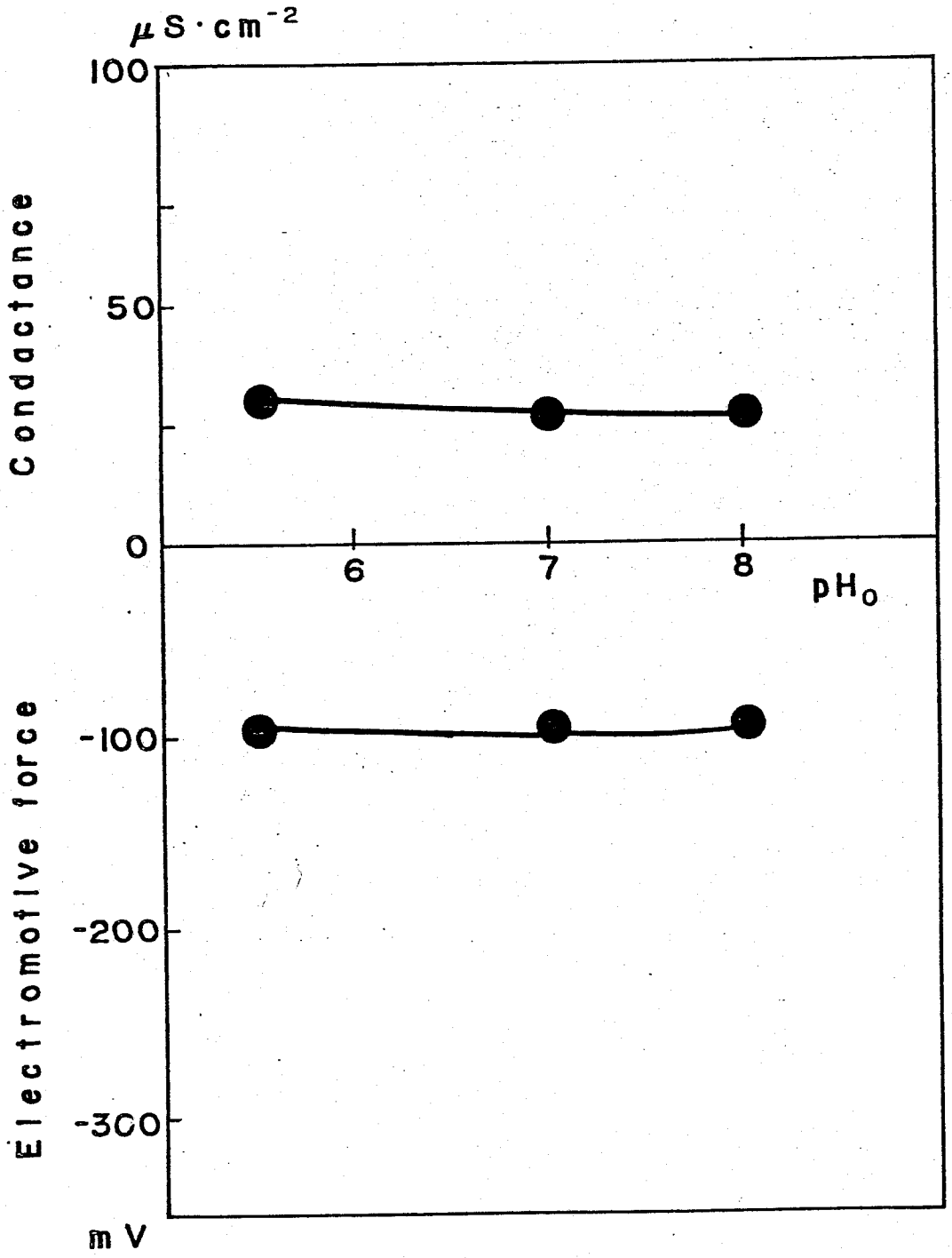


Fig. 17 The pH dependency of the conductance and emf of the plasmalemma of the Chara, the vacuole of which was perfused with 1 mg/ml hexokinase and 5 mM glucose added to the normal perfusion medium.

Discussion

The pump emf (E_p) changed with a slope of 52 mV/pH at 20°C in the unperfused cell and 60 mV/pH at 10°C in the perfused cell. E_p was between -200 mV to -300 mV at pH 7. This value of E_p is far from the electrochemical potential of H^+.

Mitchell(1967) described the mechanism of the H^+-pump of mitochondria. If the similar H^+-ATPase exsists in the plasmalemma of the Chara, the free energy of ATP hydrolysis can be transferred to the electrochemical potential of H^+. This idea was applied to the H^+- pump of Chara corallina. At the thermodynamic equilibrium E_p can be expressed as follows. If m mol of H^+ is driven by the hydrolysis of 1 mol ATP.

$$E_P = 1/(mF) \quad G_{ATP} + (RT/F) \ln ([H^+]_o/[H^+]_i)$$

I choose the concentrations of ATP was 1 mM, ADP 0.5 mM and Pi 0.5 mM (The bases of this assumption was described in the next chapter). Then,

$$G_{ATP} = G_oATP - (RT/F) \ln [ATP] / [ADP][Pi] \fallingdotseq 10 \text{ Kcal/mol}$$

Accordingly E_p at 20°C is,

$$E_p = 1/m \quad (-525) - 58 \log [H^+]_o / [H^+]_i \quad (mV) \qquad (1)$$

If m=2 in equation (1), E_p was about -250 mV when the external pH was at 7.0. The pH dependency of E_p of the plasmalemma of the perfused cell was almost equal to -58 mV. This indicates that m is about 2 in the plasmalemma of the Chara corallina. That is, this electrogenic pump carries out 2 mol H^+ following the hydrolysis of 1 mol ATP.

The problem is why E_p was hyperpolarized by TPC in the dark. Generally the pump emf is expected to decrease during the pump inhibition because of the decrease of ATP concentration. When E_p is hyperpolarized, it might be caused either by the decrease of stoichiometry ratio of H^+ with ATP or by the increase of internal H^+ concentration (Eq.1). The latter possibility is scarce, since the internal H^+ concentration need to increase more than

100 times. If the efficiency of driving 2 H^+ by 1 mol ATP decreased to 1 H^+ by 1 mol ATP, E_p would hyperpolarize from -250 mV to -500 mV.

In this experiment the conductance of the passive channel (g_d) changed little by external pH. This fact is inconsistant with the Kitasato's (1968) assumption that the conductance of passive H^+ channel was very large. Passive emf (E_d) changed 20 mV/pH in the case of unperfused cell. I assume here that ions which contribute to the diffusion potential are K^+, Cl^-, and H^+. Each equilibrium potential are calculated by Nernst equation and are shown in Fig. 18. The equilibrium potential of K^+ and Cl^- are -190 mV and +150 mV respectively. Diffusion potential of H^+ is 0 mV at pH 7.0 and should change with a slope of 58 mV/pH. Since each ion channel distributes in parallel in the membrane, the experimental data should be simulated using these values. As shown in Fig.18, $g_K > g_{Cl} > g_H$, and g_H is small so long as the external pH was smaller than 8.3. It is worth noting that g_{Cl} decreases with the increase of the external pH_o, while g_k increases. As the external solution became more alkaline than pH8, g_K and g_{Cl} both increased greatly (Fig. 18). This result seems interesting, since the Chara membrane often shows an action potential when the external pH was increased from 7 to 9-10. In this case E_d also depolarized by the alkaline treatment and did not recover to the hyperpolarized state. This suggests some qualitative changes in the plasmalemma might occur at pH_o larger than 8.0.

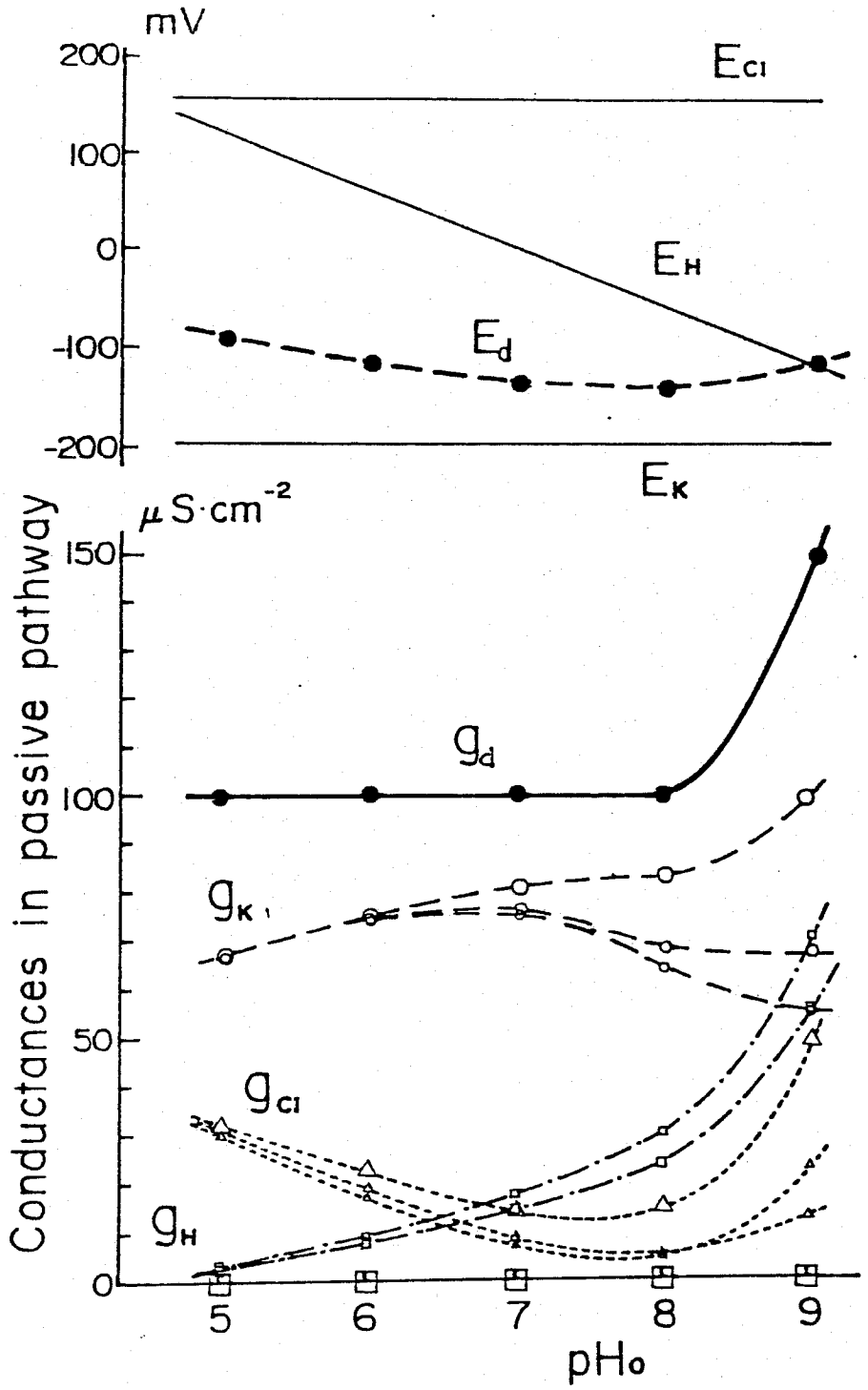


Fig. 18 Slelectivity change of the passive diffusion channel of the Chara membrane for K^+ and Cl^- and/or H^+ against external pH change. The largest symbols (o, △ , and □) are conductances for K^+, Cl^-, and H^+, respectively, decided by simulation of changes of E_d and g_d under an assumption of no H^+ flow through the passive diffusion channel. The smallest symbols are conductances determined similarly under an assumption that all the pump H^+ current flowed back into the cell through the passive diffusion H^+ channel. The middle-sized symbols are conductances determined similarly under an assumption that 4/5 of the pump H^+ current flowed back through the passive H^+ channel. Actual situation may be between the two extreme cases. Anyway, the selectivity of the Chara membrane tended to be lost at pH above 8.3.

Chapter3

Internal ATP level and electrogenic pump in Chara corallina

Introduction

It is very important to know the relationship between the activity of electrogenic pump and the internal ATP concentration. Slayman et al. reported a close correlation of the extent of depolarization with decrease of internal ATP level during CN^- treatment in Neurospora hyphae (1973). They analysed membrane potential and ATP content using a first order Michaelis-Menten kinetics on the base of current source model and found that apparent K_m value was 2 mM.

Similar correlation was found also in red beet K^+-ATPase by Mercier & Poole (1980). In this case the internal ATP level at which the membrane potential was much depolarized was 0.4 mM. Shimmen & Tazawa (1977) reported that in Chara australis, Nitella axillaris and Nitella pulchella, the pump activity reached saturation at the internal ATP larger than about 50 μM. According to Keifer & Spanswick (1979) 5 μM CCCP depolarized the membrane potential of the Chara corallina to the diffusion potential and decreased ATP concentration down to 0.13 mM.

To know the relation between the pump activity and ATP concentration, it is not necessarily a good way to compare ATP concentration simply with the membrane potential. We know that the E_p sometimes showed a transient hyperpolarization during pump inhibition with TPC poisoning. So in this Chapter, I try to compare the change both of E_p and g_p with ATP level.

Smith & Walker (1981) described that 2mM ADP added to 2mM ATP inhibited 50 % of the pump activity of perfused internode of Chara corallina and decreased g_m from 33 to 24 μS cm^{-2} and decreased pH sensitivity of the

membrane potential. Tazawa et al. (1982) reported that K_m value, which was obtained by the plot of membrane potential against the $[ATP]_i$ with perfusion method, increased from 0.08 mM to 0.25 mM by addition of 1 mM ADP. These results suggest a possibility of direct inhibitory effect of ADP. In this Chapter changes of concentrations of ATP, ADP and AMP are compared with the change of pump activity during inhibition.

Material & method

Chara corallina was cultured in our laboratory with a photoperiod of 12 hr. light (Ca. 2000 lx) and 12 hr. dark. The internodes which were 0.7 mm in diameter and 6 cm in length on the average were isolated from adjacent cells and kept in the APW for at least one day in a natural sun light condition beside the window.

The diameter and length of each sample were measured to calculate the cell volume , then put into a test tube. These test tubes contained 3 ml APW with or without inhibitor. Inhibitors are 2 μM TPC, 0.1 mM monoidoacetic acid (MIA) , 0.2 mM 2-4-dinitrophenol (DNP), 50 μM dicyclohexylcarbodiimide (DCCD) or 1 mM sodium azide. APW was the same as described in Chapter 1. These test tubes with samples were incubated at 20°C and was taken out of a water bath at desired time, then boiled for 10 min at 100°C to stop the ATPase activity in the sample, and then frozen quickly. They were stored in a deep-freezer before use.

ATP concentration in the sample was determined with a luciferin-luciferase assay (Strehler, 1952; Miyamoto et al., 1976). One vial of the buffered firefly lantern extract FLE-50 (Sigma, Chem. Co) was dissolved in 5-10 ml distilled water and to which about 1 g calcium phosphate tribasic was added. After 10 min incubation at room temperature, they were centrifuged to remove the ATP contamination in FLE-50. Then the supernatant was used as the assay medium for ATP measurement. Luminescence reaction was performed with a mixture of equal volumes (0.4 ml) of four reagents. They are 0.2 M sodium arsenate (pH 8.0), supernatant of FLE -50, 0.08 M $MgSO_4$ and sample or standard ATP solution. This luminescence was detected by a photomultiplier. Calibration curve for the ATP luminescence was linear for ATP concentration from 10^{-8} to 10^{-5} M. The ATP concentration per cell was calculated under the assumption that the volume of cyto-

plasm was one tenth , on the average, of the total cell volume (Tazawa, Kishimoto & Kikuyama, 1974)

ADP concentration was measured according to the method of Kimmich et al. (1975) in which ADP was converted to ATP enzymatically. In this method phosphoenolpyruvate was used as substrate and pyruvate kinase converted ADP to ATP by about 95%. 0.2 ml assay medium was added to 2 ml of the sample or standerd ADP solution and incubated for about 40 min at room temperature. Then this solution was boiled at 100°C for 10 min and frozen quickly to stop the reaction. Assay medium contained 50 mM phophoenolpyruvate, 35 mM KNO_3, 6 mM $MgSO_4$ and 150 International Unit(IU)/ml pyruvate kinase (Behringer-Mannheim). The pH of the assay medium was buffered at 7.4 with 100 mM TES.

AMP was also measured by the enzymatic method. ATP was used as the substrate and myokinase which convert the AMP to ADP was added, and ADP was then converted to ATP by the ADP assay method . AMP assay medium contains 0.02 μM ATP and 100 IU/ml myokinase(Sigma,Chem.Co.) added to ADP assay medium. The concentration of ATP which was converted from AMP or ADP was measured with a luciferin-luciferase assay as described above.

Result

In Chara corallina it has been reported that the concentration of ATP did not change much by dark treatment in spite of the existence of chloroplast and photosystem. The change of internal ATP value after dark was variable depending on the condition of pretreatment with light or dark. First, I measured the change of ATP level during long dark condition. As shown in Fig.19 the ATP concentration was decreased to half of the control level after 4 days in the constant dark. When such a long dark adapted cell in which ATP concentration decreased to half level was placed in the light condition, the ATP level recovered to near the control level after two hours. Then this cell was placed in the dark again. As shown in Fig.20 the ATP concentration of dark adapted cell which had been placed in the dark for 3 days and then placed in the light for 2 hour decreased in 1 hour dark. On the other hand, the ATP concentration of the cell of normal photoperiodism (12 hr. light and 12 hr. dark) did not change appreciably or increased a little during 1 hour dark. Later experiments were performed on the cell under the normal photoperiodism.

In Chapter 1, I showed that TPC is an ideal inhibitor of electrogenic pump. I measured the change of internal concentration of ATP during TPC poisoning in the dark. As shown in Fig.21, ATP level gradually decreased to one third of the control. If TPC was treated in the light , ATP level reduced to 0.6 mM. These decline of ATP level seemes to have a good correlation with the results of electrophysiological measurement in membrane potential. That is, TPC in the dark abolished the pump activity almost completely in an hour, while it affected much more slowly in the light.

The effect of 0.2 mM DNP, which is known as an uncoupler, on the ATP

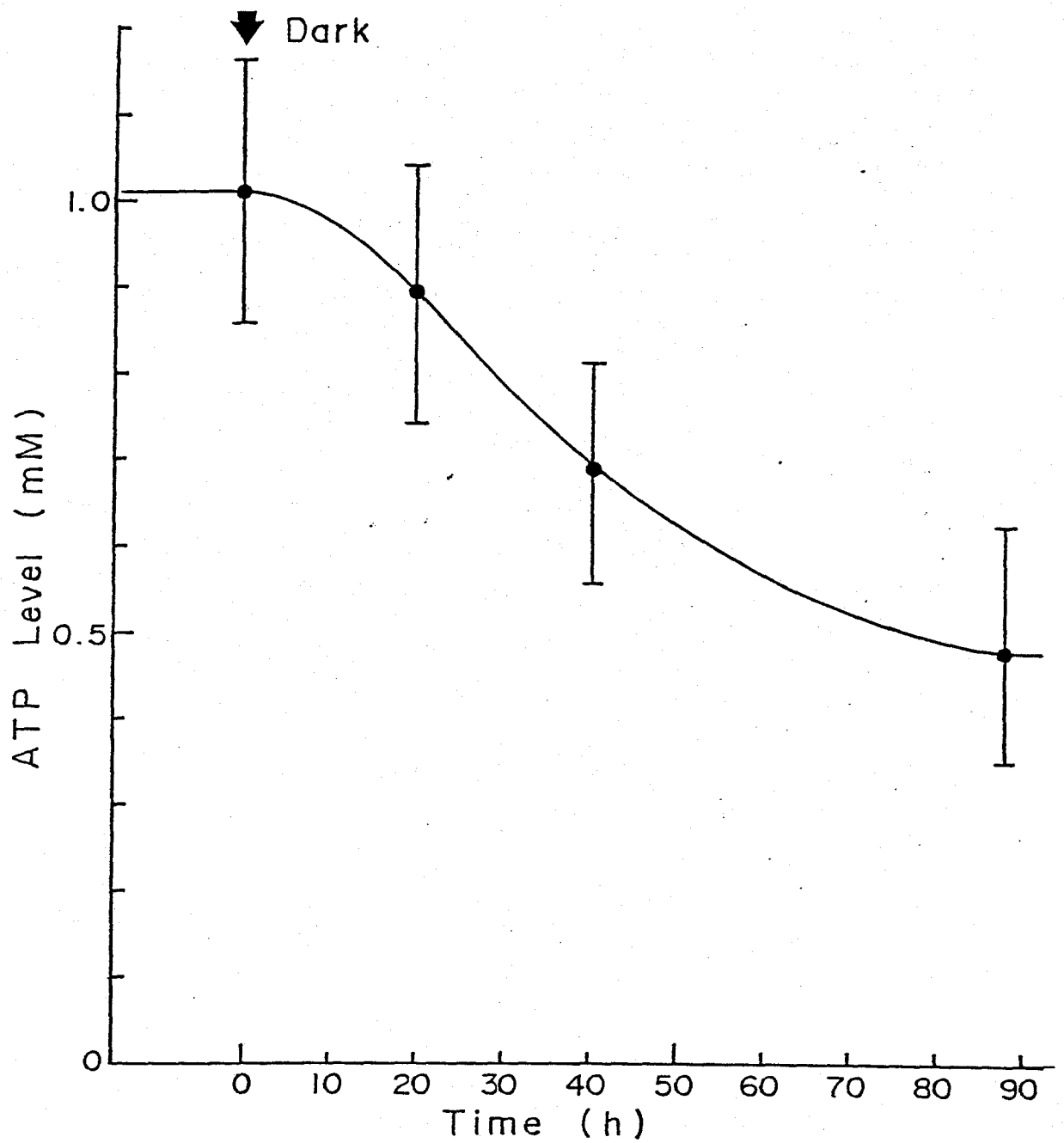


Fig. 19 The ATP level decreased to less than half of the original level, if the Chara internode was kept in the dark for 4 days or longer. Temperature, 20°C.

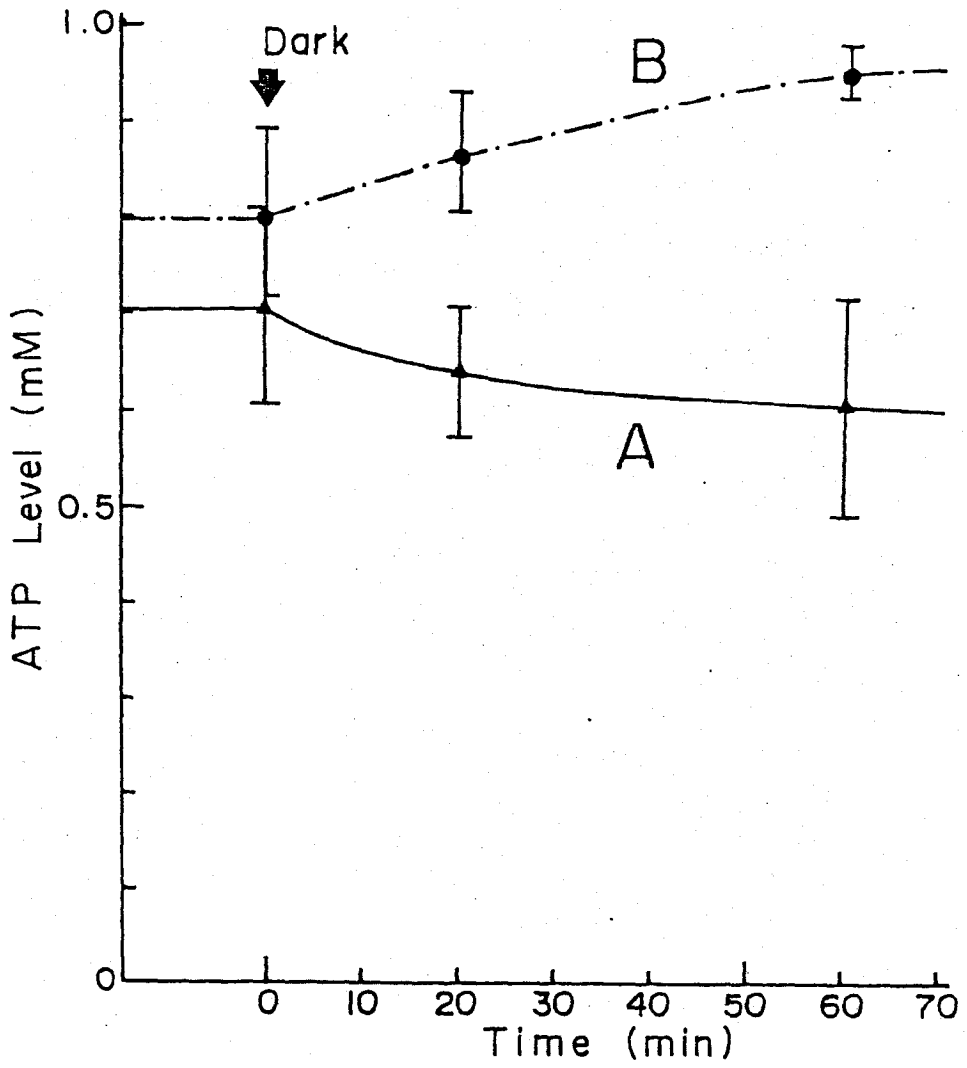
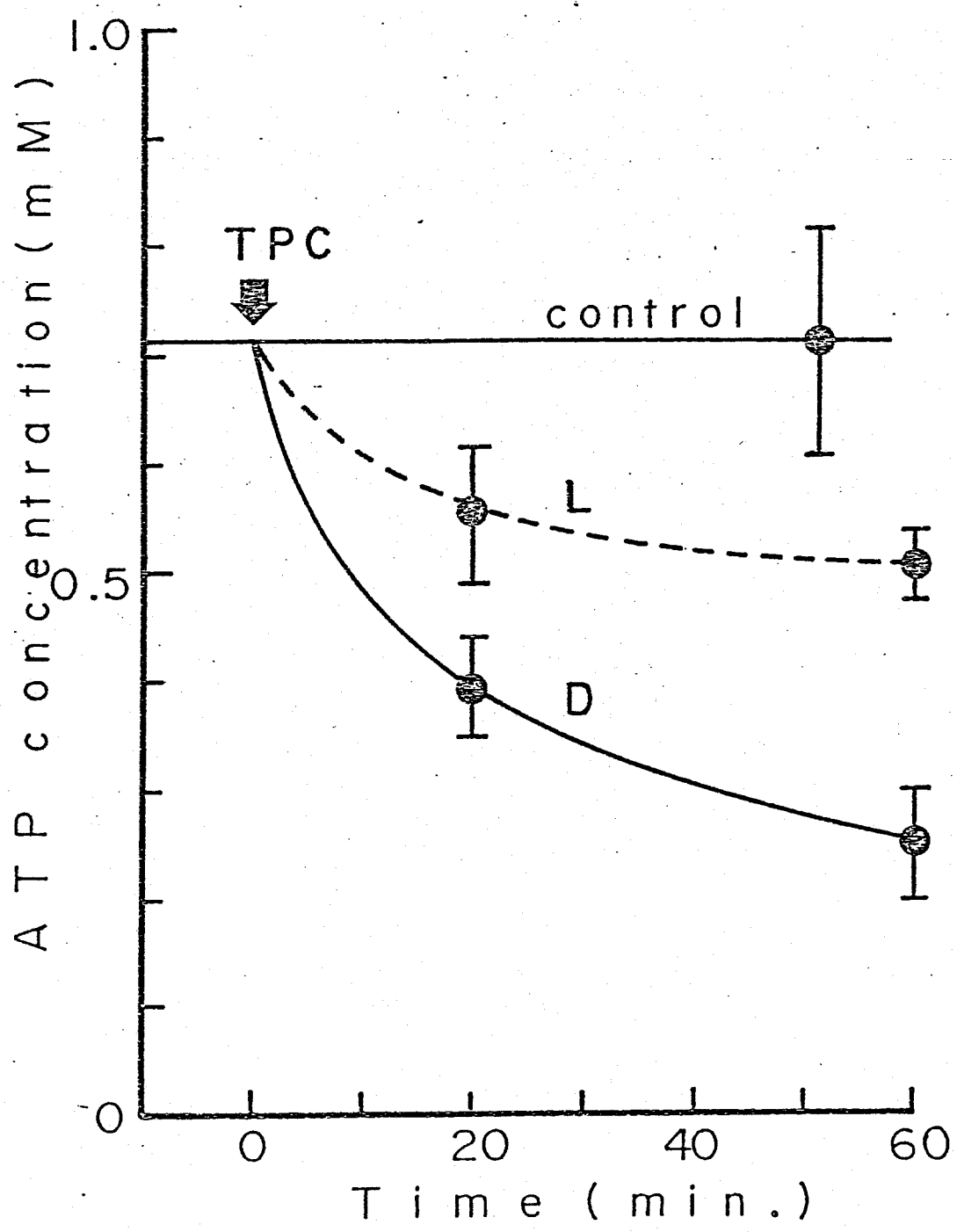


Fig. 20 Changes of ATP level of the Chara internode is not markedly large for the first 1 h in the dark. The ATP level decreased to some extent for about 1 h in the dark (Curve A). This example is on the internode which had been kept in the dark for a few days and then illuminated for 3 h before the experiment. On the other hand, if the internode had been in a normal 12 h - 12 h photoperiod, the ATP level showed either no appreciable change or a slight increase (Curve B). Temperature, 20°C.

Fig. 21 2μM TPC reduced the ATP level of the chara internode to a level which was lower in the dark than in the light. Temperature, 20°C.



level was shown in Fig.22. DNP also reduced the ATP level to half of the control.

The ATP level was not zero at all after the pump activity was abolished, but it remained at about 0.3 mM as shown in Fig.21. When I applied 1 mM MIA , which is known as an inhibitor of glycolysis, ATP concentration was reduced to 60 % of the control level. When both MIA and TPC were applied simultanously, ATP level decreased to 10-20 % of the control level (Fig.23). These results show that ATP was synthesized both by glycolysis and by electron transfer system. When TPC and MIA were applied at the same time, the velocity of cytoplasmic streaming was reduced within 30 min.

If the cell was illuminated (2000 lx) after TPC treatment in the dark, the ATP level recovered to the level of TPC in the light (Fig.24). I found that the depolarized cell with TPC in the dark hyperpolarized again by illumination, even if the APW contained TPC. This agrees with the present results of the change of ATP level.

As shown in Fig.25, the decreasing process of ATP level during TPC poisoning is similar to the decrease of pump conductance. But it does not agree with the change of pump emf, which hyperpolarized transiently with TPC in the dark.

I also investigated the effects of DCCD which is known as an inhibitor of mitochondrial H^+-ATPase. DCCD decreased the internal ATP level more slowly than TPC or DNP. As shown in Fig.26, ATP level decreased to half of the control level after 90 min with 50 μM DCCD. The progress of decrease of ATP level was practically the same in the dark as in the light. Effects of 50 μM DCCD progressed slowly also in the electrophysiological measurement. Time courses of slow decreases of pump conductance, pump emf and the pump current are very similar to that of ATP level.

I measured the changes of other nucleotides than ATP during inhibition. In the control state ATP, ADP, AMP levels in the Chara are about 0.75 mM ±

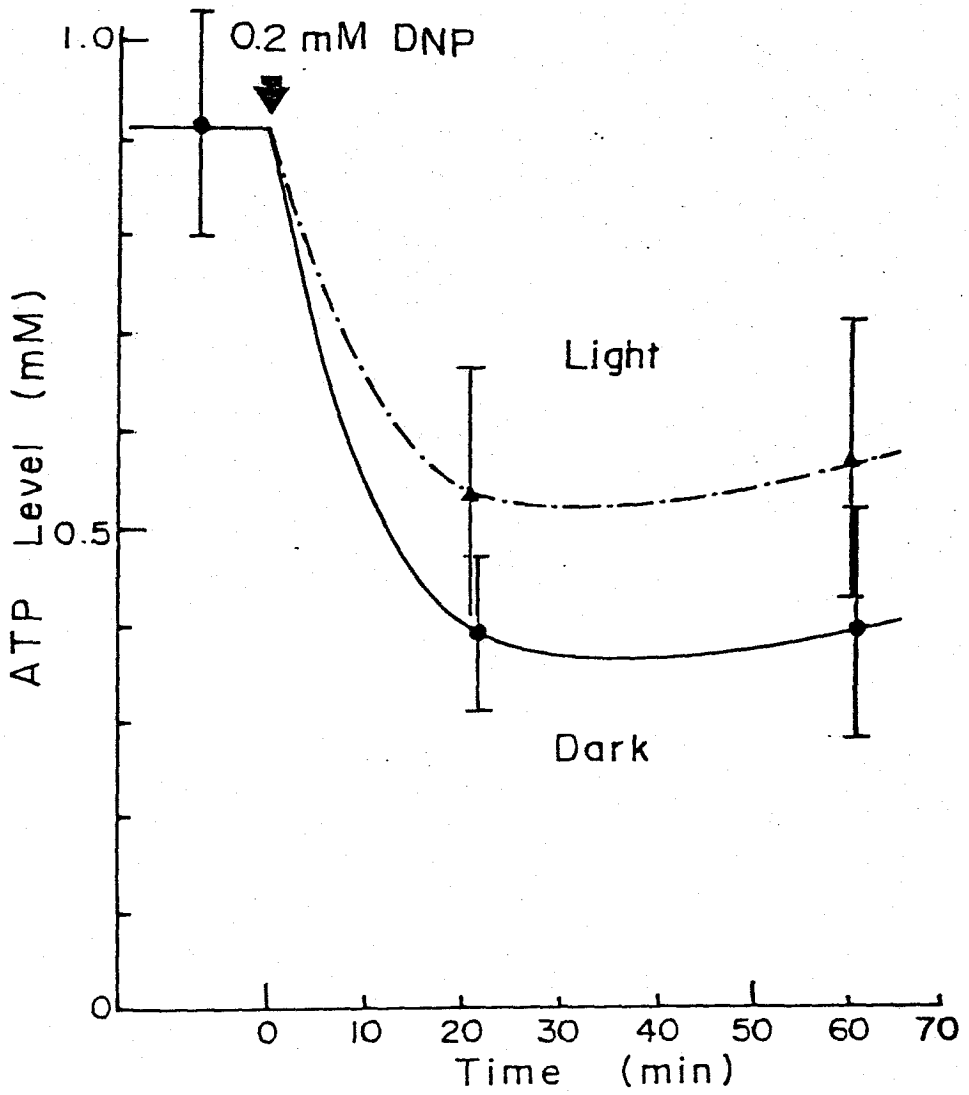


Fig. 22 0.2 mM DNP reduced the ATP level of the Chara internode to a level which was lower in the dark than in the light. Temperature, 20°C.

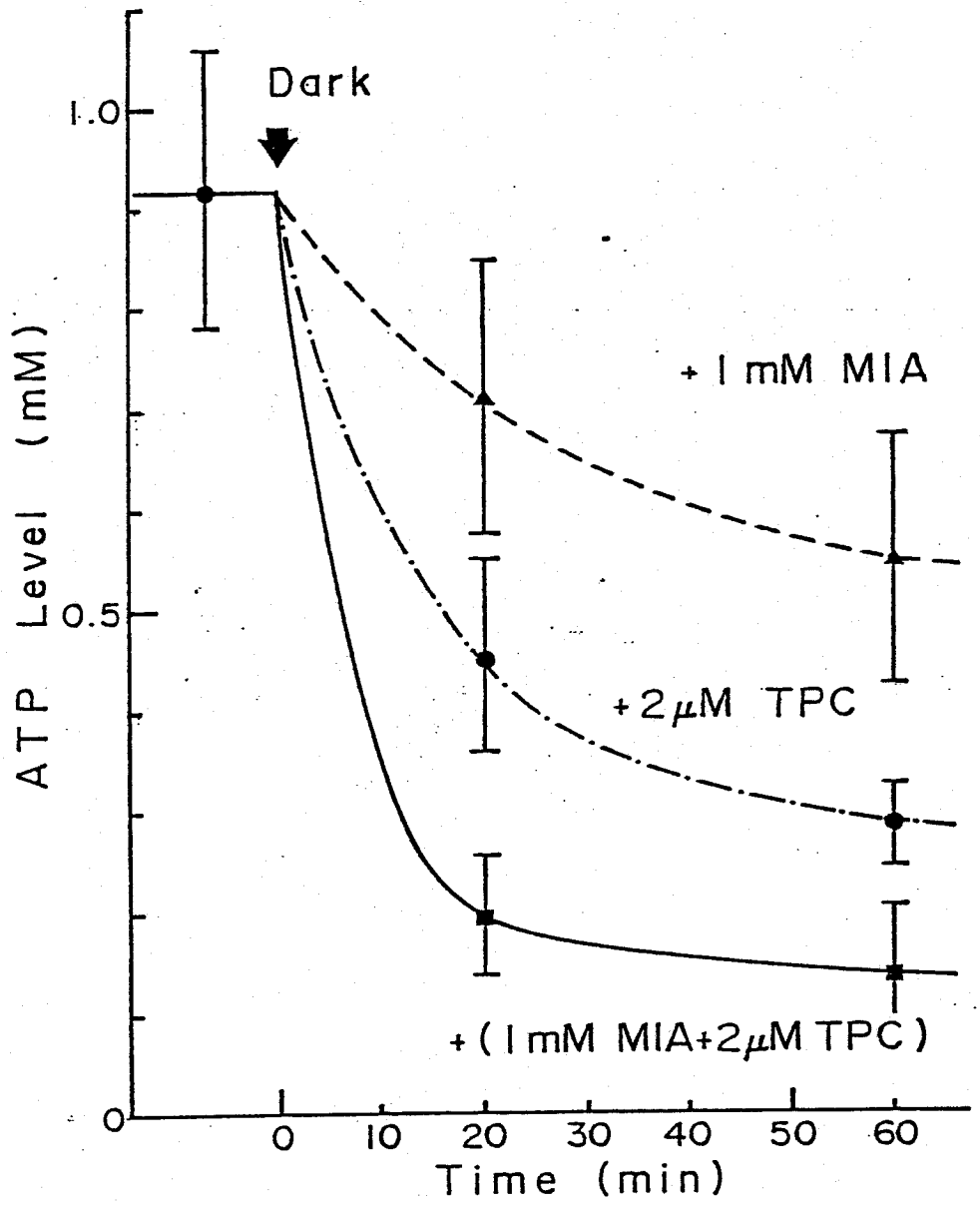


Fig. 2 3 The reduction of ATP level in the dark of the Chara internode was additive by inhibitors of glycolysis (1 mM MIA) and of energy transduction at the F_1 particles in the mitochondrial inner membrane (2 M TPC). Temperature 20°C.

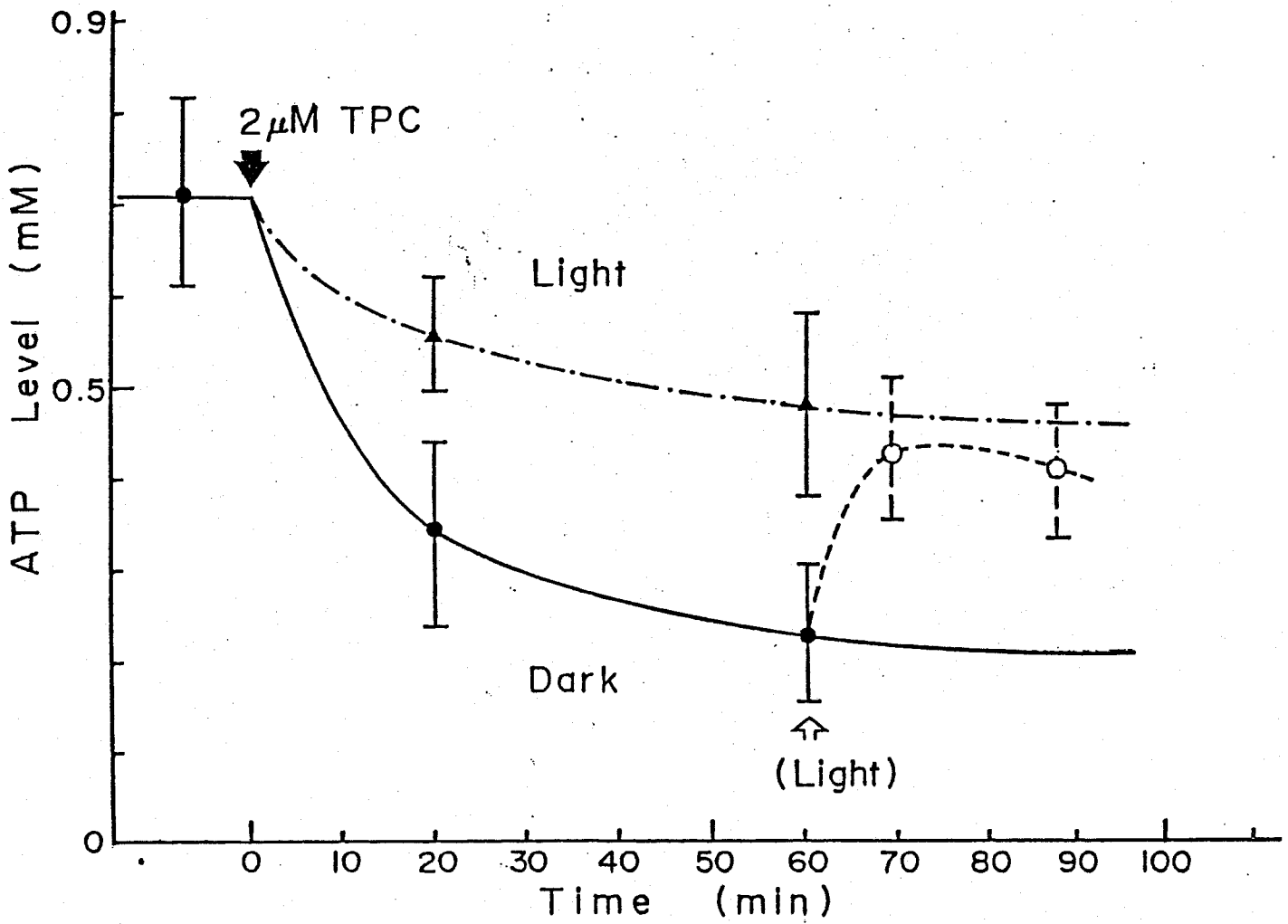
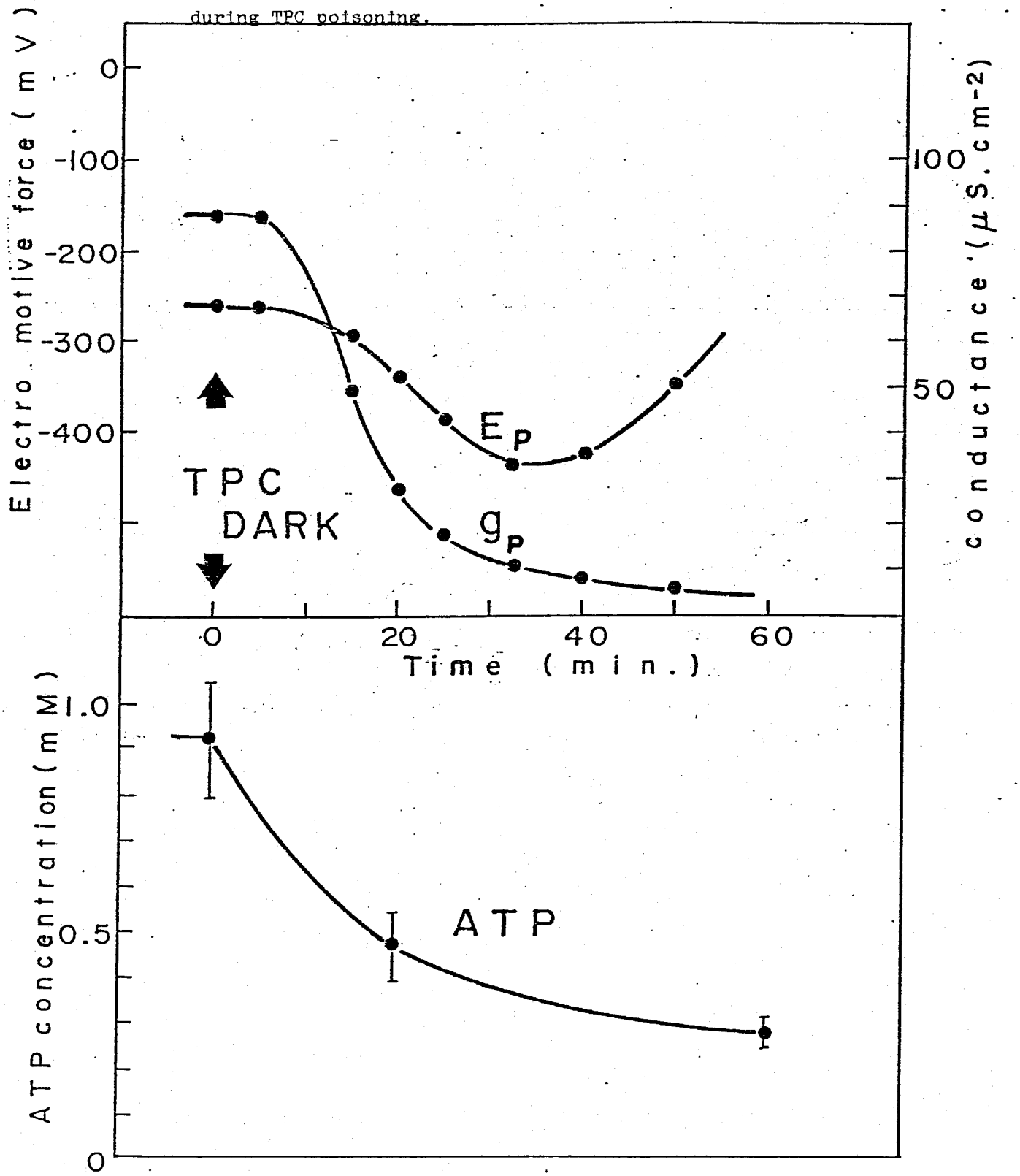


Fig. 24 2 μM TPC reduced the ATP level of the Chara internode to a level which was lower in the dark than in the light. The ATP level which was reduced by 2μM TPC in the dark recovered by illumination. Temperature, 20°C.

Fig. 25 The correlation of the changes of pump parameter and internal ATP level during TPC poisoning.



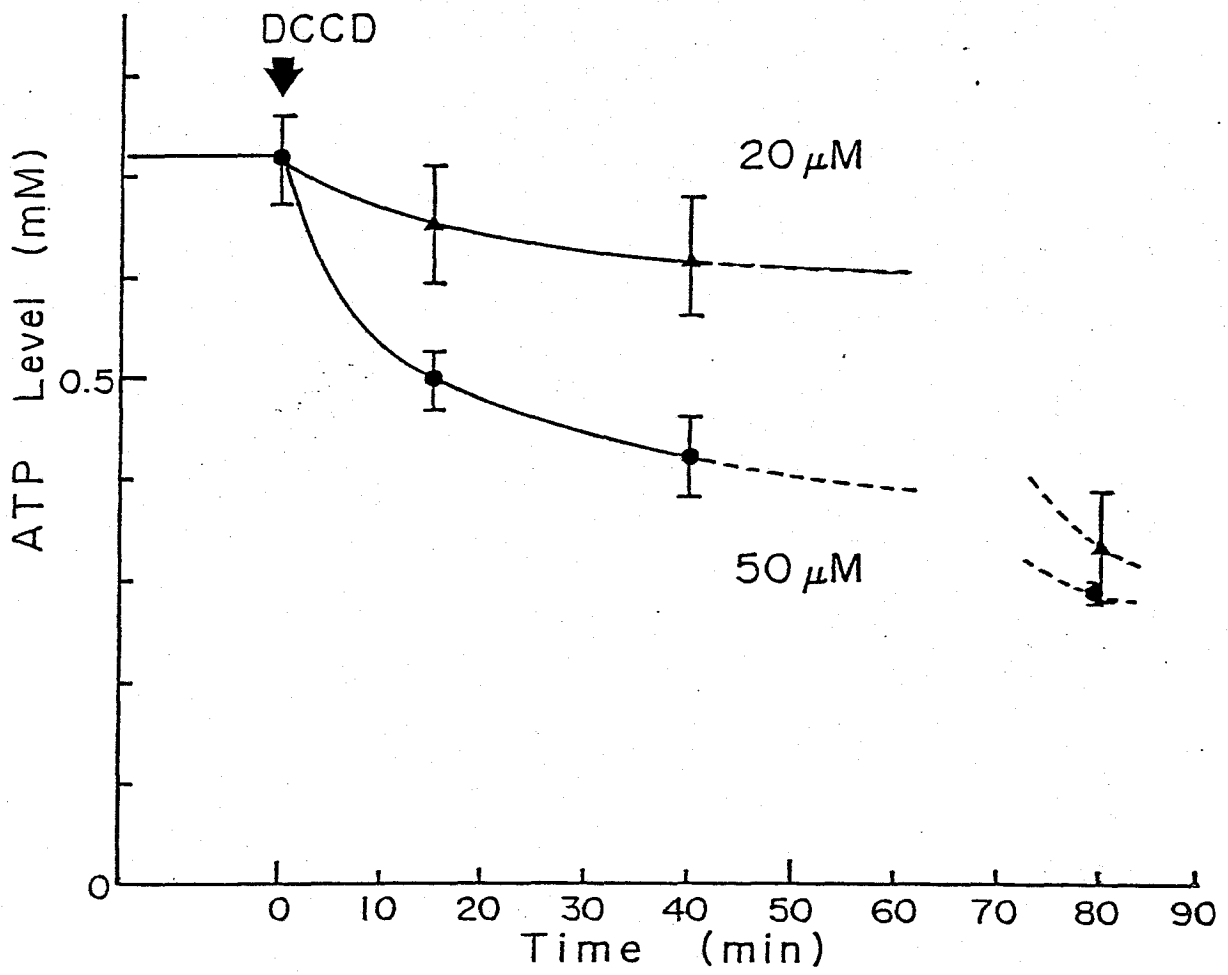


Fig. 26 DCCD reduced the ATP level of the Chara internode almost exponentially for the first one hour. About 80 min after application of DCCD, the ATP level generally decreased to lower levels. The progress of decrease of ATP level was practically the same in the dark as in the light. Temperature, 20°C.

0.07 mM, 0.35 mM ± 0.03 mM, 0.27 mM ± 0.05 mM respectively. When the ATP concentration gradually decreased to one thrid of the control level with 1 μM TPC in the dark , the ADP level increased transiently twofold during first 10 min, then decreased to the control level in 60 min (Fig.27). AMP level increased gradually during 30 min and decreased almost to the control level again. When 1 μM TPC was added to the cell in the light, the ATP decay progressed more slowly, and the change of ADP and AMP also progressed more slowly (Fig.28). For example ADP level increased twofold during 30 min, then decreased again, while AMP gradually increased during 60 min.

Treatment with 1 mM NaCN, which is an inhibitor of electron transfer, in the dark decreased the ATP level transiently and then recovered to the control level again. The ADP level also changed transiently as shwon in Fig.29. The changes of these nucleotide levels during 1 mM NaCN treatment progressed slower in the light (Fig. 30) than in the dark (Fig. 29).

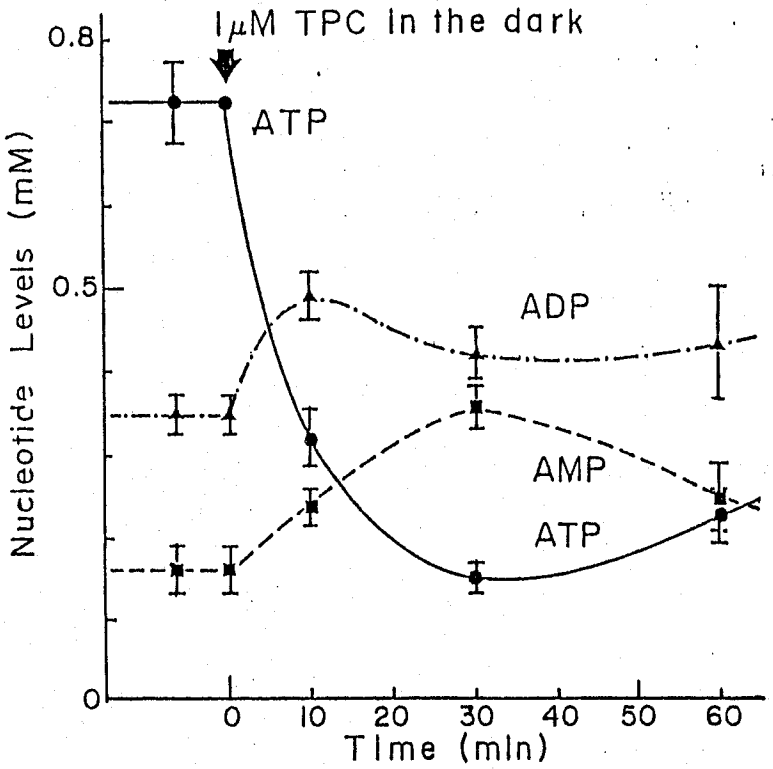


Fig.27 Changes of ATP, ADP and AMP levels of the Chara internode during inhibition by 1 μM TPC in the dark.

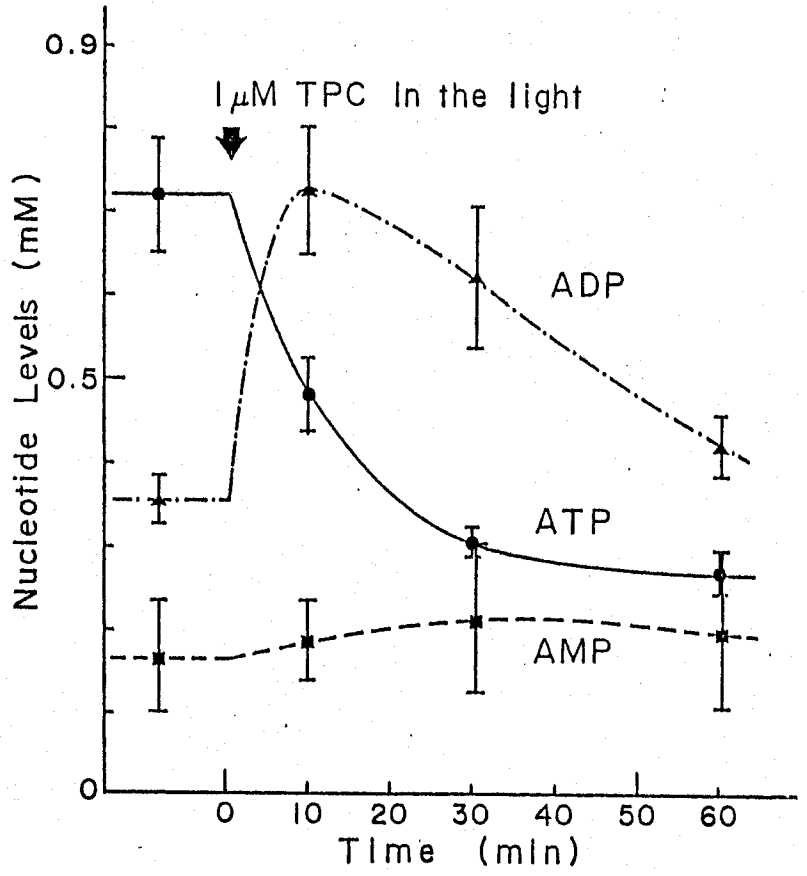


Fig. 28 Changes of ATP, ADP and AMP levels of the Chara internode during inhibition by 1 μM TPC in the light.

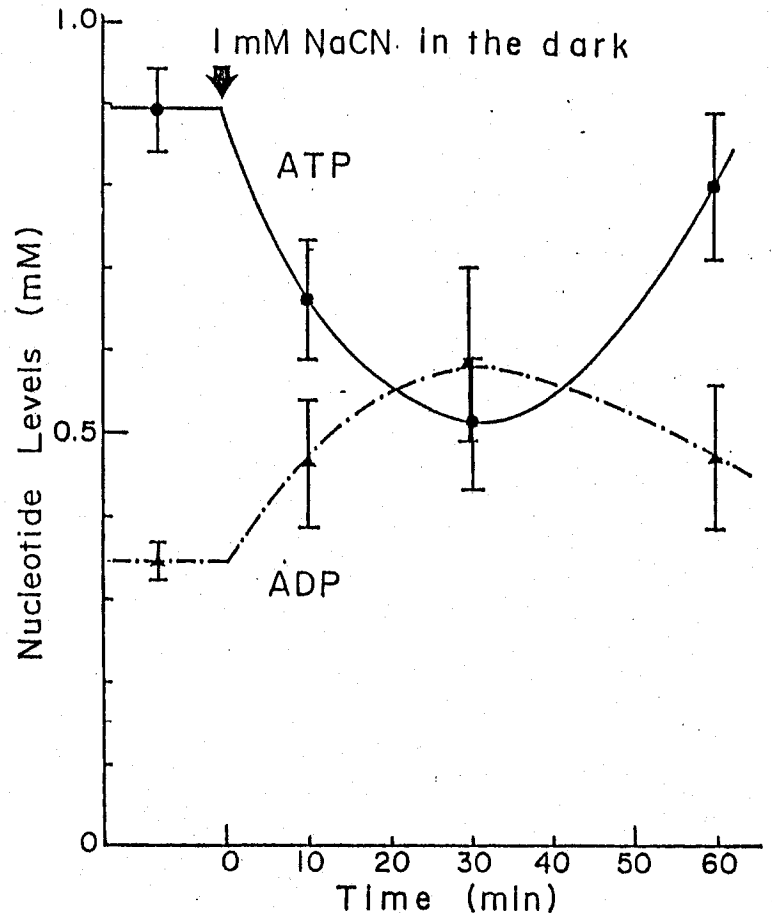


Fig. 29 Changes of ATP and ADP levels of the Chara internode during inhibition by 1 mM NaCN in the dark.

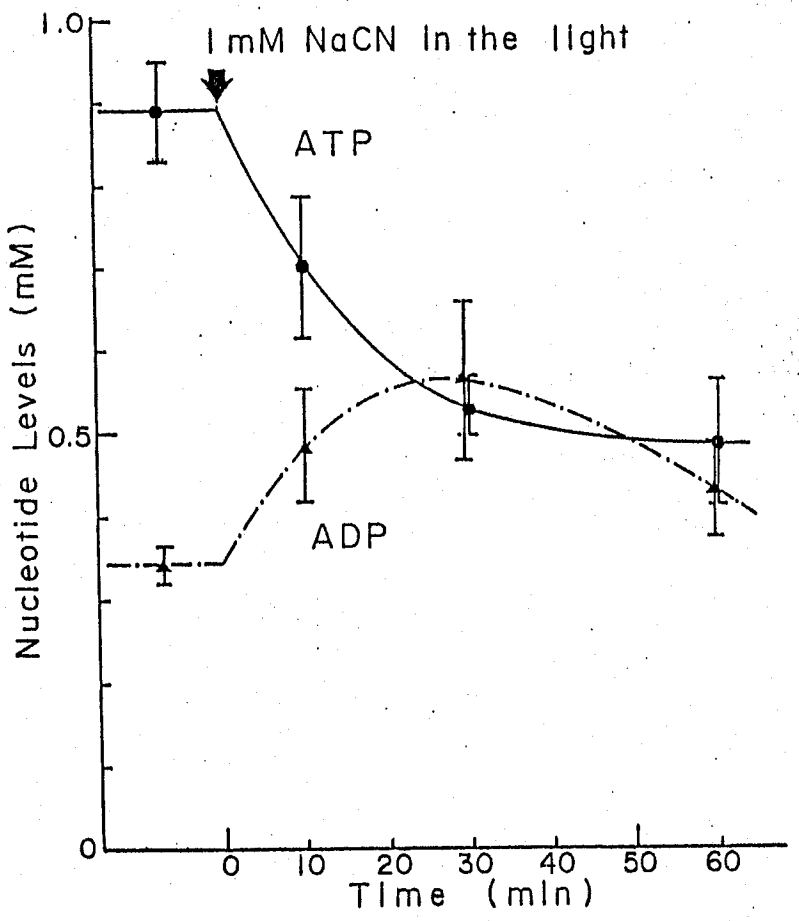


Fig. 30 Changes of ATP and ADP levels of the Chara internode during inhibition by 1 mM NaCN in the light.

Discussion

Metabolic inhibitors such as TPC, DNP, DCCD, and MIA all decreased the internal ATP level. Slayman et al. reported a close correlation of internal ATP level with membrane potential (1973). The data was simulated with Michalis-Menten equation in their report. Slayman expressed Vm as follows

$$V_m = V_{pm} [ATP]_i / (K_{1/2} + [ATP]_i) + V_o$$

V_o is the membrane potential in the absence of electrogenic pumping

V_{pm} is the apparent maximal added voltage developed by the pump.

In Neurospora membrane, resistance seemed change scarcely during pump inhibition. So Slayman proposed that V_o was passive potential and $K_{1/2}$ was equal to 2.0 mM. But in Chara corallina the resistance decreased largely by pump inhibition. I expressed the pump with the pump conductance and the pump emf as described in Chapter 1. As shwon in Fig.25, g_p and ATP level had a close correlation during TPC inhibition. Similar result was obtained with DCCD inhibition. Even if g_p is zero by inhibitor, 0.3 mM ATP remained. So the direct effect of inhibitor on the membrane conductance is also expected. The change of E_p could not be explained simply by the change of ATP level. Generally the electrogenic pump of Chara corallina possess high sensivity to H^+ and the E_p can be expressed as follows (described in Chapter 2).

$$E_p = 1/m \times RT/F (\ln k_{ATP} + \ln[ATP]/[ADP][Pi]) + RT/F (\ln [H^+]_o / [H^+]i)$$

After pump inhibition, ATP level decreased to one-third of the control level and ADP level increased to twofold. So the ratio of ATP/ADP decreased from 2 to 0.3, then E_p is expected to depolarize by about 20 mV. However, we know in some occasion E_p will hyperpolarize during pump inhibition. For such a case following possibilities can be postulated. First, if the stoichiometry may change from 2 to 1, E_p will hyperpolarize from -250 mV to -500 mV. Second, if the internal ATP level may increase or ADP will

decrease, Ep will hyperpolarize. Third, if internal pH becomes low or external pH becomes high, E_p will hyperpolarize.

As shown in my results, ATP concentration did not increase and ADP concentration did not decrease at all with many poisons, so the second possibility can be neglected. Walker and Smith measured the internal pH both in the light and in the dark (1975). In their reports cytoplasmic pH changed to 7.4 from 7.7 with the dark treatment. Moreover, in my experiment external solution was perfused with APW containing pH buffer, the pH change by the dark should not be large. So the first possibility is not likely. I will discuss about the possibility of the change of stoichiometric ratio in the next chapter.

Next question is why the several hundred μM of internal ATP still remain even after simultaneous treatment with TPC and MIA. I investigated the activity of adenylate kinase which might remain in the sample even after 10 min heat teatment. But the added ADP to the sample did not change into ATP. Such residual ATP may be the foundamental ATP to maintain the cellular structure or the compartmented ATP which was not affected directly by inhibitors. This problem remaines to be solved by future study.

The results of the changes of other nucleotides levels indicated the existence of regulatory mechanism of internal ATP level. Inhibitors block the synthesis of ATP primarily. Then the ADP and Pi level will increase by the hydrolysis of ATP in various mechanism. Actually ADP increased during first 10 min by TPC inhibition. In this case total adenine nucleotide also decreased, so the following reaction may be enhanced at the same time.

$$ATP \longrightarrow AMP + PPi$$

$$AMP \longrightarrow ADEN + Pi$$

In this case another possibility that transphosphorylation may occur between ATP and other nucleotides such as GTP or CTP.

The later changes of AMP and ADP were very similar to the result which

was described in Neurospora by Slayman (1973). This decrease of ADP may be caused by the activation of adenylate kinase.

$$2ADP \longrightarrow ATP + AMP$$

Recovery of ATP and decrease of AMP were markedly large especially in NaCN inhibition. Such a regulatory mechanism of ATP level by AMP/ATP ratio would be caused by the action of phosphofructokinase (Atkinson 1966, Conn and Stump 1976).

The ATP level of the Chara internode did not decrease appreciably during first one hour in the dark, if the cell was pretreated under normal photoperiodic condition. This result is the same as the other algae (Holm-Hansen 1970, Penth & Weigl 1971, Jeanjean 1976, Lilly and Hope 1971, Keifer and Spanswick 1979). But the ATP level decreased to half of the original level in a long dark period such as three or four days. This suggests that glycolysis may be enhanced, contributing to protect against the decrease of ATP level in the case of short dark treatment. If the level of total glucose, which is the substrate of glycolysis, become small during long dark treatment, the regulation of ATP level by glycolysis may not work anymore.

Chapter 4

Current voltage relationship of the H^+-pump in Chara corallina

Introduction

It is not yet equivocal that electrogenic pump has its own conductance. Finkelstein (1964) and Rapoport (1970) proposed that an electrogenic pump is voltage sensitive and consequently contributes to the membrane conductance from their theoretical model. Gradmann (1975) reported on the Cl^- pump in Aetabularia that the nonlinear current-voltage relationship is sensitive to light and temperature. Graves & Gutkneckt investigated the Cl^- pump in Halicystis parvula and concluded that this electrogenic pump is an ion conducting pump (1977). Slayman initially considered the H^+ pump as the current source (Slayman et al.1973). Subsequently by the analysis of current-voltage curve before and after inhibition with CN^-, he showed that conductance of the pump accounted 5-10 % of the total membrane conductance (Gradmann et al., 1978).

Recently, Hansen et al. (1981) explained the pump mechanism with a kinetic model, and presented the current-voltage relationship of H^+ pump in Neurospora. As described in Chapter 1, I concluded that H^+ pump in Chara corallina also posseses the conductance from the results of current clamp method. Anyway, it is a crucial points to demonstrate how the pump conductance is voltage dependent.

In this Chapter, the current-voltage relationship of both control and passive channels will be analysed. The I-V relationship of pump channel can be obtained from the difference of two I-V curves before and after inhibition.

The I-V curve has another advantage to know the pump mechanism. We can

choose the pump model in which I-V relation of the pump can be explained and determine kinetic parameters of the pump by simulating the pump I-V relation. Some results on the effects of various inhibitors such as TPC, DCCD, NaCN and the changes of light and pH are presented in the followings. Changes of the kinetic parameters of the pump are also analyzed.

Material Method

Internodal cell of Chara corallina was prepared as described in Chapter 1. APW and inhibitors are the same as described before.

Voltage clamp method

The basic setup is the same as described in Chapter 1 (Fig.2). Voltage clamping was achieved by the negative feedback circuit with a control amplifier, DATEL AM-303B. The total gain of the feedback system was about 6000. This output was connected to the internal platinum-coated tungsten wire electrode, which was inserted from the nodal end into the vacuole of the Chara internode. The small and short pulse was applied under the voltage clamp condition. The response was monitored with an ocilloscope or penrecorder and stored into the memories of the microcomputer for later computation.

What we need to know is the change of membrane emf and conductance from time to time during pump inhibition from the change of I-V curve. As shown in Fig.31 (a), the current response to square pulse in various voltage reached to a stable state in <20 sec . Fig.31 (b) showed the current response to the ramp voltage pulse. If we choose the rate of ramp, i.e., k = 200 mV /25 sec, the I-V curve obtained with ramp voltage clamp agreed with that of the step pulse method (Fig.32). The ramp pulse method was useful enough to analyse the steady I-V relation during inhibition.

Conductance values were calculated from the size of the current response to the square voltage pulse which was applied during ramp voltage change under voltage clamp condition. The length and amplitude of this test pulse were chosen as short and small as possible,that is,40-100 msec and 5-20 mV respectively.

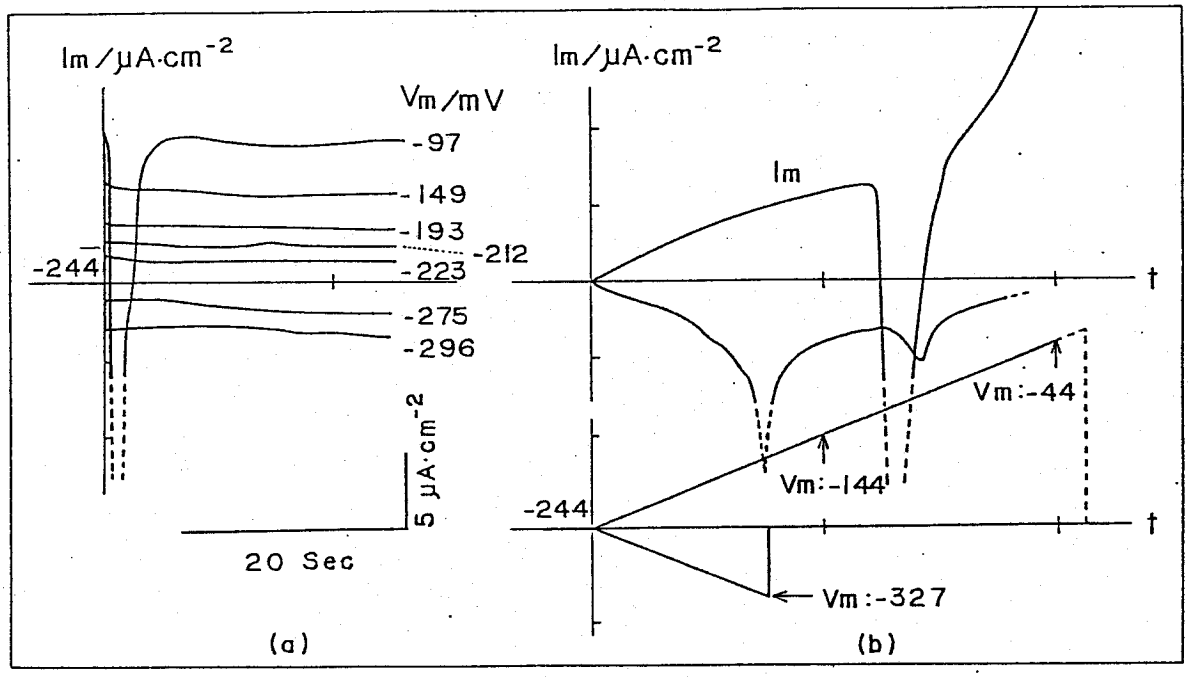


Fig. 31 The current response to square pulse (a) , or to triangle pulse (b) on voltage clamp condition. Temperature 20°C, pH 7.0 (TES)

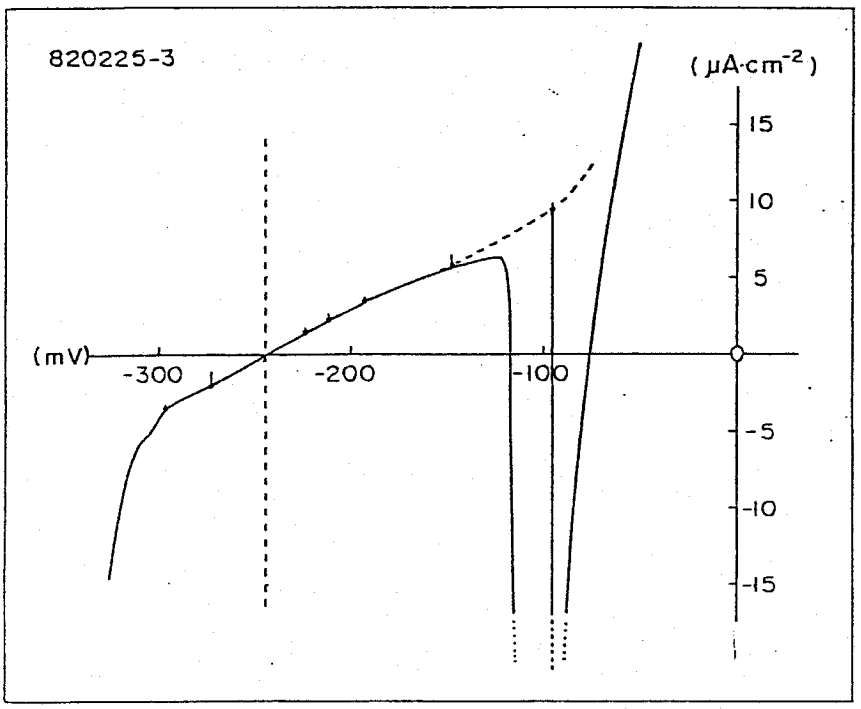


Fig. 32 I-V relation which were gained by two method in Fig.31. A solid line is the current response to triangle pulse and (↓) is the current response by square pulse.

Result

TPC effect

TPC is an ideal inhibitor of the H^+-pump of Chara plasmalemma as described in Chapter 1. When 1 μM TPC was added to the internodal cell in the light, the resting potential where I=0 gradually depolarized from -220mV to -170 mV and the slope of I-V curve became small (Fig.33). The maximum value of the membrane conductance decreased from 70 to 20 $\mu S/cm^2$ with TPC in the light (Fig.34). Then the Chara was placed in the dark. About 25 min in the dark with 1 μM TPC, the membrane potential additionally depolarized to -130 mV (Fig.33) and the maximum value of the membrane conductance decreased to 12 $\mu S/cm^2$ (Fig.34). In the final state of TPC poisoning (i.e.,65min) only the diffusion channel is acting as described in Chapter 1. As shown with an interrupted line in Fig.33④, I-V relation of the diffusion channel i.e., i_d-V curve was comparatively linear from about -250 mV to -150 mV. When membrane potential is below -250 mV and over -150 mV, the conductance of the passive channel(g_d) increases again(Fig.34). Such an increase of g_d occurs generally by the breakedown response below -250 mV and by the excitatory mechanism over -150 mV.

The change of pump current during TPC poisoning in the light was calculated from the difference between the I-V and the i_d-V. As shown in Fig.35, I-V relation curve of the electrogenic pump i.e., i_p-V curve is nonlinear and saturated in both for a large depolarization and for a large hyperpolarization i.e., sigmoidal shape. The voltage at which the pump current is zero is equal to the electromotive force of pump (E_p). In the control state E_p of Chara corallina was about -270 mV at pH 7.0. The pump current decreased from 4.5 to 0.8 $\mu A/cm^2$ during the inhibitory process in the light. E_p decreased from -270 mV to -230 mV and did not show any transient hyperpolarization during first 20 min by TPC poisoning in the

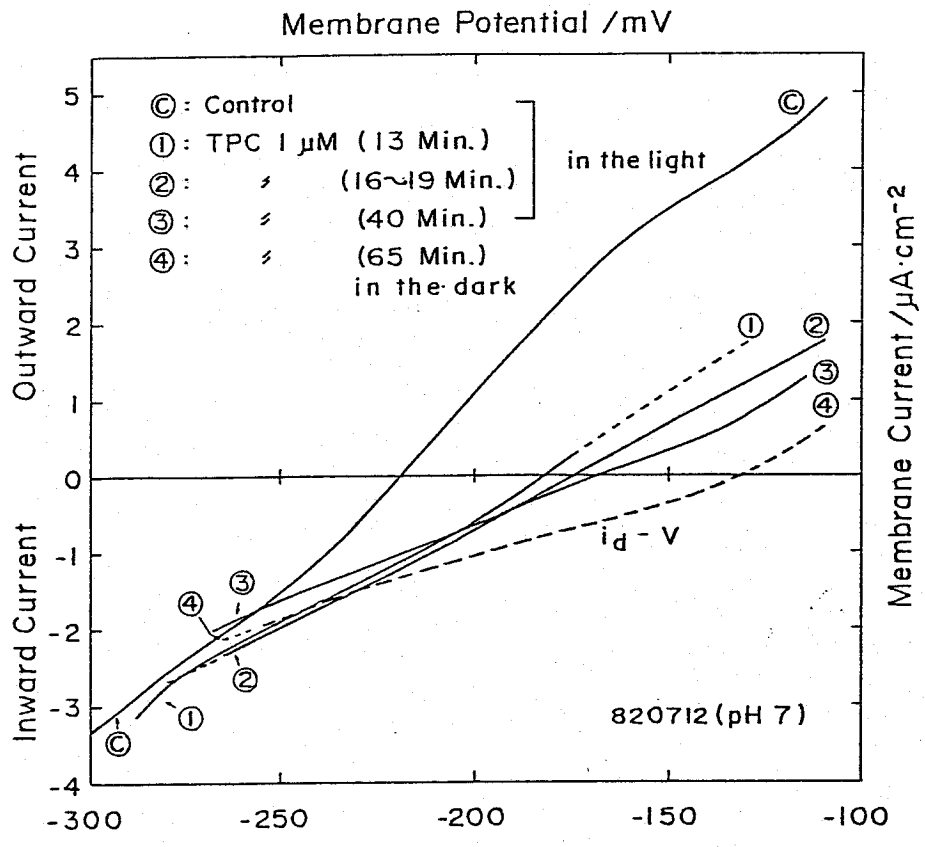


Fig. 33 I-V relation of Chara internode which was applied TPC 1 μM in the light (40 min) and then in the dark (25 min). Temperature, 19.5°C, pH 7.0.

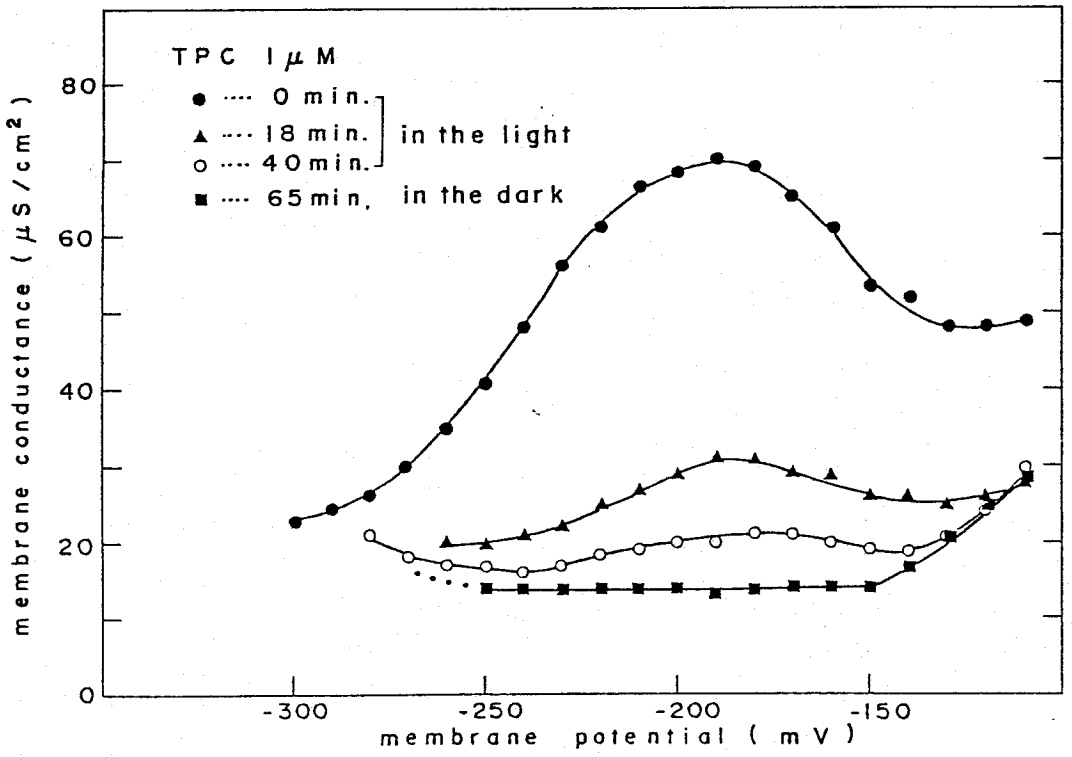


Fig. 34 The change of voltage dependency of the membrane conductance during TPC poisoning. Other situation is same as Fig.33. Membrane conductance of the diffusion channel (■ 65 min TPC in the dark) is constant between -250 mV to -150 mV.

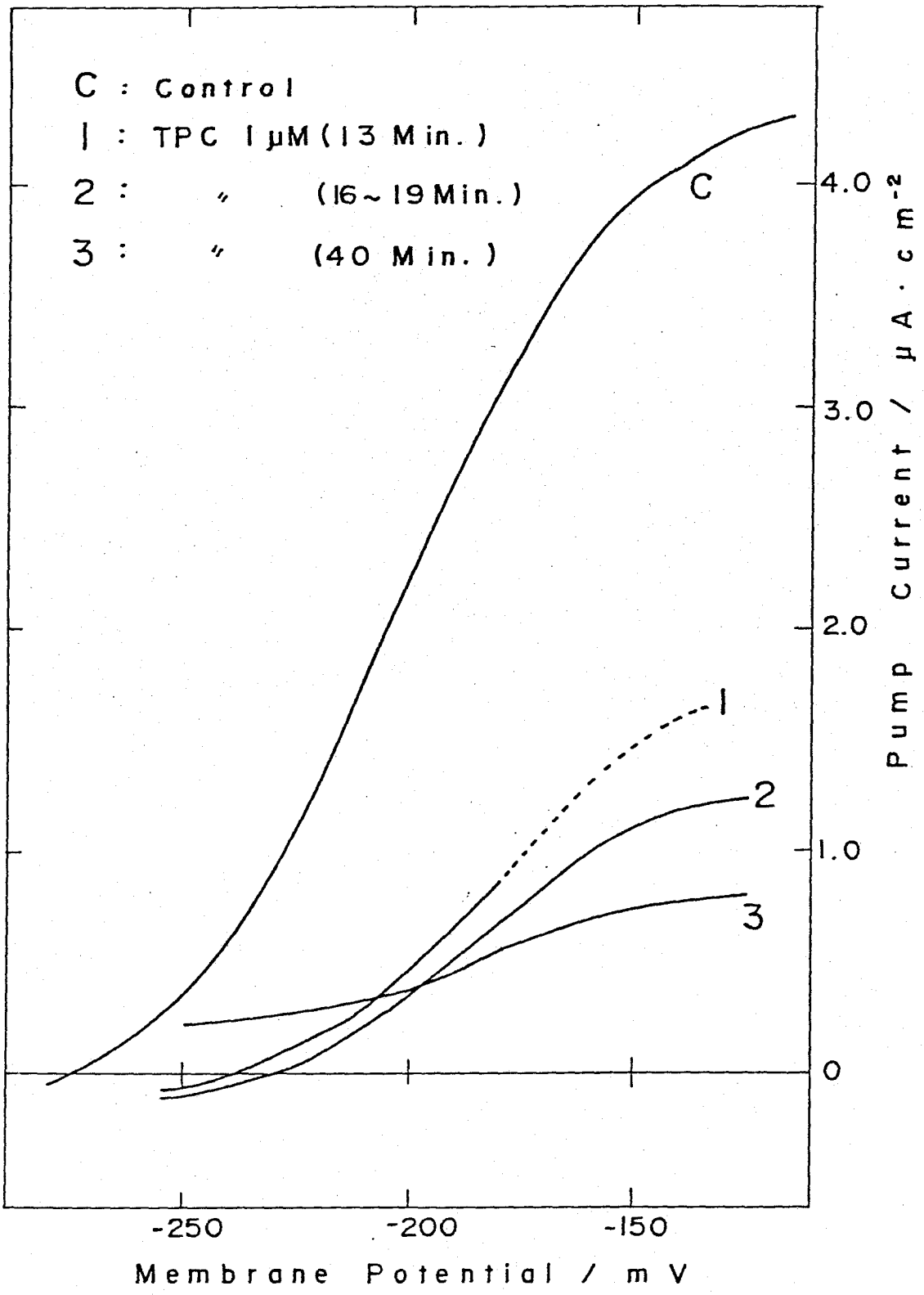


Fig. 35 The i_p-V relation of the pump channel during TPC poisoning in the light. This i_p-V relation was obtained by Fig.33.

light. But when TPC and dark treatment applied simultaneously, E_p hyperpolarized transiently after 8 min (data not shown). This transient hyperpolarization is similar to the results obtained by the current clamp method in Chapter 1(Fig.8). On the other hand, when TPC was applied to the dark adapted cell which was about 75 min in the dark before TPC poisoning, E_p depolarized gradually from -310 mV to -220 mV during 40 min TPC poisoning without a transient hyperpolarization (Fig.36). It is worth noting that E_p hyperpolarized from -260 mV to -310 mV after 75 min in the dark without TPC as shown in Fig.36. These results suggest that the hyperpolarization of E_p reflects the change of pump mechanism, which was caused mainly by the dark.

DCCD effect

DCCD is known as an inhibitor of H^+-pump and decreased the internal ATP level to half of the control level. When 50 μM DCCD was applied in the dark, membrane potential gradually depolarized from -240 mV to -130 mV and the slope of I-V relation became small during 130 min (Fig.37). The change of the membrane conductance (G) with 50 μM DCCD is shown in Fig.38. The peak conductance decreased from 180 to 30 $\mu S/cm^2$ during 130 min. The conductance of the passive channel(g_d : after 130 min DCCD) is almost constant between from -250 to -150 mV. The E_d where $i_d = 0$ reached by 50 μM DCCD is almost similar to the one obtained by 1 μM TPC inhibition. But the process of inhibition and the time needed to the complete inhibition are not necessarily the same.

Fig.39 show the i_p-V relation of the electrogenic pump calculated from the difference between the I-V curve and the i_d-V curve in Fig.37. The i_p-V relation of the pump obtained by DCCD is sigmoidal shape. E_p is about -270 mV before inhibition and gradually depolarized during inhibition. E_p reached to about -200 mV 105 min after application of DCCD. Maximum

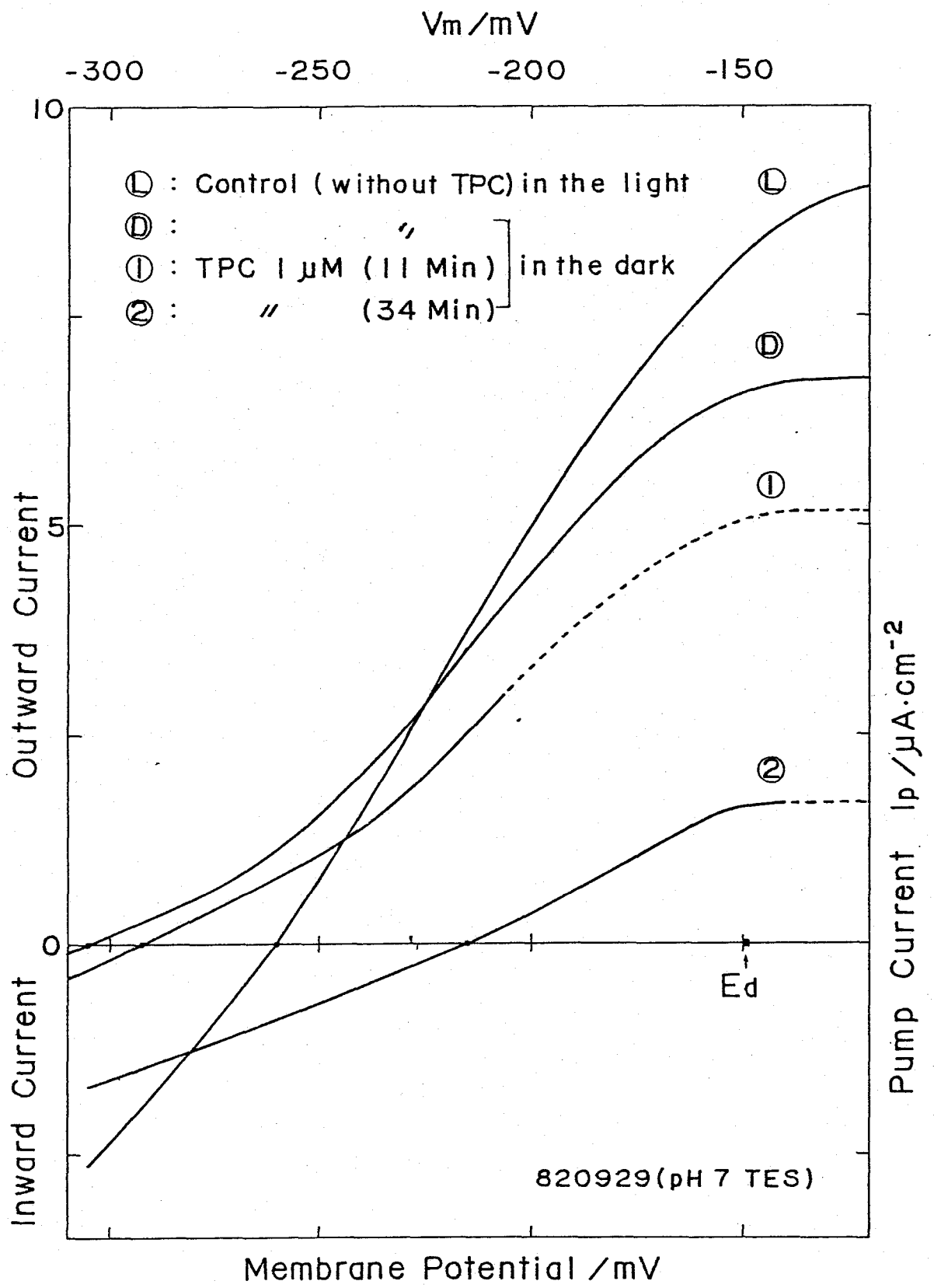


Fig. 36 The change of i_p-V relation of the electrogenic pump during TPC poisoning in the dark.

L: control
D: 75 min in the dark without TPC
1: 11 min after application of 1 μM TPC in the dark
2: 34 min after application of 1 μM TPC in the dark
Temparature 19°C, pH 7.0(TES)

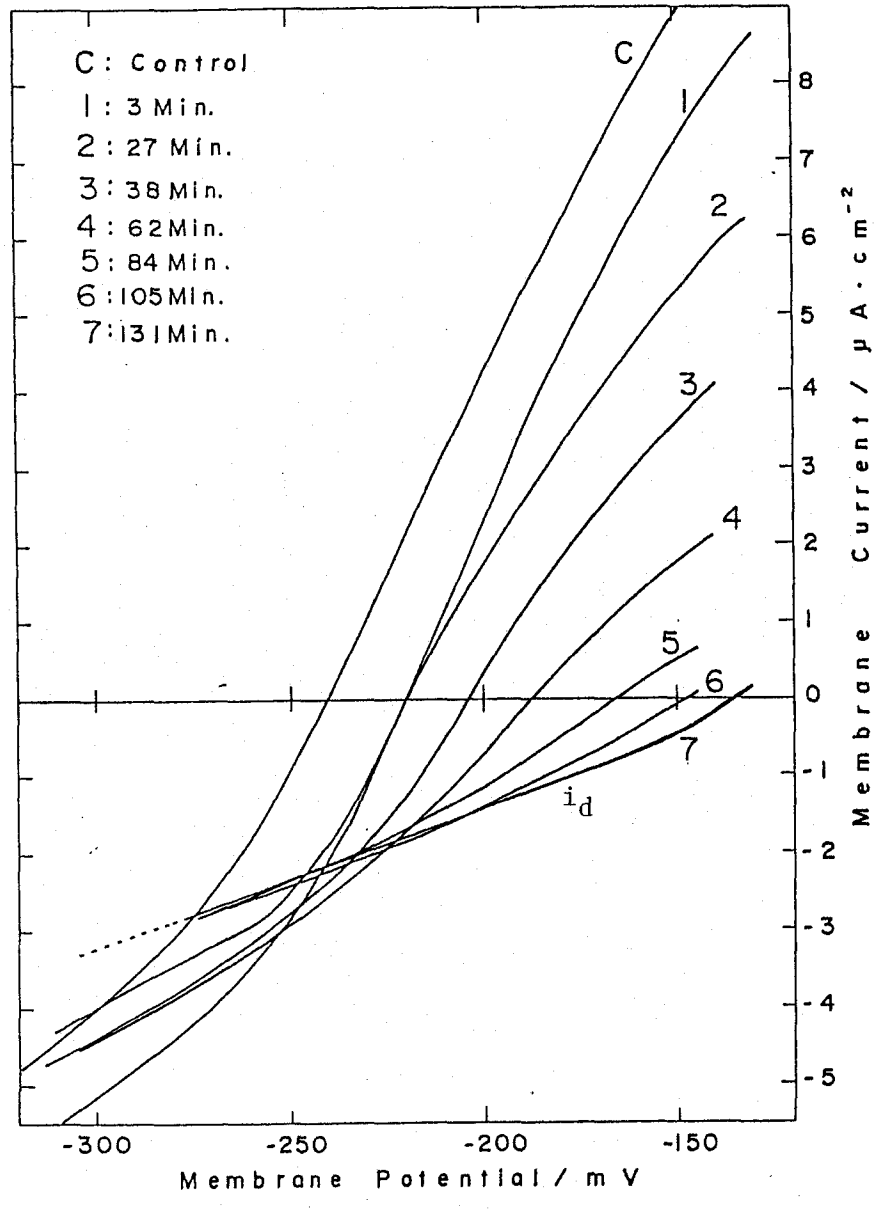


Fig. 37 The change of I-V relation of Chara internode during application of 50 μM DCCD in the dark. Tem. 19°C, pH 7.0 (TES). Each time represents the time after DCCD poisoning. Diffusion state was obtained 131 min after application of DCCD in the dark.

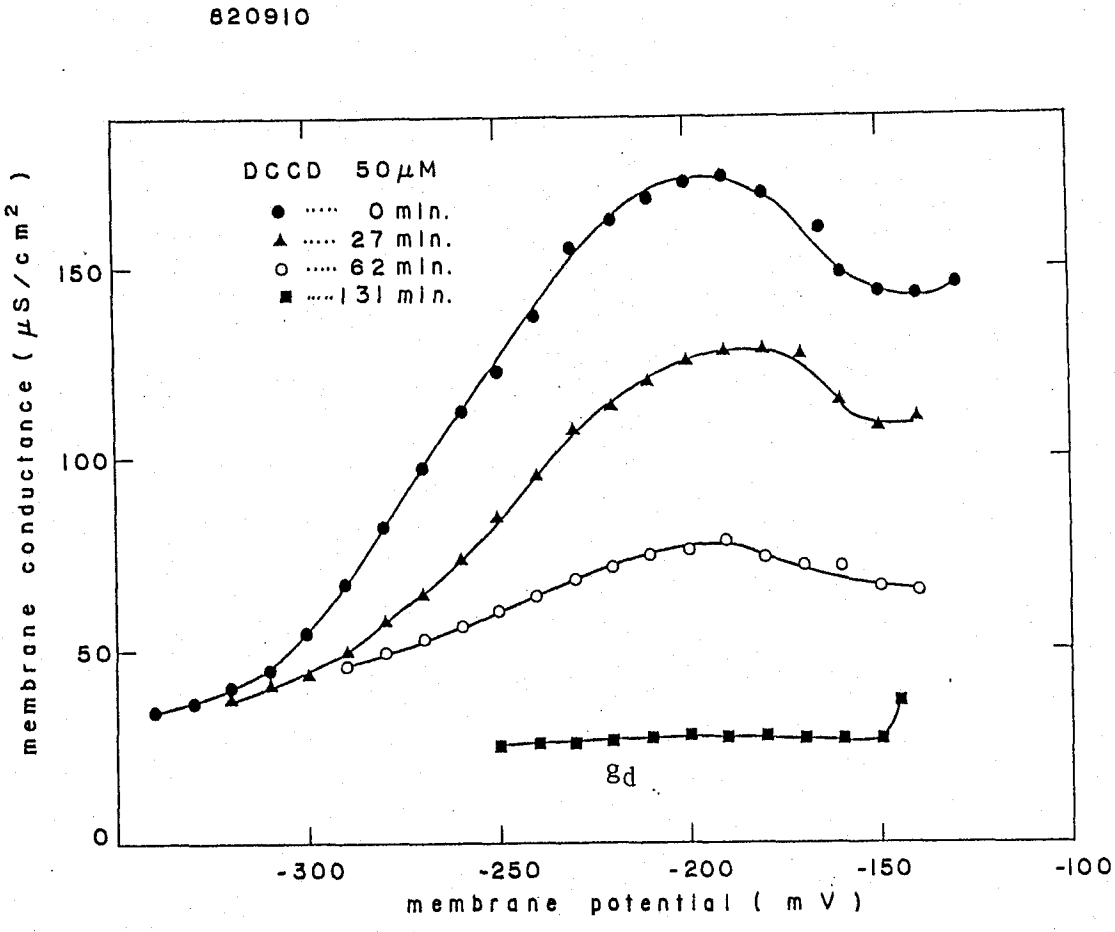


Fig. 38 The change of voltage dependency of the membrane conductance during 50 μM DCCD poisoning in the dark. Other situation is same as Fig.37.

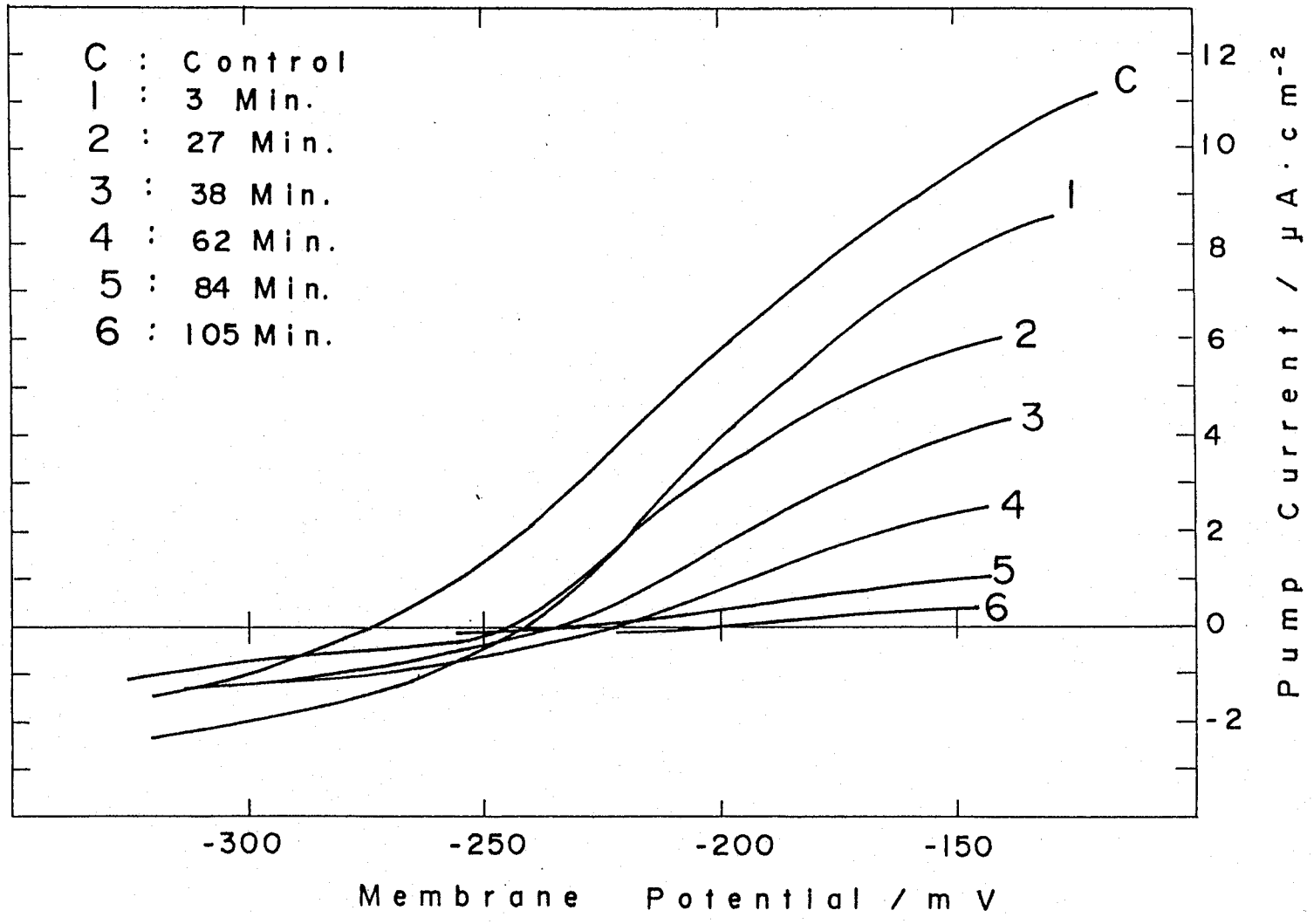


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Fig. 39 The change of i_p-V relation of the elctrogenic pump during 50 μM DCCD poisoning in the dark. Each data was calculated from I-V curve and i_d-V curve in Fig.37.

current of the pump(i_p) decreased from about 12 to 0 $\mu A/cm^2$. When 50 μM DCCD was applied, perfect inhibition of the pump reached after 90-120 min. The extent of depolarization of E_p by DCCD is almost the same as that in the dark (data not shown). The effect of DCCD was irreversible.

CN^- effect

CN^- is an inhibitor of electron transfer. 3 mM NaCN depolarized membrane potential from -240 mV to -130 mV and the slope of I-V curve decreased (Fig.40). As shown with an interrupted line in Fig.40, I-V relation of the diffusion channel was comparatively linear from -300 mV to -150 mV. The I-V relation of the pump (i_p-V) obtained from the difference of control state and final state during CN^- poisoning is shown in Fig.40(o). In the control state, E_p is -275 mV and i_p-V relation of pump is sigmoidal shape.

Now the common points obtained with these three inhibitors TPC, DCCD and CN^- are follows.

1) The relationship between the voltage and pump current is a typical sigmoid.
2) E_p is about -250 mV at pH 7 before inhibition and gradually depolarized to -200 mV except for the TPC treatment in the dark.
3) Pump conductance decreased gradually almost to zero during inhibition.
4) Conductance of the passive channel remains almost unchanged from -250mV to -150 mV.

The effect of external pH change

The pH sensitivity of the pump was obtained by the current clamp method as described in Chapter2. E_p changed with a slope of about 50 mV/pH at 20^oC.

Following the change of external pH , the I-V curve moved almost paral-

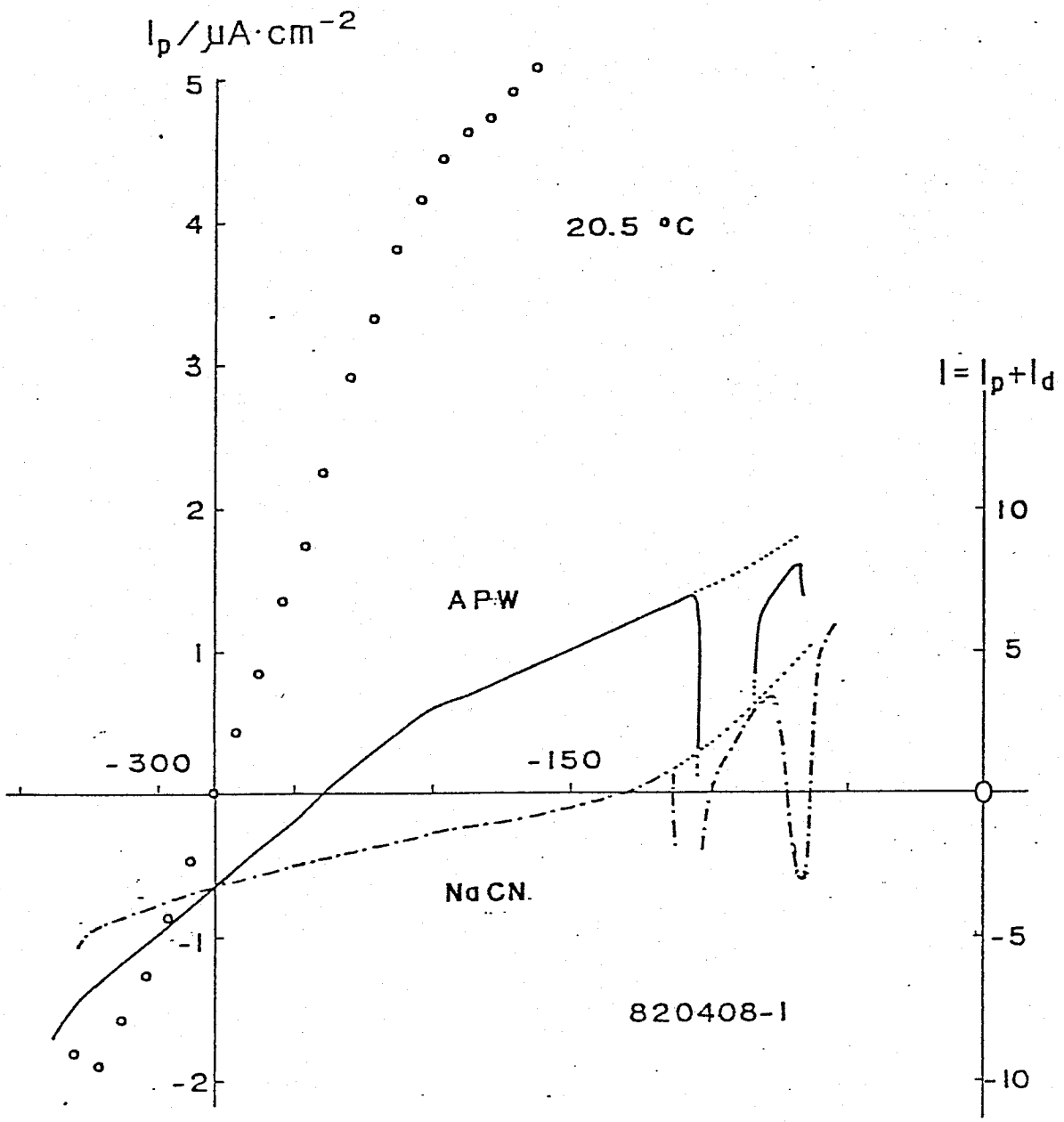


Fig. 40 I-V relation of Chara membrane before (——) and after (—·—·—) 3 mM NaCN poisoning.

i_p-V relation of the elctrogenic pump (o) was calculated from the difference of two I-V curves. Tem.20.5°C, pH 7.0(TES).

lel along the pH axis. Resting potential changed with a slope of about 30-40 mV/pH (Fig.41). In this data only TES buffer was used. Fig.41 also demonstrates the effect of external pH change on the i_d-V curve obtained after pump inhibition with 50 μM DCCD. The pH sensitivity of the passive channel was very small. The conductance of the passive channel was practically constant from -150 to -250 mV at various pH value. This finding agreed with the result of current clamp method (Chapter 2, Fig. 12). The change of emf of the diffusion channel after inhibition was only about 10mV/pH. From the difference of I-V curves between before and after inhibition in Fig.41, the i_p-V curve of the pump channel at various pH can be drawn. As shown in Fig.42 the shapes of the pump i_p-V curves at three different pH's resembled each other. In this data the value of E_p is -270 mV at pH 7.0 and changed with a slope of about 40 mV/pH between pH 7.5 and 6.5.

When the cell was in the alkaline condition above pH 8.0 in the light, the change of E_p became to small. For example, the difference of E_p between pH 7.5 and 8.0 was about 10 mV (data not shown). Generally the membrane potential depolarized in the dark by about 10-20 mV and the conductance in the dark decreased to half that in the light. This tendency seemed to be common in various pH. When I examined the pump i_p-V curve in the dark at various pH, very interesting result was obtained. That is , at pH8 E_p hyperpolarized in the dark largely comparing with at smaller pH. In the dark, E_p changed by 50 mV/pH in both acid and basic condition (data not shown). These results suggest that other mechanism than the H^+-pump of stoichiometry =2 will work, when the external solution becomes alkaline above pH 8.0 in the light.

The effect of darkness on the pump activity

As described in Chapter 3, the ATP level scarcely changes by the dark

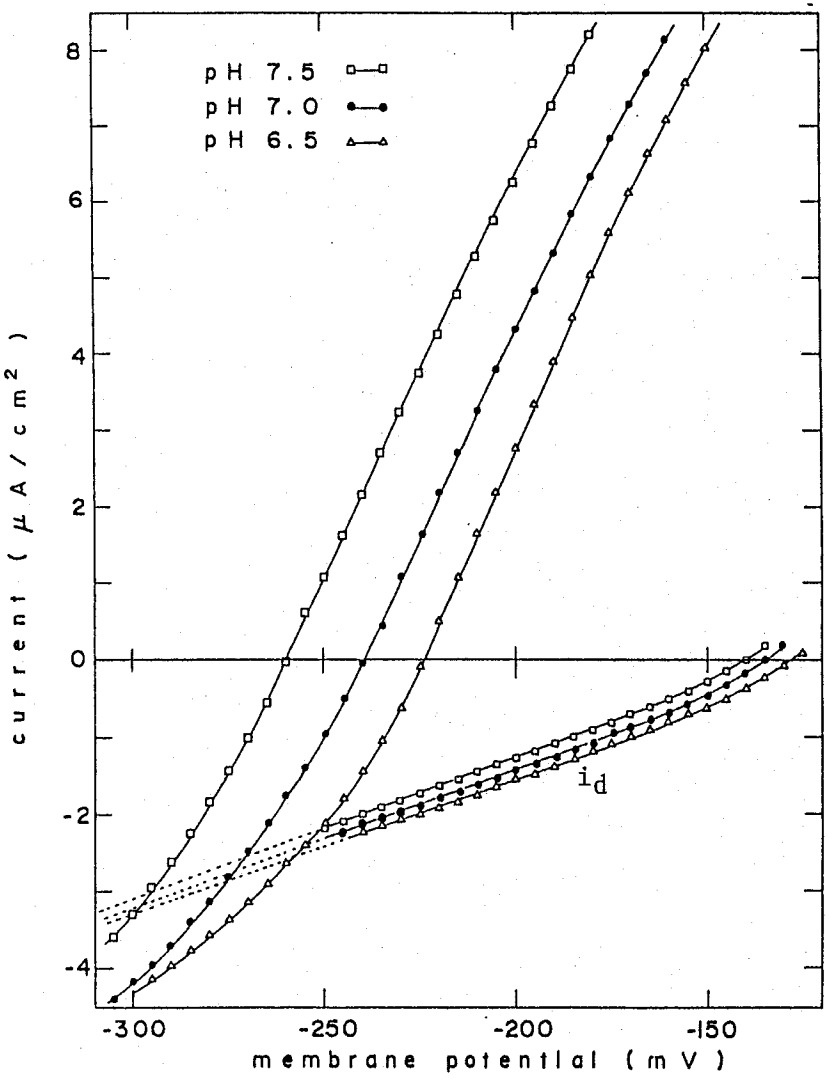


Fig. 41 The pH effect on the I-V relation in Chara membrane.
In the control state pH was changed by APW contained with 2 mM TES buffer in the light.
I-V relation of the passive channel (i_d-V) was obtained 131 min after application of 50 μM DCCD in the dark as shown in Fig.37.

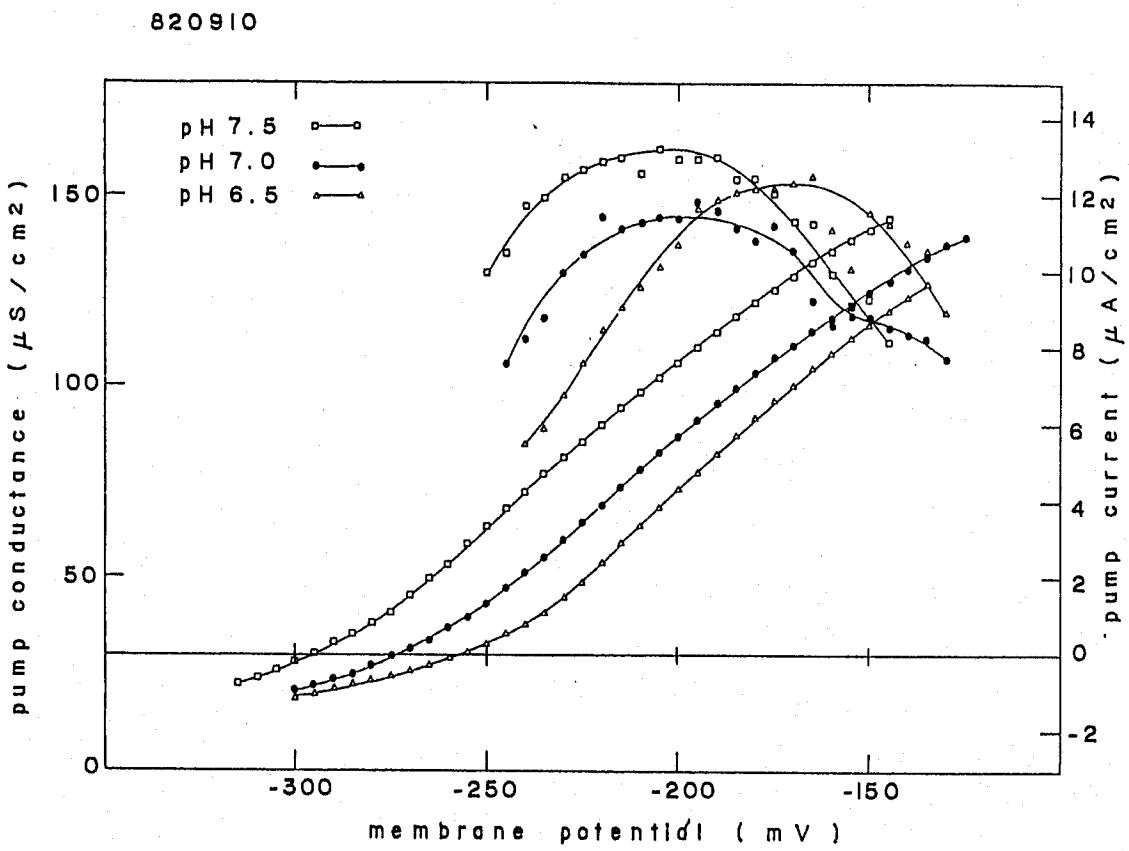


Fig. 42 The changes of i_p-V curves of the elctrogenic pump and the voltage dependency of the pump conductance with the change of external pH.
These i_p-V curve were obtained from Fig.41.

treatment, while the membrane conductance decreases to half the control level.

As shown in Fig.43, the effect of darkness during 1 hour on the membrane potential could be classified in three types, that is, depolarization type (from -210 mV to -130 mV), stable type (from -220 mV to -200 mV) and hyperpolarization type (from -200 mV to -240 mV). This hyperpolarization type was often found, when the cell was pretreated in the long dark. On the other hand, the membrane conductance (G) decreased in all three type from 30-180 to 30-50 μS /cm^2 during 1 hour in the dark. The main difference in these cells is in the E_p change as shown in Fig.44, in which E_p was calculated by current clamp method described in Chapter 1. E_p depolarized from -260 mV to -180 mV in the depolarization type, and hyperpolarized from -230 mV to -260 mV in the stable type and also hyperpolarized from -250 mV to -320 mV in the hyperpolarization type.

Then I investigated the change of I-V relation in Chara corallina by dark treatment. The change of i_p-V curve of pump is demonstrated in Fig.45. In this stable type the membrane potential(E) did not change much, while conductance(G) decreased largely in the dark. However, E_p hyperpolarized from about -250 mV to below -300 mV and maximum pump current decreased from 10 to 3 $\mu A/cm^2$. In the depolarization type the membrane potential depolarized largely in the dark and the conductance decreased largely. However, E_p depolarized from below -300 mV to -180 mV and maximum pump current also decreased from 6 to 0.5 $\mu A/cm^2$(Fig.46). These results suggest the imperfect inhibition with dark and the existence of another pump mechanism having a stoichiometric ratio not 2 but 1 in the Chara.

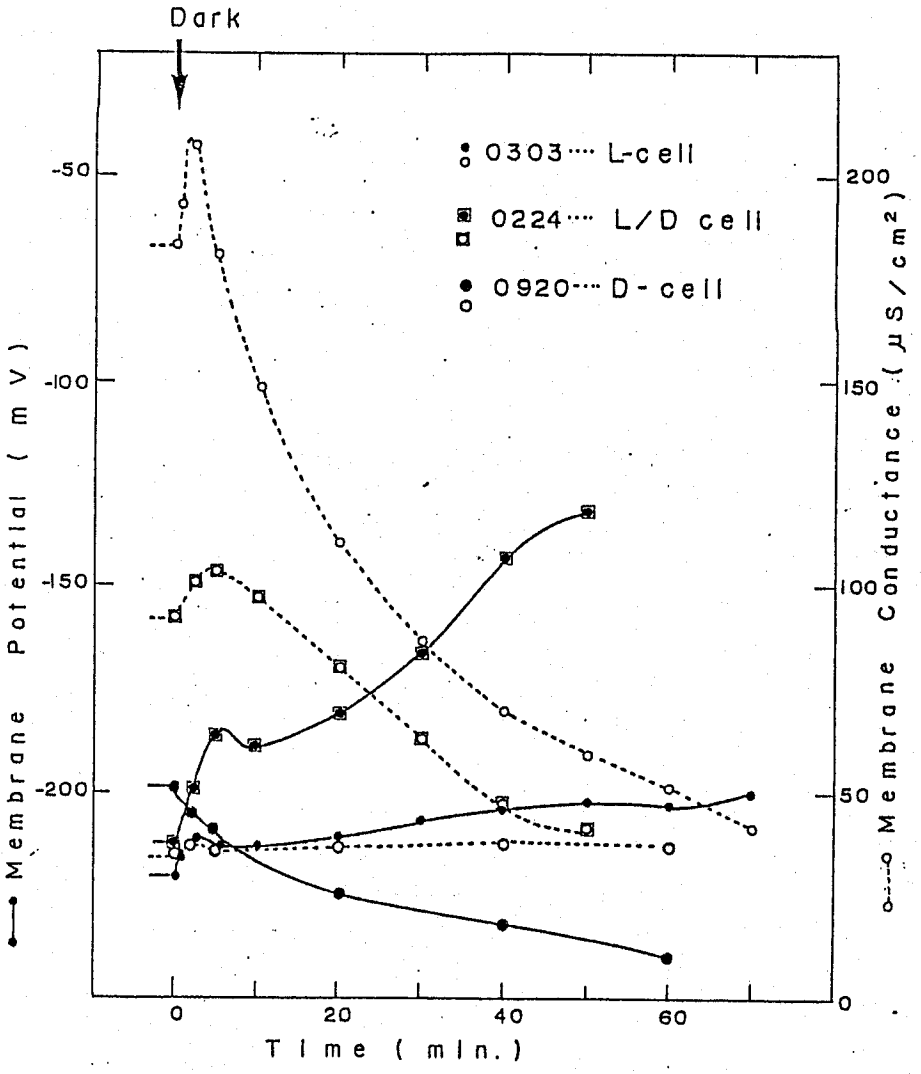


Fig. 43 Changes of the membrane potential (a solid line) and the conductance (a dotted line) of Chara membrane during 1 hour in the dark.
●:are stable type (this cell was in the light continously).
◙:are depolarization type (this cell was 12 hour in the light and 12 hour in the dark).
●:are hyperpolarization type (this cell was in the dark 3 days).

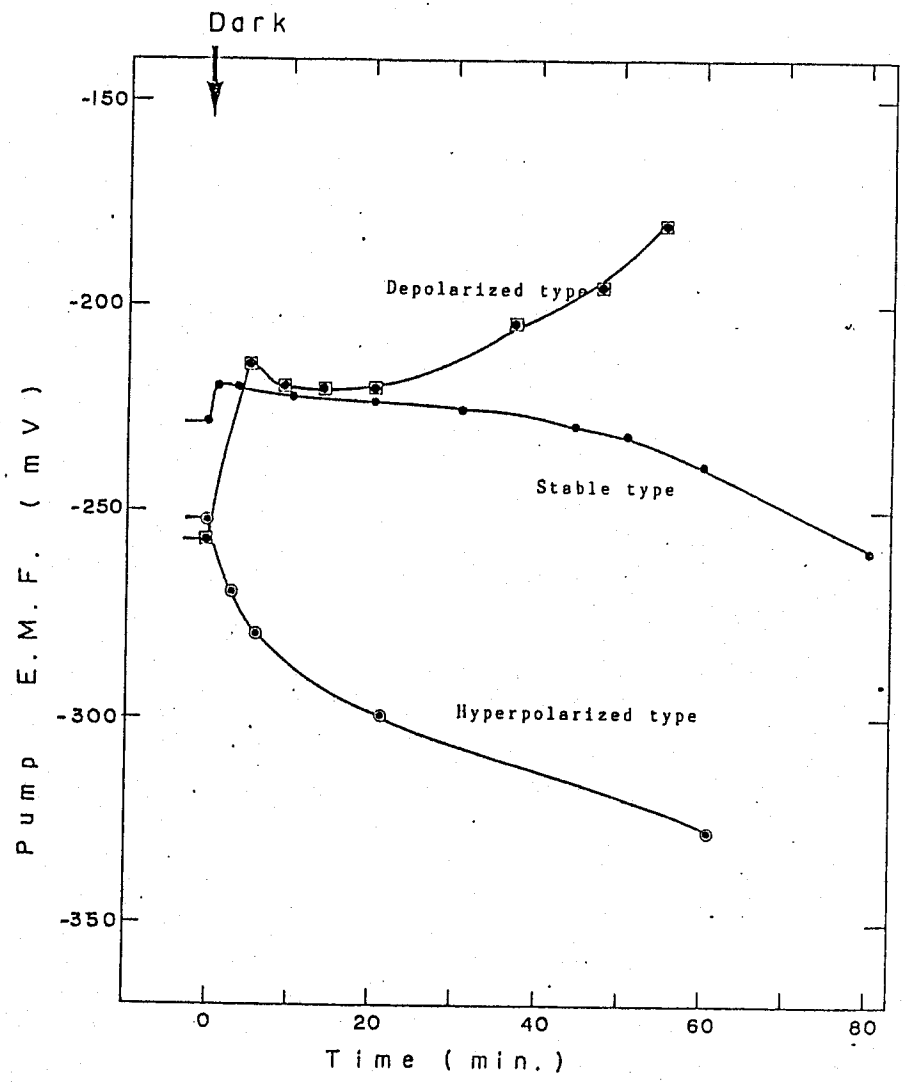


Fig. 44 The change of electromotive force of the pump channel during 1 hour in the dark. This value was calculated from the data in Fig.43 using Eq. 5 and 6 in Chapter1 (E_d and g_d were obtained with DCCD in the dark).

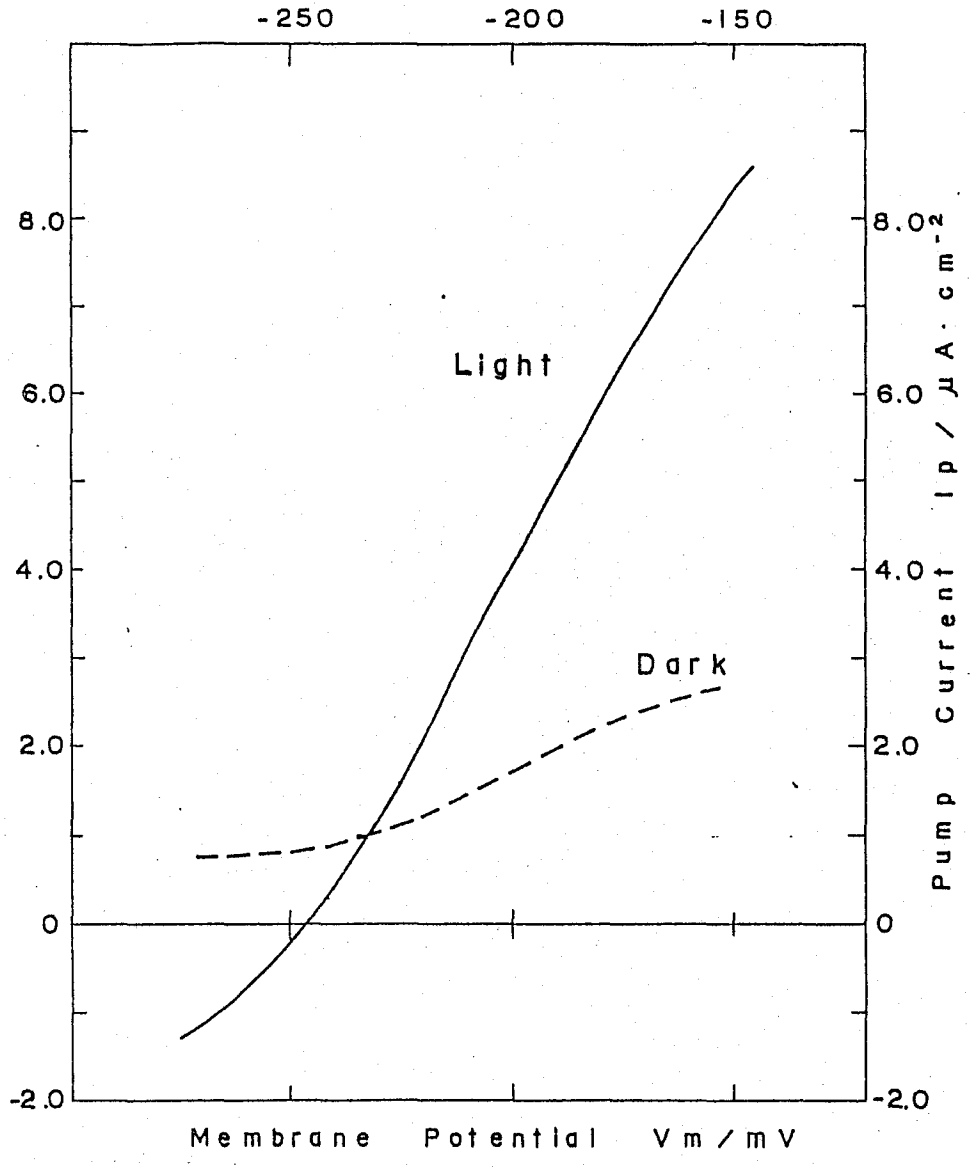


Fig. 45 i_p-V relation of the pump channel in the light (a solid line) and 65 min after in the dark (a dotted line).
Diffusion state was obtained 73 min after 75 μM DCCD poisoning.
Temp.20°C, pH 7.0(TES).

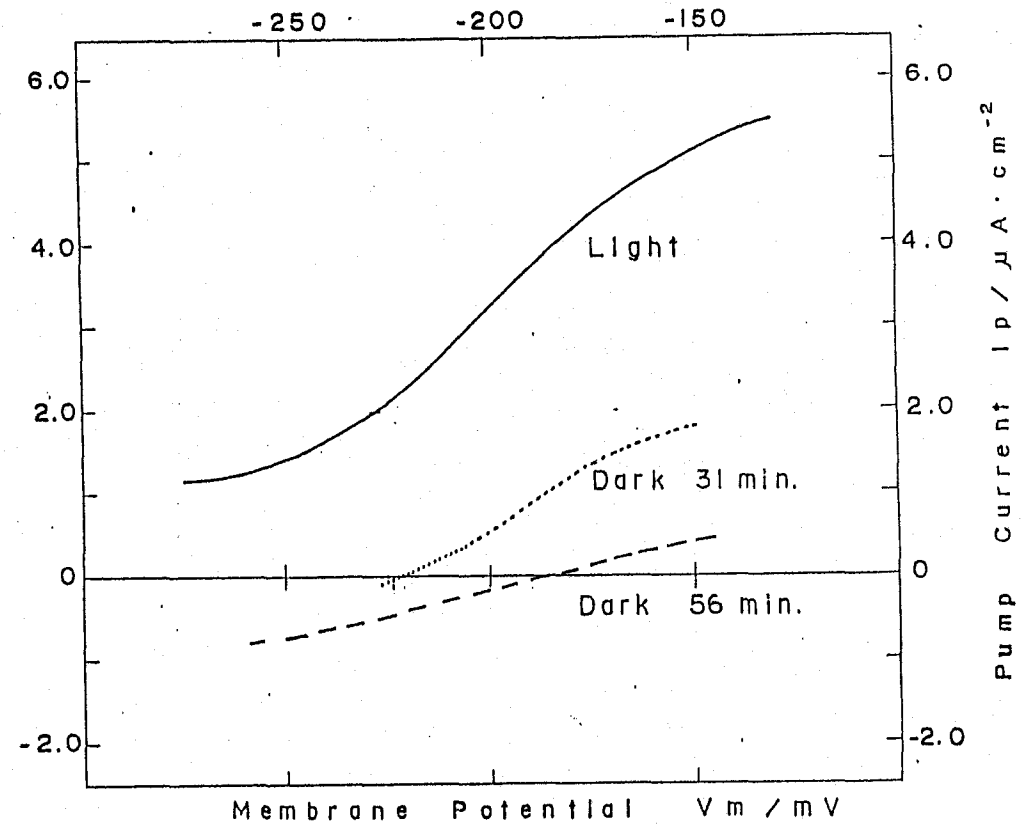


Fig. 46 i_p-V relation of the pump channel in the light (a solid line) and in the dark (a dotted line).
Diffusion state was obtained with 75 μM DCCD poisoning (68 min) in the dark.
Temp. 20°C, pH 7.0 (TES).

Discussion

The relationship between pump current and the voltage is a sigmoid type. These I-V curve can be simulated with our kinetic model satisfactory. Our kinetic model for the H^+-pump gives not only the size of E_p but also a quantitative expression for the pump conductance (g_p) as a function of voltage.

A kinetic model for the electrogenic H^+ pump in the Chara

We assume a cyclic change of five states of H^+- ATPase, i.e., E_1, E_2, E_3, E_4 and E_5 (Fig.47). Schemes of reaction in this change are listed in the followings.

1) The transformation of ATPase from E_1 into E_2 is mediated by using energy from ATP hydrolysis.

$$E_2 = K_{ATP} [ATP_i] [ADP_i]^{-1} [P_i]^{-1} [E_1] = M [E_1] \quad (1)$$

K_{ATP} is the dissociation constant of ATP. K_{ATP} is equal to 4.247×10^{-6}, if the standard free energy change of the ATP hydrolysis is taken as -7.2K cal/mol

2) When mH^+ ions are incorporated in the ATPase

$$E_3 = \alpha [H_i]^{+m} [E_2] \quad (2)$$

where α is the ratio of forward and backward rate constants for incorporation of mH^+ ions into an ATPase molecule.

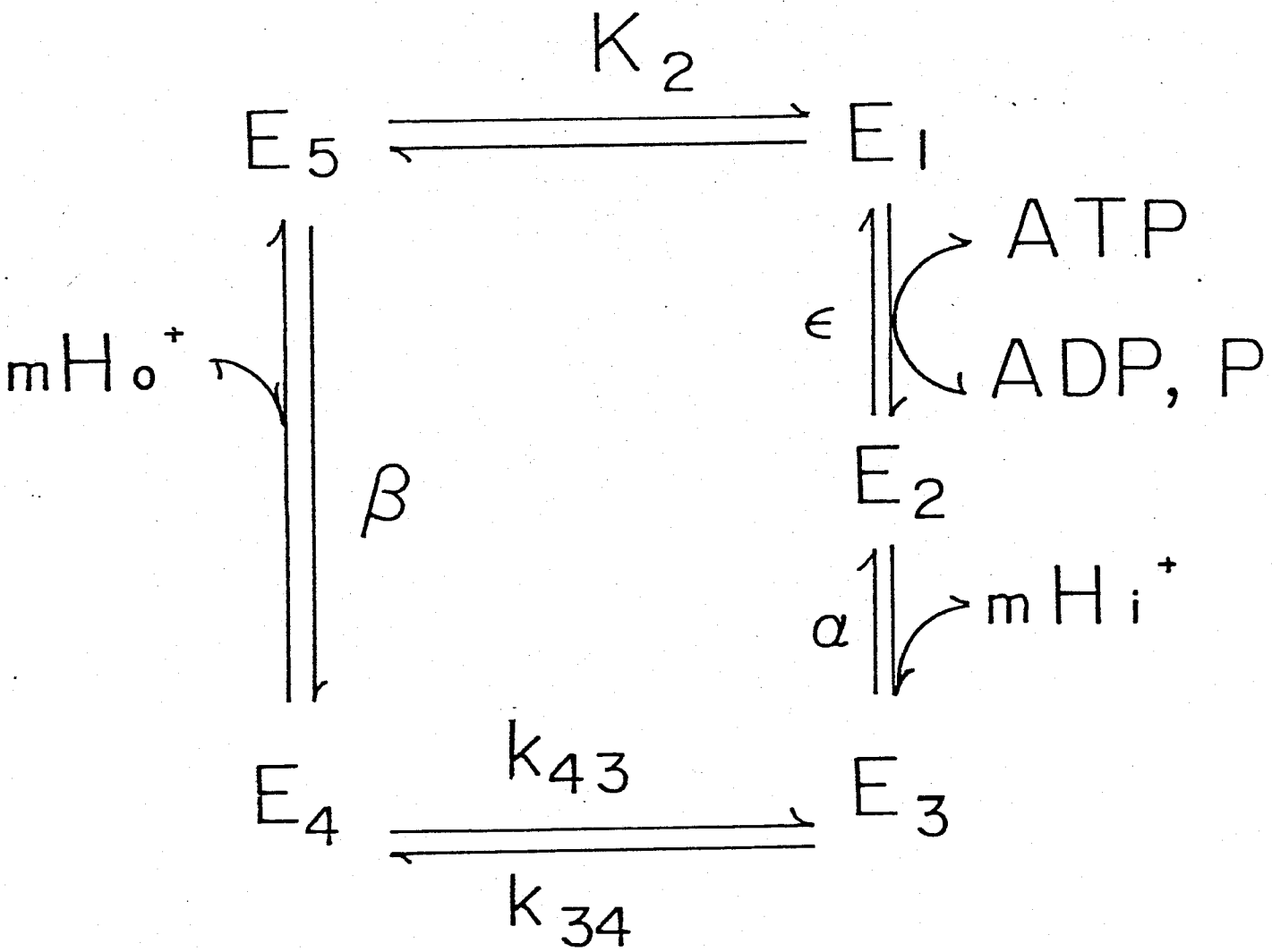
3) The transition of ATPase from E_3 into E_4 is a charge carrying process and therefore its rate is voltage dependent. For simplisity, I assume here the ATPase itself is uncharged. Then, the effulux of H^+ can be written with Eq.3

$$f_{34} = k_{34}[E_3] - k_{43} [E_4] \quad (3)$$

where k_{34} is the rate constant for the transition from E_3 into E_4, while k_{43} is the one for the opposite transition. If I assume a symmetry of

Fig. 47

A kinetic model for the electrogenic proton pump.



energy barrier in the pump cahnnel (Läuger, 1979),

$$f_{34} = K_1 \left(M[H_i]^m \exp(mFV/(2RT))[E_1] - [H_o]^m \exp(-mFV/(2RT))\, [E_5]\right) \quad (4)$$

R, T and F have usual meanings.

4) mH^+ ions are realesed outside during transition of ATPase from E_4 into E_5. Then,

$$E_5 = \beta\, [H_o]^m\, E_4 \quad (5)$$

where β is the ratio of forward and backward rate constants for the release of mH^+.

5) The transition from E_5 into E_1 is assumed here for simplicity as a electroneutral process and the rate is voltge independent. Then, the rate of transition is given by

$$f_{51} = k_{51}\,[E_5] - k_{15}\,[E_1] = K_2\,([E_5] - [E_1]) \quad (6)$$

Supposing that the H^+ pump is working steadily by a cyclic transition of ATPase, i.e., $E_1 \rightarrow E_2 \rightarrow E_3 \rightarrow E_4 \rightarrow E_5 \rightarrow E_1 \cdots$, then f_{34} should be equal to f_{51}. The total amount of the ATPase $[E_0]$ remains constant. Thus,

$$[E_1] + [E_2] + [E_3] + [E_4] + [E_5] = [E_0] \quad (7)$$

The current carried by H^+, i.e., I_p (=pump current), is proportional to f_{34} ($=f_{51}$). Then, I_p can be calculated with Eq. (1) - (7)

$$I_p = (D - A_1/D)\, A_4 \,/\, (D + A_2/D + A_3) \quad (8)$$

where $D = \exp(mFV/(2RT))$, $R_2 = [E_0]^m$, $R_1 = M[H_i]^m$,

$$A_1 = R_2/R_1, \qquad A_2 = (R_2/R_1)\,(1 + R_1\alpha) \,/\, (1 + R_2/\beta)$$

$$A_3 = (K_2/K_1)(1/R_1)(1 + R_1\alpha) \,/\, (1 + R_2/\beta)$$

$$A_4 = K_2[E_0]F/(1 + R_2/\beta) \quad (9)$$

E_p can be calculated at the voltage where $I_p = 0$.

$$E_p = (1/m)\,(RT/F)\,(\ln([ADP_i]\,[P_i] \,/\, [ATP_i]) + \ln(1/K_{ATP}) + (RT/F)\ln([H_o]/[H_i]) \quad (10)$$

Then, the conductance of the H^+ pump, i.e., g_p can be given by

$$g_p = I_p \,/\, (V - E_p) \quad (11)$$

This conductance is given not as a slope conductance but as a chord

conductance in our model.

The expression for E_p can also be derived theoretically with thermodynamics, while g_p can be derived only from the reaction kinetics like the one shown above. Similar kinetic analysis on the active transport mechanism has been also reported by Läuger (1979), Hansen et al. (1981) and Chapman et al.(1983).

The experimental i_p-V curves were simulated using this kinetic model as shown in Fig.48. This data is the change of i_p-V relation of the pump channel during 50 μM DCCD poisoning in the dark from Fig.39. The data are satisfactory simulated by the theoretical curves (solid line) using Eq.(8) and four parameters in Eq.(8) ,i.e., A_1, A_2, A_3 and A_4 was determined by computer simulation. Then, each parameters in our kinetic model ,i.e., K_1, K_2, k°_{34}, k°_{43}, α and β were determined using Eq.(9). Fig.49 and 50 show the results of analysis of the data of Fig.35 and 39 during poisoning with TPC and DCCD. In the case of pump inhibition, such as 50 μM DCCD in the dark and 1 μM TPC in the light, the factor of β, which is the ratio of rate constants for releasing mH^+ decreased largely, while α, which is the ratio of rate constants for incorporation of mH^+ ions into ATPase increased. The decrease of the factor "M" during inhibition corresponds to the decrease of the internal ATP level.

Changes of kinetic parameters with pH was also calculated using the data in Fig.42. In the case of alkaline treatment α did not changed much while β decreased markedly (Fig.51).

E_p was hyperpolarized frequently during dark treatment. As described in Eq.10, E_p was determined with the concentrations of ATP, ADP, P_i, m and the equilibrium potential of H^+. ATP level did not change much during short dark treatment as described in Chapter 3. The external H^+ concentration will not change much, because outer medium which was buffered was perfused with high speed. Walker & Smith reported that the cytoplasmic pH changed

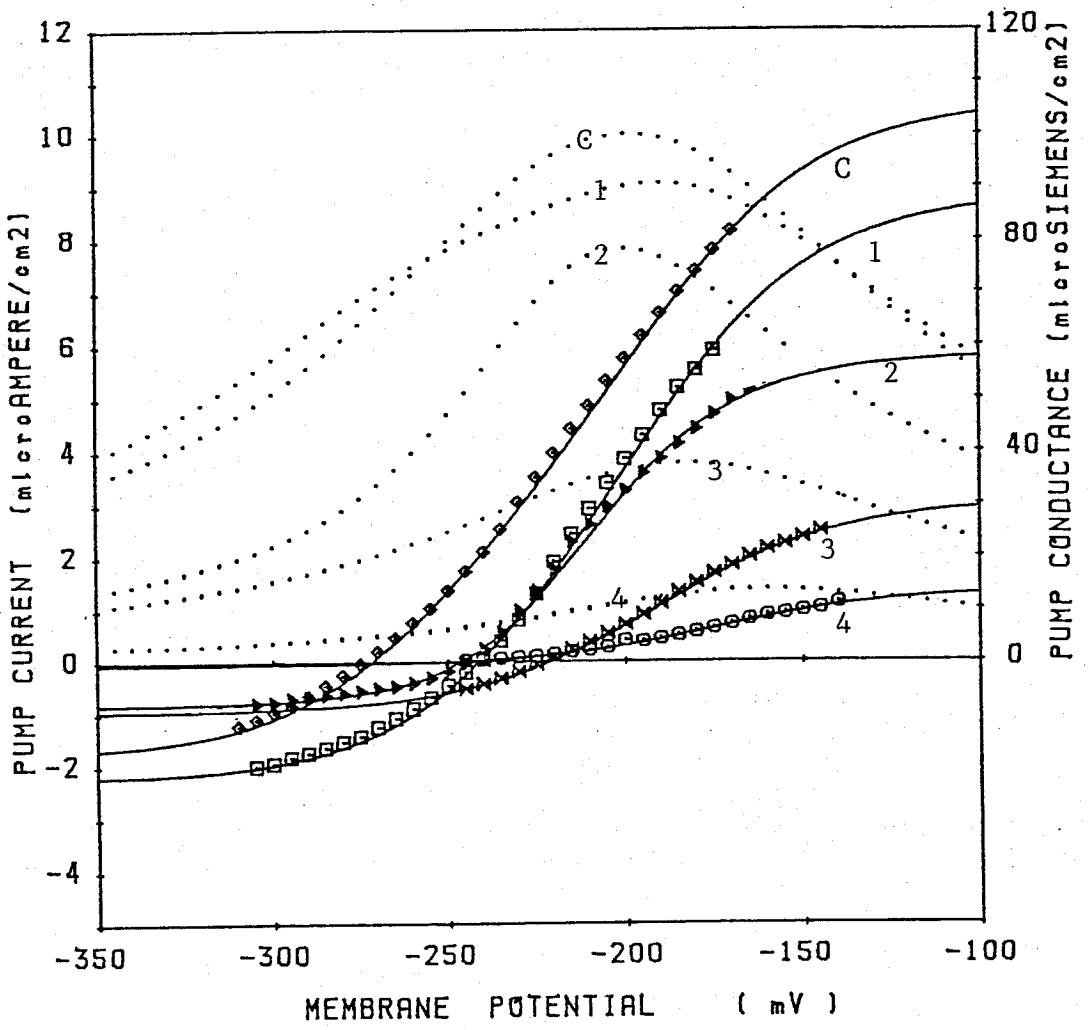


Fig. 48 The experimental i_p-V curves were simulated using kinetic model. Each mark show the real data from Fig.39 and each solid line is the simulating curve.

c: Control

1: 3 min after DCCD

2: 27 min after DCCD

3: 62 min after DCCD

4: 84 min after DCCD

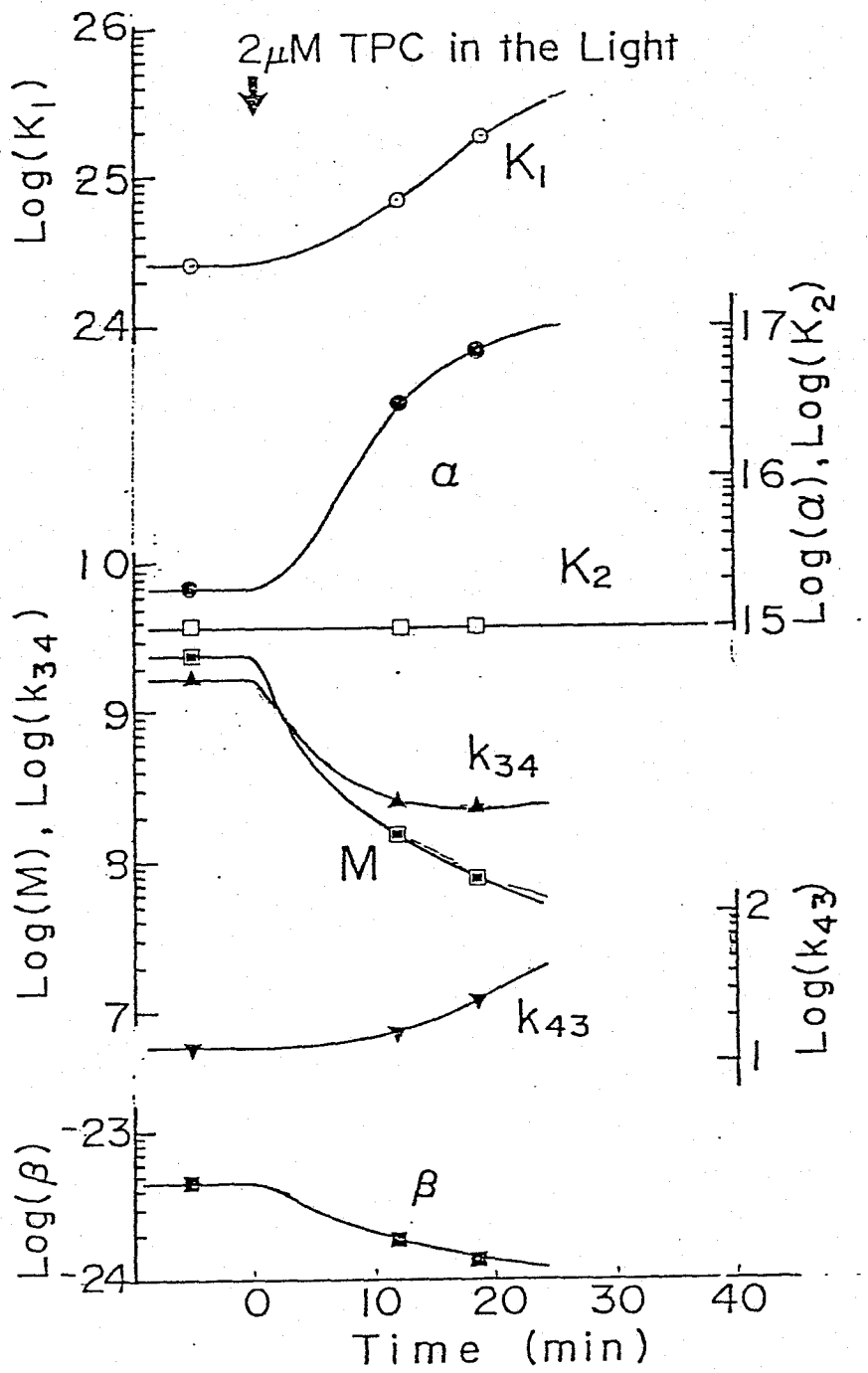


Fig. 49 The change of parameters in the kinetic model during TPC poisoning in the light. These parameters were calculated from the data of Fig.35.

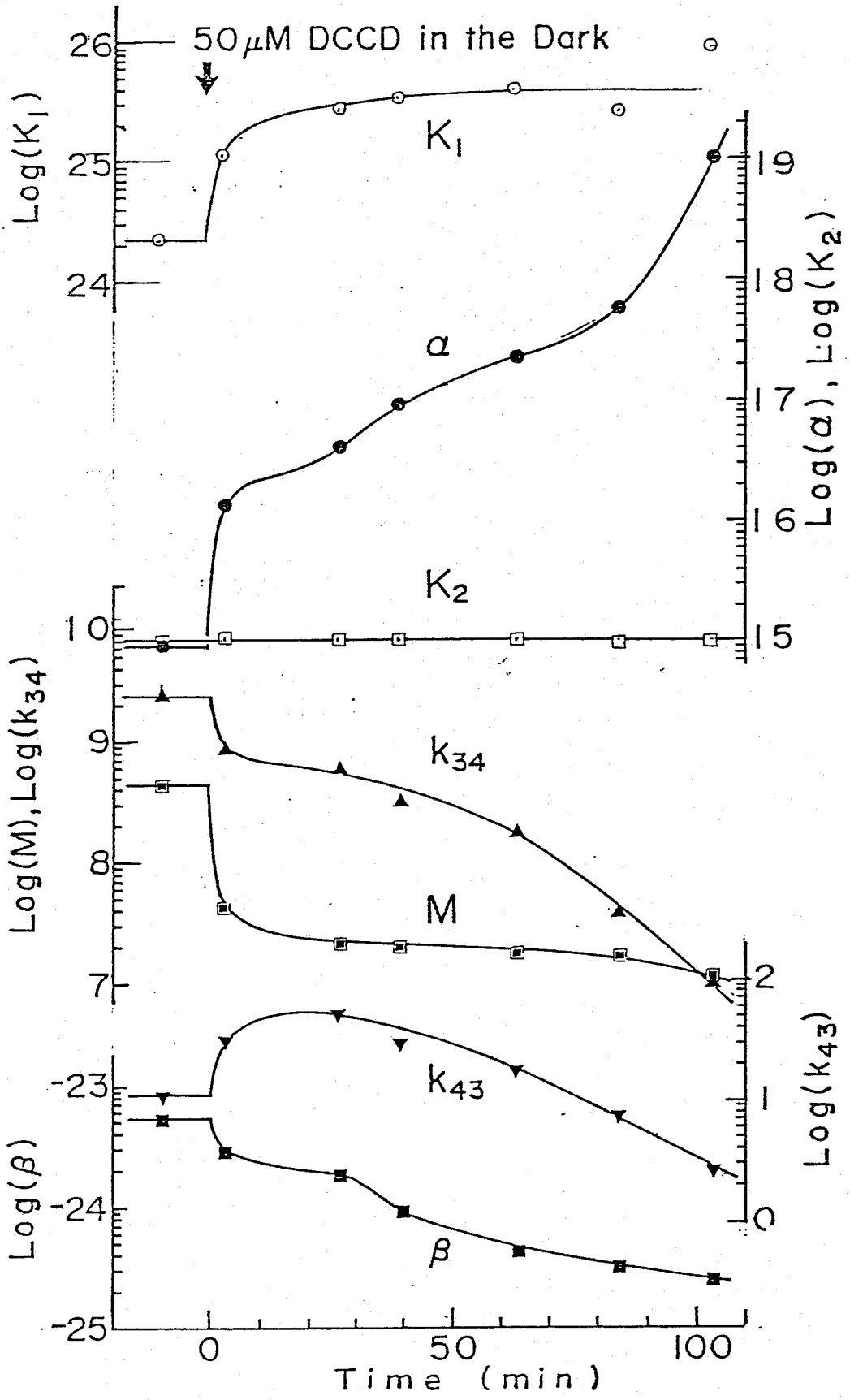


Fig. 50 The change of parameters in the kinetic model during DCCD poisoning in the dark. These parameters were calculates from the data of Fig.39.

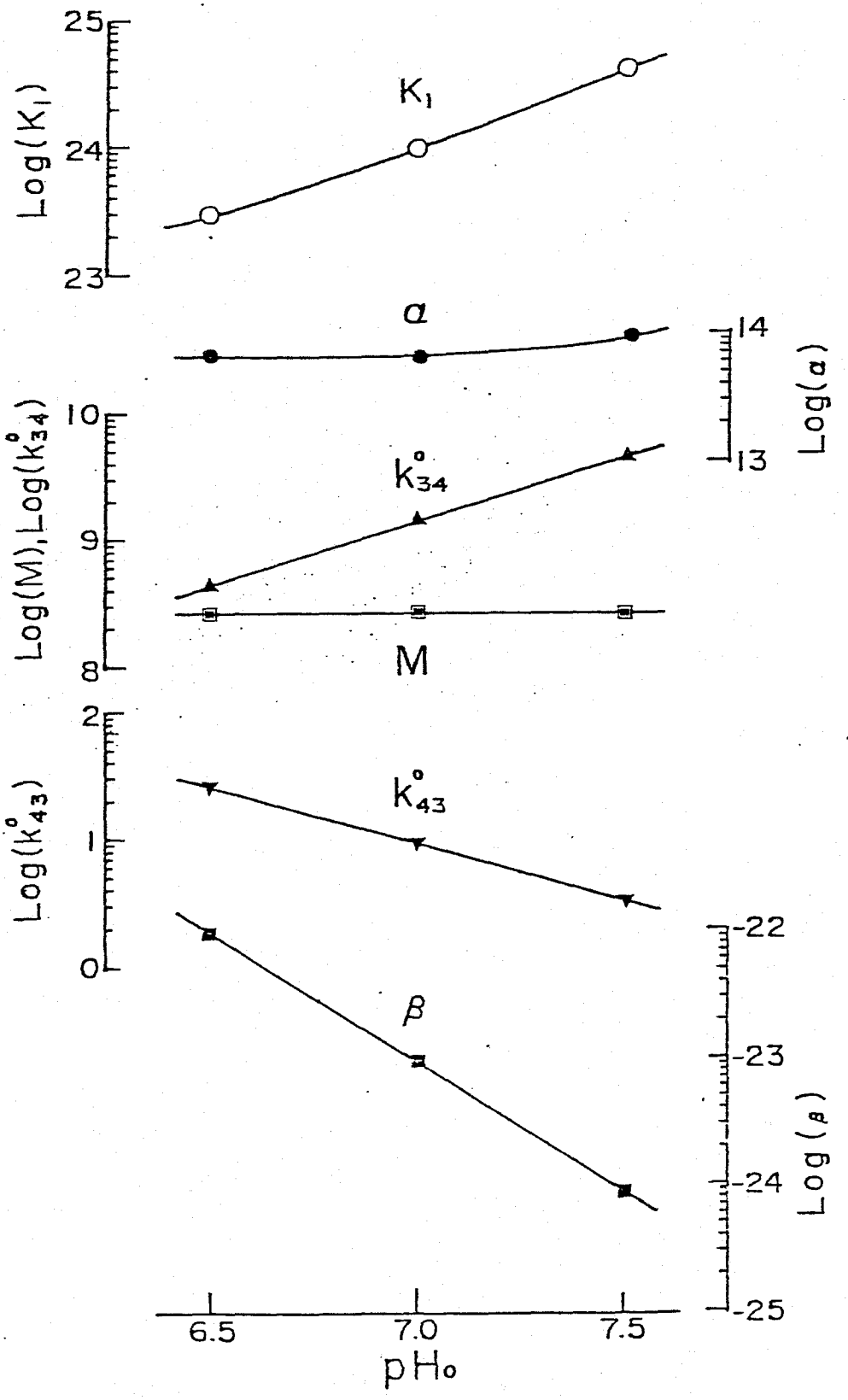


Fig. 51 The change of parameters in the kinetic model with external pH change. These parameters were calculated from the data of Fig.42.

only a little with dark treatment (1975). So in the case of dark treatment , I must consider another contribution to explain the hyperpolarization of E_p. As shown in Fig.52, I assumed that both H^+ pumps of stoichiometric ratio 1 and 2 exist in Chara corallina. Practically the i_p-V curve could be simulated under an assumption that in the range from -300mV to -150mV another constant outward current exist in addition to the H^+ pump of stoichiometric ratio 2. The simulated curve of the data and the parameters of two electrogenic pumps were shown in Fig.53 and 54 using the data from Fig.45 and 46. When E_p was hyperpolarized during dark treatment, the contribution of Ip_1(pump current of the stoichiometric ratio 1) became comparatively greater than Ip_2 (pump current of the stoichiometric ratio 2) compared with control (Fig.53). On the other hand , in a case when E_p depolarized during dark treatment, Ip_1 decreased as shown in Fi.54.

Recently the effect of fusicoccin was analysed on the plasmalemma of Sinapis alba (Felle ,1982). From the current-voltage analysis of this root hair cell, the pump current increased and E_p shifted to hyperpolarize with fusicoccin. On the other hand, Lucas reported a large hyperpolarization in membrane potential such as from -250 mV to -400 mV by dark treatment in Chara corallina (1982). This result suggests that the existence of the pump, the emf of which is more negative than -400 mV.

There are many reports about other transport system beside the H^+-ATPase such as $2H^+$:$1Cl^-$ co-transport in Chara (Sanders:1980). On the other hand, the light dependent mosaic characteristics of Chara membrane were studied electrophysiologically (Lucas & Dainty:1977, Walker & Smith:1977). In their reports HCO_3^- influx is associated with acid and alkaline band formation. In the alkaline zone formation of OH^- (H^+) channel was proposed (Bisson & Walker:1981). The relationship between H^+-ATPase and other transport system and the relationship between two types of H^+-ATPase which have the different value of stoichiometric ratio remain to be studied.

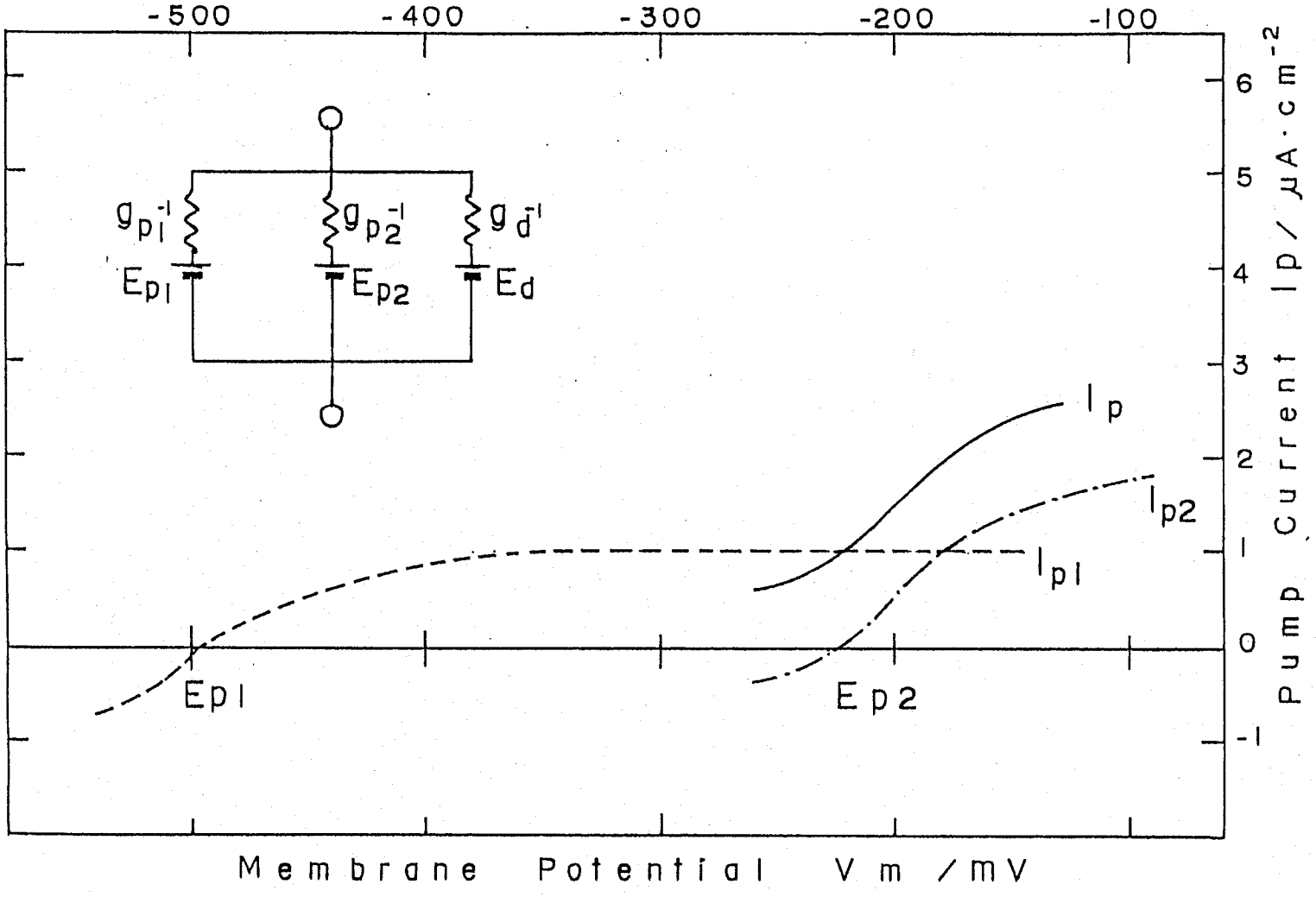


Fig. 52 The model of two proton pumps.

When both of stoichiometric ratio = 1 (E_{p1}, g_{p1} -----) and 2 (E_{p2}, g_{p2} —·—·—) exsist in parallel, I_p-V curve of the pump channel (——) is the total of these two I_{p1}-V and I_{p2}-V curves.

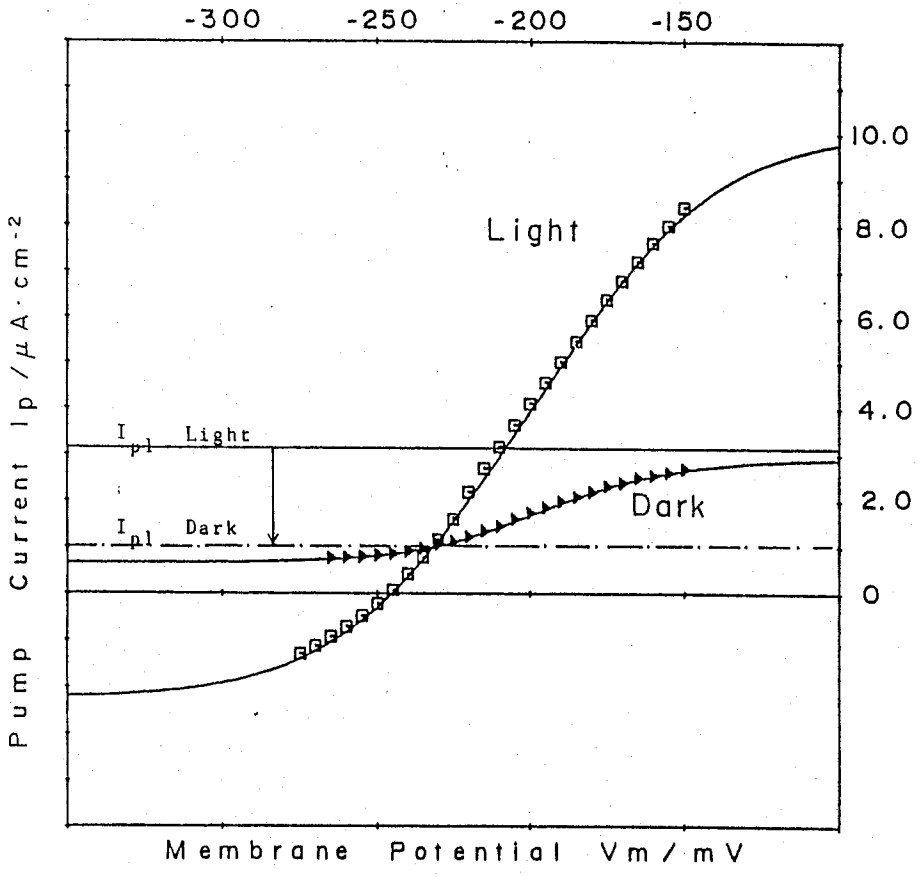


Fig. 53 The pump current of stoichiometric ratio = 1 (I_{pl}) calculated from the data of Fig.46.

Both I_{pl} and I_{p2} decreased in the dark, but the contributio I_{pl} increased in the dark compared with I_{p2}.

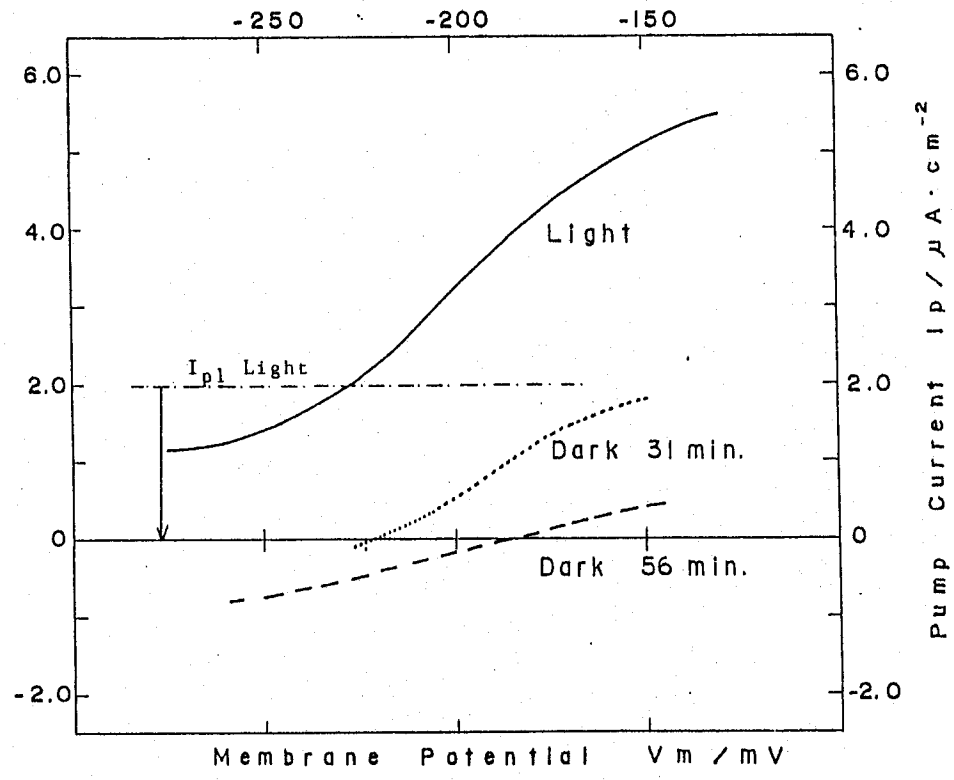


Fig. 54 The pump current of stoichiometric ratio = 1 (I_{pl}) was calculated from the data of Fig.47.

I_{pl} decreased largely in the dark, while I_{p2} still remained.

Summary

1. Membrane conductance and membrane emf could be obtained accurately by the method of curve fitting of the voltage response caused by the test current pulse under current clamp condition.
2. The emf change of the pump in Chara corallina against external pH was about 50 mV/pH in unperfused cell and 60 mV/pH in perfused cell. This value suggests that the pump in Chara is almost consisted with H^+ extrusion pump and the H^+-pump of plasmalemma mainly contributes to the membrane potential.
3. The H^+-pump in Chara is driven by the energy of ATP hydrolysis.
4. The i_p-V relation of pump current is sigmoidal.
5. The i_p-V curve of Chara membrane could be simulated by a kinetic model. This model is based on the assumption that H^+-ATPase incorporated mH^+ using the energy of hydrolysis of one molecule of ATP and then mH^+ was translocated by the membrane potential. These mecahnism are working by a cyclic transition of ATPase.
6. The value of E_p is about -250 mV at pH 7.0. This suggests that the H^+-pump of stoichiometric ratio =2 is acting mainly in the Chara membrane. But in Chara corallina in the dark another H^+ pump having stoichiometric ratio one seems working. Different contribution of two pumps might cause the hyperpolarization of the electromotive force of the pump in the dark.
7. DCCD and TPC seem to be ideal inhibitors of the pump. These reagents inhibit the activities both of these two pumps by mainly inhibiting the H^+ releasing reaction.
8. Dark treatment reduces the kinetic parameters of the pump mechanism. However, the extent of reduction of the pump activity is not large. In other words, the effect of darkness is far from complete inhibition of

the pump. ATP level did not decrease by the dark. The membrane conductance decreased to half level of the control, while the membrane potential did not depolarize much. Dark treatment is suggested to reduce the contribution of the H^+-pump, the stoichiometry of which is 2.

Acknowledgement

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Some of the results in this experiment were already published in following papers.

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Kishimoto, Kami-ike, Takeuchi and Ohkawa (1982) An inproved method for determining the ionic conductance and capacitance of the membrane of Chara corallina. Plant & Cell Physiol. 23:1041-1054.

Chapter2-----Kishimoto, Kami-ike and Takeuchi (1981) A quantitative expression of the electrogenic pump and its possible role in the excitation of Chara internodes. In The Biophysical Approach to Excitable Systems. Edited by W. J. Adelman, Jr. and D. E. Goldman. p.165-181. Plenum Publishing Corp, New York.

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