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Hysteresis in the Responses of Membrane Potential, Membrane Resistance, and Growth Rate to Cyclic Temperature Change[1]

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ABSTRACT

Measurements of electrical potential, membrane resistance, and elongation rate have been carried out on the developing pollen tube of *Oenothera drummondii*.

The plasmalemma potential was observed to be −138 millivolts ± 19 (SD). Approximately 70% of this potential was apparently due to the operation of an electrogenic pump(s). KCN rapidly and reversibly depolarized the potential to about −40 millivolts. Lowering the temperature from 20 to 4 C brought about similar rapid depolarization. The effects of KCN and of temperature were in no degree additive. KCN had only a small effect on membrane resistance. In contrast, the latter was markedly increased by lowering the temperature.

When the pollen tubes were submitted to cyclic temperature changes striking hysteresis effects were observed in the response of all three parameters, membrane potential, resistance, and growth rate. The hysteresis pattern for potential differed from that for resistance but resembled that for growth rate (measured simultaneously on the same pollen tube). The correlation coefficient between potential and growth rate was very high.

The probable relevance to our results of the hysteresis phenomena associated with "clustering" and phase transition in lipids is pointed out. Attention is also drawn to the possible significance of the large difference between the electric potentials at the start of the cooling and heating paths, respectively.

The germinating pollen tube has a number of important advantages from the point of view of electrophysiological studies. It is a single cell of dimensions far exceeding those of most higher plant cells, reaching a length of many mm *in vitro* and a diameter of about 18 μm. This facilitates the insertion of microelectrodes. Since it is an isolated cell, its electric phenomena can be studied without interference from neighboring cells (*cf.* 20). Moreover, it is fast growing; cell elongation can be followed over short time intervals and compared with the electric potential measurements made simultaneously on the same cell. Growth and potential measurements have hitherto rarely been collected simultaneously; and where they have been, growth has been assessed from the aggregate growth rate of a tissue segment (*e.g.* 19). These considerations led us to undertake a simultaneous study of the electrical properties and the growth of the developing pollen tubes of *Oenothera drummondii*.

During the course of our investigation Jaffe, Weisenseel, and their collaborators have published a number of interesting observations (13, 27, 28) with regard to electric currents traversing the length of lily pollen tubes as measured by external electrodes. They have reported a rapid and parallel reduction in growth and current when the K^+ concentration was reduced.

In this first communication we present evidence for the operation of an electrogenic pump and report on marked hysteresis patterns discernible in the responses of membrane potential, membrane resistance, and cell elongation rate to cyclic temperature changes.

MATERIALS AND METHODS

Flowers of *O. drummondii* Hook were picked on the campus of the University immediately after their evening opening. They were stored for a maximum of 3 days at 2 C, during which period full viability of the pollen grains was maintained. Before experiments pollen grains were removed from the flowers and placed on blocks of 1% agar of the following standard composition: 1 mM K^+, 1.3 mM Ca^{2+}, 1 mM Mg^{2+}, 3.7 mM NO_3^-, 1 mM SO_4^{2-}, 1.7 mM H_3BO_3, 300 mM sucrose. The pH of this culture medium was between 5.5 and 6.0. The pollen grains germinated within 20 min at 26 C and when the pollen tubes had reached a length of 30 to 100 μm they were transferred to the laboratory and used for the experiments at 20 C.

Electrical Measurements. Micropipettes were prepared and filled with a filtered solution of 2 M KCl in 1% agar (3). The high viscosity of the latter countered the tendency of the cytoplasm to plug the electrode tip. The pipettes used were less than 0.5 μm in diameter with a tip potential of 20 to 30 mv and a resistance of 20 to 40 mΩ as measured in the culture medium. They were held by electrode holders (EH-2R, W. P. Instruments). The reference electrode was of the type RC-1 supplied by the same company. The electrodes were connected to a high impedance amplifier (locally constructed). The voltage was displayed on a Tektronix storage oscilloscope, model 5103 N/D 15 with 5 A 20N dual trace amplifier and 5 B 10N time base amplifier. It was also recorded on an X-Y recorder (Yokogawa Technicorder 3078).

Membrane resistance was measured by application through an inserted microelectrode of a sinusoidal current (frequency 1 Hz, amplitude 3 $\mu amp \cdot cm^{-2}$) supplied by an HF VGG generator (Wavetek model 12). This current electrode was connected to a high impedance input electrometer amplifier (Keithley 603) and to X axis of the recorder. A second electrode, which measured the potential, was connected to the Y axis. It was inserted into the pollen tube approximately 150 μm from the first. The resistance was calculated from the slope of the long axis of the elliptical curve relating voltage to current. Correction for phase angle (equation 4.6 of ref. 4) would only raise the estimated resistance by at most 5%. On the other hand, since the space constant was estimated to be 1.5 mm, the membrane resistance will have been underestimated by about 10% because of the cable effect. (The space constant observed is intermediate between that reported for *Acetabularia* [4] and that quoted for higher plant cells [4]. It is possible to insert two electrodes in pollen tubes considerably further apart than is feasible in most higher plant cells, and this

[1] The data are taken from a dissertation to be submitted by H. M.-H. to the Hebrew University of Jerusalem in partial fulfillment of the requirements for a Ph.D. degree.

may lessen membrane injury and therefore current leakage.)

Routinely, the microelectrodes were inserted into the pollen tubes under a binocular microscope (Nikon) at a magnification of ×40, using micromanipulators (Narishige).

Growth Measurements. Elongation of the pollen tubes was followed by photographing them through the binocular microscope with a Nikon semiautomatic microflex EFM M355 camera. Substage illumination was provided by a flashlight (Metz Mecablitz III). Elongation rate was assessed by comparison of sequential photographs taken at known time intervals (Fig. 1). The operation of the flashlight induced a brief change in current in the recording system, thus indicating on the tracing the timing of the photographs and providing a means of synchronizing growth and potential measurements.

Experiments Involving Changes in Temperature. A glass cell was constructed with inlet and outlet tubes through which water at various temperatures could be circulated from a Hetofrig thermostatted bath. A deep depression in the upper surface of this glass cell contained the thin agar block (1 mm thick) bearing the pollen tubes. A thermistor (RFL Industries 27687-5) was fixed to the upper surface of the glass cell next to the agar block and connected to an electrometer (Keithley Par-TM model 134). The temperature was calculated from the measured resistance.

RESULTS

The membrane potential of *Oenothera* pollen tubes germinating on our standard agar was −138 mv ± 19 (SD). This figure is based on over 100 separate determinations. The standard deviation indicates that the potential varied comparatively little over the seasons and the years of this investigation. This potential can be regarded as the plasmalemma potential, since first, it was observed when the electrode was inserted within 200 μm of the tip, where no vacuole or tonoplast is yet observable; and second the tonoplast (vacuolar) potential is in any case small compared with the plasmalemma potential, as will be demonstrated in a future communication (Melamed-Harel and Reinhold, in preparation). Its contribution to the sum of the two potentials in cases where the electrode penetrates the tonoplast is small enough to lie within the experimental error of the determination of the plasmalemma potential. The potential accords well with that observed by Matschkal and Weisenseel (27) in lily pollen tubes.

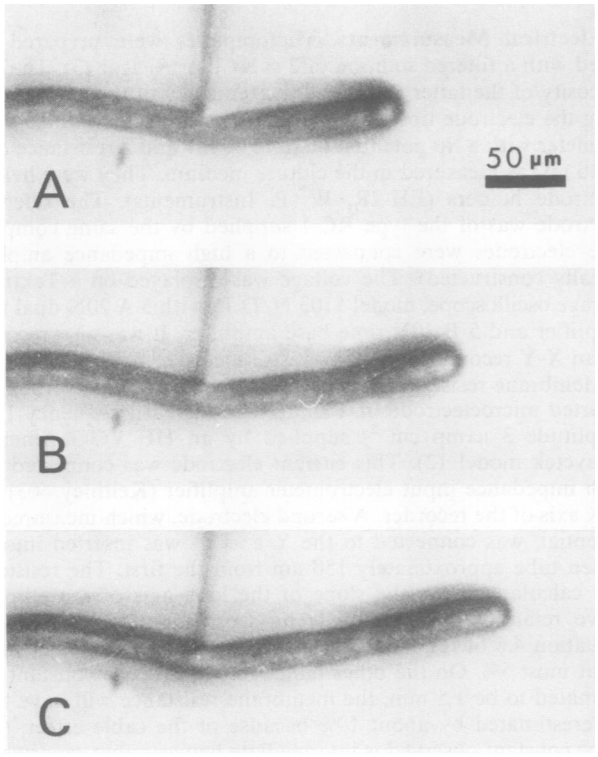

FIG. 1. Sequential photographs of tip of elongating pollen tube as used for estimating elongation rate. Time interval between A and B, 100 s; between B and C, 70 s.

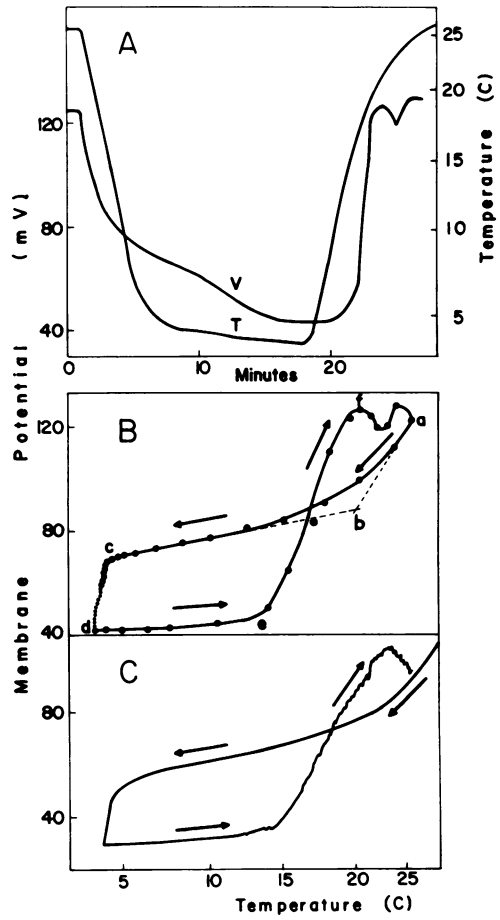

FIG. 2. Effect of temperature on membrane potential. A: tracings of potential (V) and of temperature (T) from experiment where temperature was first lowered and then raised again. B: analysis of potential shown in A as a function of temperature. Points indicate half-min intervals. For explanation of letters and dotted lines, see text. C: potential as a function of temperature obtained in another experiment by simultaneous recording on an X-Y recorder. Note: In all three figures the temperature scale is nonlinear.

Effect of Temperature on Membrane Potential. In the experiment shown in Figure 2 the temperature was lowered from 26 to 4 C over the course of about 6 min (see thermistor trace in Fig. 2A). An immediate effect is seen on the potential which dropped at first rapidly and then more slowly to reach a level approximately 30% of the initial value. When the temperature was raised again, a rise in potential followed only after a lag period of about 2.5 min. The rise in potential was then steep.

When the changes in potential described above were analyzed as a function of temperature, a hysteresis pattern clearly emerged (Fig. 2B). Figure 2C is an example of a pattern obtained in a subsequent experiment by direct recording of potential *versus* temperature on an X-Y recorder. The cooling pathway differs from the heating pathway. The former shows three phases: a steep drop in potential, followed by a more gradual drop, followed again by a steeper drop. On reheating, on the other hand, there is at first a phase of little or no rise in potential, followed by a sharp rise. (We judge it desirable to present the actual recorder tracings in these experiments, though this necessitates a temperature scale

nonlinear at its extremes, due to the characteristics of the thermistor. Replotting the data on a linear temperature scale changes the picture to only a very slight extent.)

That the hysteresis effect is not solely a result of a time lag in response to a rise in temperature (Fig. 2A) is indicated by the fact that the hysteresis pattern was also observed when temperature was raised more slowly. The points of inflection of the curve were determined in 10 separate experiments, and, in spite of the fact that the time taken for the full temperature cycle ranged from 15 to 60 min in these experiments, the inflection points varied remarkably little. They were as follows (for assignment of letters to the various points see Fig. 2B. The point b has been obtained from the point of intersection of the extrapolated dotted lines as shown in this figure): b, 20.5 C ± 0.7; c, 5.5C ± 0.5; d, 4.2 ± 0.2; e, 14.2 ± 0.5; f, 21.4 ± 0.4.

Effect of KCN on Membrane Potential. Figure 3A shows that when 5 mM KCN was added to the pollen tubes, the potential dropped immediately, reaching a level approximately 30% of the initial value. This effect may also be seen in Figure 3C. Provided treatment with the inhibitor was not too prolonged, washing away the KCN resulted in an immediate rise of the potential which regained its initial level (Fig. 3A).

KCN and Temperature Effects are Nonadditive. In the experiment shown in Figure 3B the temperature of the pollen tube was lowered to 5 C resulting in a drop in potential to a steady low level. Application of KCN at this stage brought about no further fall in potential (see arrow in Fig. 3B). In the experiment shown in Figure 3C the order of the treatments was reversed. KCN treatment produced a drop in potential; subsequent lowering of the temperature had no further effect (compare thermistor and potential tracings in Fig. 3C).

Effect of KCN and Temperature on Membrane Resistance. KCN had only a small effect on the electrical resistance of the membrane. In a typical experiment the resistance was initially 19.5 $k\Omega \cdot cm^2$, and 22.5 $k\Omega \cdot cm^2$ after depolarization. This result recalls similar observations by other workers. Slayman (23) observed only a slight effect on resistance in *Neurospora* at a point where depolarization by azide was maximal; and Anderson *et al.* (1) also reported that after depolarization was completed, resistance in CN-treated roots was approximately equal to the initial value.

In contrast to KCN, lowering the temperature had a marked effect on electrical resistance. The effect of low temperature on resistance was approximately the same in the presence or absence of KCN. For example, a fall in temperature from 24 to 5 C raised resistance from 15 to 60 $k\Omega \cdot cm^2$ in the absence of KCN, and from 12 to 54 $k\Omega \cdot cm^2$ in its presence.

When the resistance was followed during cyclic temperature change, a hysteresis pattern was again observed (Fig. 4). The pattern, however, differed from that for the membrane potential (compare Figs. 2 and 4). The resistance rose sharply on cooling between 24 and 16 C, then flattened between 16 and 4 C. As cooling continued beyond 4 C, the resistance dropped again. On warming, resistance decreased relatively steeply until approximately 10 C, above which a gradual decline was observed. The resistance of the warmed-up pollen tubes was strikingly lower than that observed before cooling.

Hysteresis Pattern for Effect of Temperature on Growth. In a number of experiments the rate of elongation of the pollen tube was observed together with membrane potential as the temperature was first lowered and then raised again. When the rate of elongation was analyzed as a function of temperature, hysteresis was observed. (Fig. 5, A and C, shows two such experiments.) The hysteresis pattern resembled that for membrane potential measured simultaneously in these experiments (Fig. 5, A and C). Both elongation rate and potential fell with decreasing temperature; on reheating virtually no change was observed in either potential or elongation rate as the temperature rose from 4 to about 13 C.

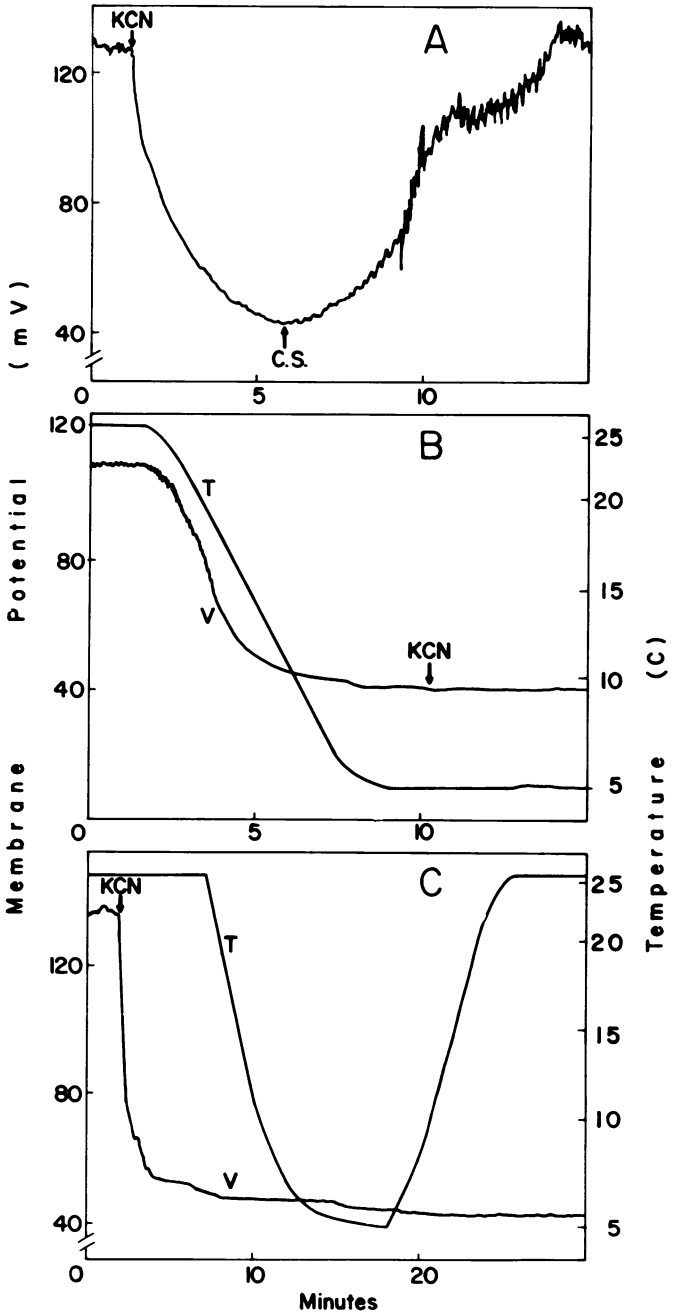

FIG. 3. A: effect of KCN on membrane potential. Arrows mark placement of a drop of 5 mM KCN in culture solution on the pollen tube, and washing away the KCN with culture solution (C.S.), respectively. B: effect of KCN after lowering temperature. C: effect of lowering temperature on potential of KCN-treated tissue. V: potential; T: temperature.

Above the latter temperature both parameters rose sharply, the elongation rate starting its rise at a somewhat lower temperature than the potential.

When potential was plotted against log growth rate in these experiments, the points for the cooling pathway were observed to fall on a straight line (Fig. 5, B and D). The correlation coefficients are 0.99 in both figures.

DISCUSSION

The plasmalemma potential of the pollen tubes apparently results to a large extent from the operation of an electrogenic pump or pumps. This conclusion is based on the rapid and

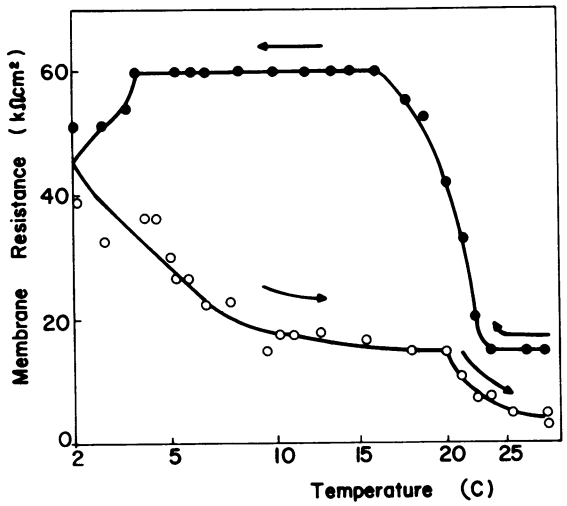

FIG. 4. Membrane resistance as a function of temperature. Pollen tube was first cooled (●) and then heated again (○).

reversible polarization brought about by treatment with KCN, and by lowering the temperature from 20 to 4 C. Electrogenic pumps have now been implicated in a number of fungal, algal, and higher plant cells (5, 8, 9). Weisenseel and Jaffe (27) inferred from indirect evidence that most of the steady-state growth current through lily pollen tubes was carried in by a K^+ leak and out by a proton pump.

When the membrane had been depolarized by KCN, lowering the temperature did not reduce the potential further (Fig. 3C). Hogg *et al.* (10) and Hope and Aschberger (11) have concluded that low temperature decreases the passive permeabilities to ions of the plasmalemma in *Nitella* and *Chara*. We have observed an increase in membrane resistance with decrease in temperature (Fig. 4) which suggests a similar effect on passive ion permeabilities in the case of pollen tubes. The fact that low temperature affected resistance to the same extent in the absence of KCN as in its presence, *i.e.* when the electrogenic pump was abolished, confirms that the *passive* ion permeability was decreased. In the *Chara* work it was further concluded (11) that the drop in membrane potential observed at low temperature was attributable to the altered and unequal changes in ion permeabilities. In our inves-

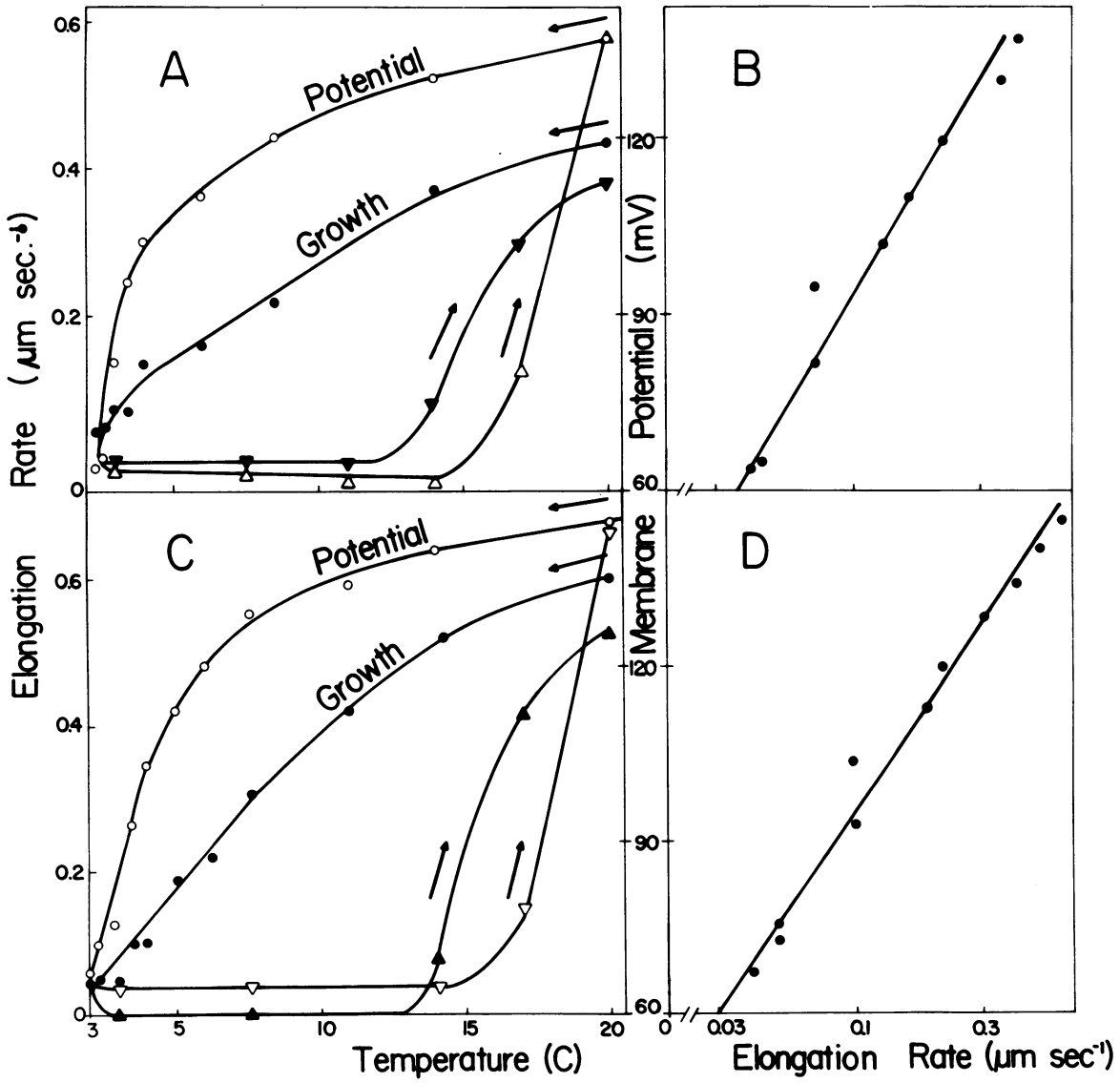

FIG. 5. Effect of temperature on growth and on membrane potential, measured simultaneously on same pollen tube. (○, ●): Cooling path; (△, ▲): heating path. A and C show two different experiments. B and D give the relationship between potential and log growth as taken from A and C, respectively (cooling path). Line drawn is calculated regression line through points.

tigation, on the other hand, the altered passive permeabilities do not appear to have contributed appreciably to the depolarization observed. The residual potential remaining in the presence of CN was presumably a passive diffusion potential, and lowering the temperature had no detectable effect on this residual potential. (The very slight downward drift in potential seen in Figure 3C when the CN-treated pollen tube was cooled, was not due to the drop in temperature, since reheating did not reverse it.) The question arises as to why the fraction of the total potential attributable to passive diffusion should not be affected by changes in passive permeabilities. A reasonable answer would seem to be that the decrease in permeability affected all of the permeating ions responsible for this diffusion potential equally, thus leaving the latter unchanged.

Closer study of the effect of lowering and raising the temperature on membrane potential revealed clear-cut hysteresis (Fig. 2, B and C). The fact that raising the temperature almost 10 C between 4 and 14 C had little or no effect on potential, whereas a subsequent increase produced a sharp and sudden rise, is suggestive of a phase transition in certain of the membrane components. Lee (15) has emphasized that the transition from the gel to the liquid-crystalline state in lipids is not identical to that for the transition from the liquid-crystalline to the gel. Hysteresis is, therefore, not unexpected in a membrane phenomenon; it is particularly likely in complex mixtures of lipids and even more so when the latter are charged (16). Pronounced hysteresis has in fact already been reported in the case of the potential of the squid axon membrane when subjected to cyclic temperature change (12). It is probable that initiation of "clustering" of lipids (see 17) rather than actual phase transition is involved in our experiments since the inflections observed in our curves occur at relatively high temperatures. Evidence has already been obtained (17) for cluster formation in native sarcoplasmic reticulum membranes at temperatures of about 20 C.

In addition to phase changes or clustering in membrane components, it is possible that time lags may also have contributed to our observed hysteresis patterns. Such time lags would reflect periods needed for the restitution of critical subprocesses after cold treatment (*e.g.* 21).

Two steep falls in potential are distinguishable on the cooling path (a → b and c → d in Fig. 2B) whereas only one steep rise (e → f) is clearly evident on the heating path.

The hysteresis curve for resistance (Fig. 4) differs markedly from that for potential. One very prominent change is evident during cooling as the resistance increases sharply between 24 and 16 C. Below 4 C it drops again, an effect possibly connected with leakage of ions through discontinuities in the packing of the membrane formed as a result of the phase transitions (6). The second drop discernible in the curve for potential (c → d) may relate to the same cause. No marked transition is visible in the heating pathway for resistance.

The low resistance of the warmed-up pollen tube, as compared with resistance before cooling, may possibly result from cold injury. It is postulated that in cold-sensitive plants certain membrane lipids undergo irreversible phase change at low temperatures (18). Such phase changes might be reflected in lower resistance.

The difference between the hysteresis patterns for potential and for resistance need not be regarded as surprising. The potential depends largely on an electrogenic pump(s), and it has been shown that the activity of membrane-bound ion-dependent ATPase in animal cells is controlled by the nearest neighbor or "annular" lipids (14, 26). Membrane resistance on the other hand may largely reflect passive ion movements (although see ref. 25) and the latter may be controlled by lipids in other regions of the membrane with different phase transitions or different points of initiation of clustering.

The magnitude of the hysteresis effect which our results show (more than 10 C) is impressive. A factor to be taken into account is that at the start of cooling the membrane potential is high, whereas at the start of heating the potential is low. The electric field itself may possibly have a marked effect on the physical state of various membrane components. Shuldiner and Kaback (22) have emphasized that evidence is accumulating for changes in the conformation of membrane proteins as a response to changes in membrane potential.

A hysteresis pattern is not only visible in the curves for potential and for membrane resistance; it is also striking in the curve for growth rate. These two effects, on electrical properties and on growth rate, might well be indirect. Nevertheless, the high correlation exemplified in Figure 5, B and D (correlation coefficients of over 0.9) tempts one to speculate that a direct relationship may be involved. An explanation that readily suggests itself, based on current views of auxin action (2) is that a dominant factor rate-limiting growth is a proton extrusion pump. This pump would then be a major contributor to the potential and to growth. The hysteresis pattern both for potential and for growth would reflect the effect of cyclic temperature changes on the operation of this membrane-bound system. However, growth is not necessarily tightly coupled to the operation of the pump under all conditions, as follows from the following considerations. The potential at 20 C during warming is equal to or greater than that during cooling; the resistance, on the other hand, is only half that during cooling. Since the pump contribution to potential is theoretically proportional to the product of pump rate and passive resistance, it would appear that the pump rate at 20 C during warming is about twice that during cooling (which may be a homeostatic mechanism to restore potential). The elongation rate at 20 C by contrast, is, if anything, lower during warming than during cooling.

An alternative explanation for the correlation between potential and growth may be that the membrane potential is itself one of the driving forces for growth, in that it may control co-transport of sugars and ions into the cell (24). However, this explanation would not predict that on rewarming, growth rate would start to rise at least 2C below the temperature at which potential starts to rise (Fig. 5, A and C). The latter effect suggests that the change in potential results from the change in growth rate, and not vice versa. Possibly the explanation lies in the observation (7, 29) that a drop in turgor can serve as a signal to activate ion pumps. If we assume that growth involves primarily a decrease in wall pressure, the drop in turgor resulting from such decrease might bring about increased activity of the electrogenic pump, thus raising membrane potential. It is to be assumed that the increased pump activity would lead to the homeostatic maintenance of internal ion concentrations, as the cell elongates and takes up water.

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