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15 June 1977; revised 9 August 1977

Osmotically Induced Changes in Electrical Properties of Plant Protoplast Membranes

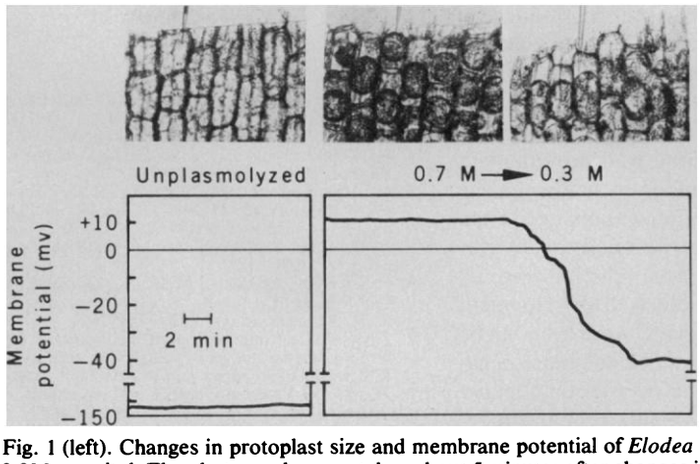
Abstract. *The internal electrical potential of protoplasts from six different plant species was positive. Plasmolyzed cells of leaves had positive voltages of similar magnitude. Both* Elodea *leaf cells and tobacco protoplasts with regenerated cell walls became more electronegative during deplasmolysis. These findings suggest that cell turgor affects membrane components that determine cellular potential.*

Since the first mass preparation of plant protoplasts by enzymatic techniques (*1*) and the regeneration of an entire plant from a protoplast (*2*), there has been much interest in the possible use of protoplasts for plant improvement (*3*). Introduction of new genetic material into the plant protoplast through transformation, transduction, or somatic hybridization becomes more feasible once the physical barrier presented by the cell wall has been removed by enzymatic digestion, but many types of protoplasts, including those from cereals, fail to develop in culture (*4*). We now report that protoplasts are electrically positive with respect to the external solution, although the leaf cells from which they were derived are electrically negative. Since isolated protoplasts must be maintained in a plasmolyzed condition during and following removal of the cell wall, we examined the effect of plasmolysis and deplasmolysis on the membrane potential of intact leaf cells, of freshly isolated protoplasts, and of older protoplasts.

For impalement of cells with microelectrodes, sections of mature leaves from oat (*Avena sativa*), corn (*Zea mays*), tobacco (*Nicotiana tabacum*), and petunia (*Petunia hybrida*) were cut and mounted in a Plexiglas chamber and bathed in 1X nutrient solution for 1 to 3 hours before impalement (*5*). Protoplasts were isolated from leaf tissue of oat, corn, tobacco, petunia, and *Datura stramonium* and stem tissue of soybean (*Glycine max*). After surface sterilization in 0.5 percent hypochlorite, the upper epidermis was stripped from the leaves and the soybean stems were split longitudinally. The areas of cells thus exposed were placed face down in an enzyme solution consisting of 1.5 percent cellulysin (Calbiochem, San Diego), 0.5 percent pectinase (Nutritional Biochemicals Corp., Cleveland), and 0.57*M* mannitol at *p*H 5.7 for 3 hours at 30°C. The released protoplasts were collected by low-speed centrifugation (75*g*) and cultured in a B-5 medium (*6*) and 0.7 percent agar. For impalement of protoplasts with microelectrodes, a 0.5 cm by 1.0 cm section was cut from the agar and placed in a Plexiglas chamber. Most measurements were made within 2 hours to 4 days of isolation; tobacco protoplasts were monitored over a 3-week period and during changes in external osmoticum.

Elodea densa was maintained in a glass beaker in 1X nutrient solution. Room air was bubbled through the medium and a combination of fluorescent and incandescent lights provided illumination at 1.2×10^4 erg cm^{-2} sec^{-1}. A leaf near the apical meristem was removed and the tip of the leaf (0.5 to 2 mm) was excised. The leaf, with the cut end up, was held in the Plexiglas chamber by a thin rubber strip on the wall of the chamber so that individual cells could be impaled with microelectrodes.

Microelectrodes with tip diameters of less than 0.5 μm were made from glass capillary tubing and filled with 3*M* KCl. The microelectrodes and a reference electrode were connected with Ag-AgCl wire to an electrometer and a chart re-



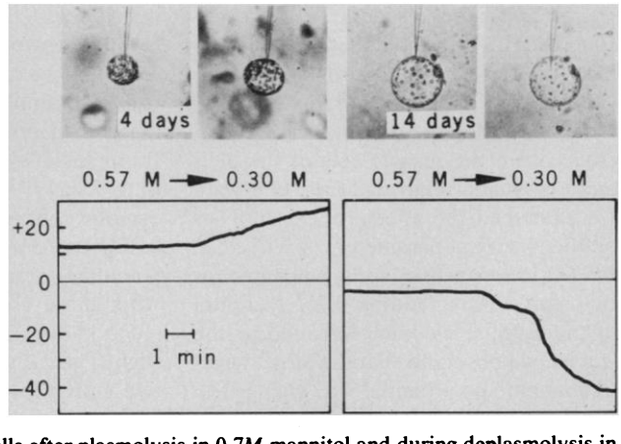


Fig. 1 (left). Changes in protoplast size and membrane potential of *Elodea* cells after plasmolysis in 0.7*M* mannitol and during deplasmolysis in 0.3*M* mannitol. The photographs were taken about 5 minutes after changes in the external osmoticum. The response of the membrane potential to external osmoticum is condensed from original chart records. This experiment was repeated three times. Fig. 2 (right). Response of young (4-day-old) and older (14-day-old) tobacco protoplasts to changes in external osmoticum. The photographs were taken about 5 minutes after perfusion of protoplasts, which were embedded in agar in 0.57*M* mannitol, with 0.30*M* mannitol. During enlargement of the 4-day-old protoplasts the potential becomes more positive. The older protoplasts have cell walls which prevent their expansion and the membrane potential becomes more negative when the osmotic concentration is lowered. Data are redrawn from chart records; these experiments were repeated six times.

corder. The system was also equipped for measuring membrane resistance by passing current through the microelectrode (*7*). The electrodes were positioned with micromanipulators and viewed under a microscope at a magnification of ×250.

Table 1 shows the levels of membrane potential and resistance for intact leaf cells and protoplasts from leaf tissue of six different plants (*8*). In the conversion of leaf cells to protoplasts, the interior potential changed from a high negative value to a low positive value and the specific resistance was lowered.

Although reduction of the negative potential could be accomplished by general lowering of resistance, induction of positive voltages cannot be explained solely on this basis. To interpret positive voltages as a membrane-related response one must postulate that the relative rates of some cation and anion transfers have been altered. However, it may be argued that the appearance of junction potentials at the tip of the microelectrode (*9*) during the passage of the electrode from an area of low salt concentration (agar) into an area of higher salt concentration (protoplast) could account for the positive potentials. To test this possibility, we examined our microelectrodes in solutions of KCl ranging from 1 m*M* to 3*M* with the reference electrode in 1 m*M* KCl. The largest tip potentials in this system were 3 to 5 mv with the microelectrode in 3*M* KCl, a concentration well above those found in most plant cells. Thus tip potentials could only contribute slightly to the positive condition of protoplasts. Furthermore, the integrity of the membrane is important in maintaining the positive voltages in protoplasts. Any disruption of the membrane-electrode sealing process, such as by slight side-to-side movement of the electrode, collapsed the potential.

Since the main perturbations to the leaf cells during the production of protoplasts were the plasmolysis of the cells and enzymatic removal of the cell wall, we examined the effects of plasmolysis on the electrical parameters of leaf cells. *Elodea* leaves proved most amenable for this study. After adding 0.7*M* mannitol to the bathing medium surrounding the leaves, we observed that the large, negative membrane potential was changed to a positive voltage following plasmolysis of the leaf cells. Because the electrode became dislodged from the membrane during this manipulation of the osmotic environment, cells were impaled in the unplasmolyzed state and the electrode was removed before plasmolysis. The electrode was reinserted after plasmolysis in 0.7*M* mannitol and remained implanted during deplasmolysis in 0.3*M* mannitol. Figure 1 shows changes in membrane potential of the leaf cells during these changes in external osmoticum. The electrode was again withdrawn and further deplasmolysis was accomplished by lowering the osmotic concentration in 0.1*M* increments over a period of 30 minutes. Measurements of potential of a number of cells were taken about 5 minutes after each change. At 0*M* mannitol the average potential of the deplasmolyzed cells was −88 mv. After 4 hours at this level of osmoticum, the potential was −104 mv. The osmotic induction of a positive voltage and the return of the voltage to more negative levels during deplasmolysis in *Elodea* cells suggest that turgor pressure influences the level of potential in plant cells.

Table 1. Membrane potential and resistance measurements of intact leaf cells and leaf protoplasts. See the text for experimental details. Values are the averages of 3 to 18 determinations.

Plant	Intact cells		Protoplasts	
	Potential (mv)	Resistance (kilohm/cm²)	Potential (mv)	Resistance (kilohm/cm²)
Oat	−85.7	2.4	+11.1	0.6
Tobacco	−78.1	3.1	+ 7.7	1.6
Corn	−65.0	3.6	+ 6.4	0.4
Petunia	−71.8	2.8	+ 9.3	0.9
Datura			+12.5	0.3
Soybean			+10.1	0.5
Av. S.D.*	5.8	0.7	1.7	0.2

*Average standard deviation.

The results with *Elodea* leaves were supported by experiments with tobacco protoplasts. In testing the effect of a reduced external osmotic concentration on tobacco protoplasts, we found that the extent of the regeneration of the cell wall played an important part in the ability of the cell to restore a negative potential. Protoplasts with little wall development (0 to 7 days) expanded in size when the osmotic concentration was changed from 0.57*M* to 0.3*M* or lower, and the interior potential became more positive (Fig. 2). After more complete wall development (10 to 14 days), the cells were found to be slightly negative and became more negative with a lowering of osmotic concentration (Fig. 2). We interpret these results in the following manner. Deplasmolysis of protoplasts without cell walls causes the protoplasts to expand and thus to develop little additional internal pressure. The resulting extension of the membrane enhances an electropositive condition. Deplasmolysis of protoplasts with complee cell walls causes an increase in internal pressure which favors processes that increase electronegativity. The finding that the protoplasts have spontaneously reverted from a positive to a slightly negative condition after several days suggests that cell turgor has increased slightly or that some membrane–cell wall association is also important in the maintenance of negative voltages.

Although pressure-related changes in membrane potential have been detailed in large algal cells (*10*), in *Drosophila* mitochondria (*11*), and recently in oat coleoptile cells (*12*), we do not know what alteration of membrane properties causes higher plant cells to change from a negative to a positive potential during plasmolysis of cells or removal of the cell wall. The generation of negative potentials in plant cells probably involves contributions from ion diffusion and an electrogenic pump (*13, 14*). Either changes in membrane permeability or active transport, or both, may be involved in the pressure-induced changes in membrane electrical properties. Measurement of membrane electrical properties during protoplast development may be a useful indicator of the subsequent ability of the protoplast to divide, differentiate, and ultimately regenerate an entire plant.

Note added in proof: It has come to our attention that similar changes in electrical properties of protoplasts have recently been reported by Heller (*15*). Also, Lin *et al.* (*15*) reported that isolated plant vacuoles have positive potentials.

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16. We thank G. Geballe for help in initiating this work. Aided by grants from the National Aeronautics and Space Administration and the RANN program of the National Science Foundation to A.W.G.

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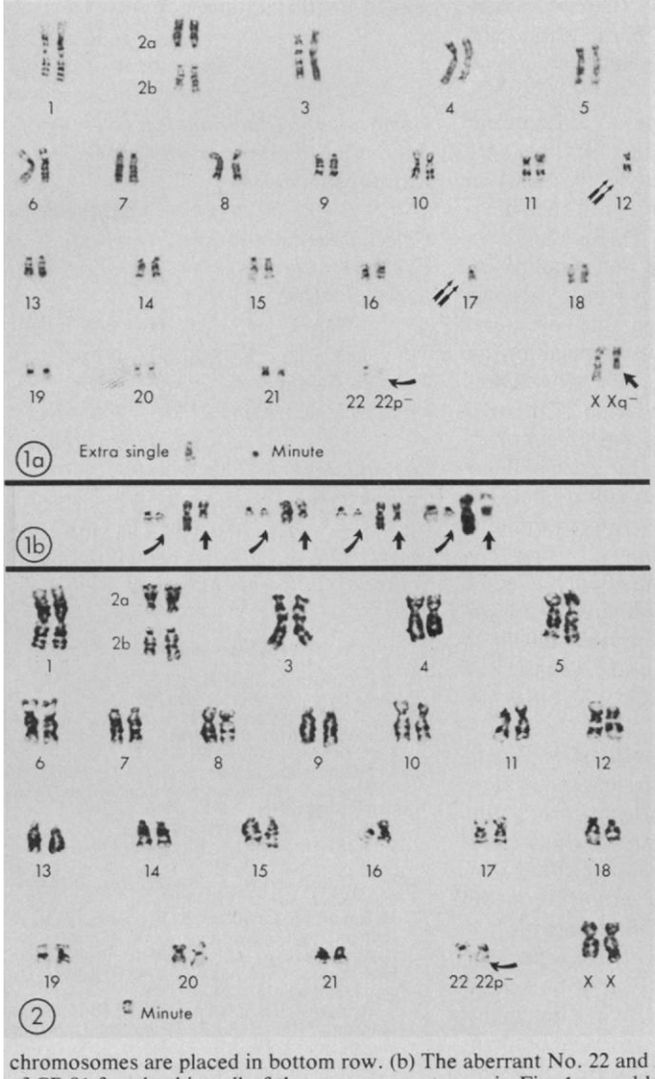
Establishment of a Cell Line with Associated Epstein-Barr–Like Virus from a Leukemic Orangutan

Abstract. *An Epstein-Barr virus like herpesvirus has been isolated from a lymphoid cell line derived from an orangutan with spontaneous myelomonocytic leukemia. Herpesvirus has not previously been isolated from this species of higher ape.*

We have isolated and partially characterized a herpesvirus similar to Epstein-Barr virus (EBV) (*1*) produced by a lymphoid cell line derived from an orangutan (*Pongo pygmaeus*) with subacute myelomonocytic leukemia. The orangutan was a 13-year-old female, born in the wild, and housed at the Los Angeles Zoo for the past 10 years. The diagnosis of myelomonocytic leukemia was made in April 1976 by hematological and cytochemical studies of bone marrow and peripheral blood smears and was confirmed at necropsy in October 1976. The animal received neither chemotherapy, radiotherapy, nor blood transfusion.

Six months before the animal died, leukocytes were obtained from defibrinated peripheral blood by separation on a Ficoll-isopaque mixture at 20°C (*2*). The cultures were maintained in RPMI 1640 media containing 20 percent fetal bovine serum, 2 m*M* glutamine, 50 μg per milliliter of gentamicin, and 25 μg per milliliter of fungizone and incubated at 37°C in humidified atmosphere containing 5 percent CO_2. During the first 10 days of culture, the medium was also supplemented with 20 percent autologous plasma.

After an initial 4-week period of slow growth, the cells began proliferating rapidly in suspension and reached a doubling time of approximately 20 hours.



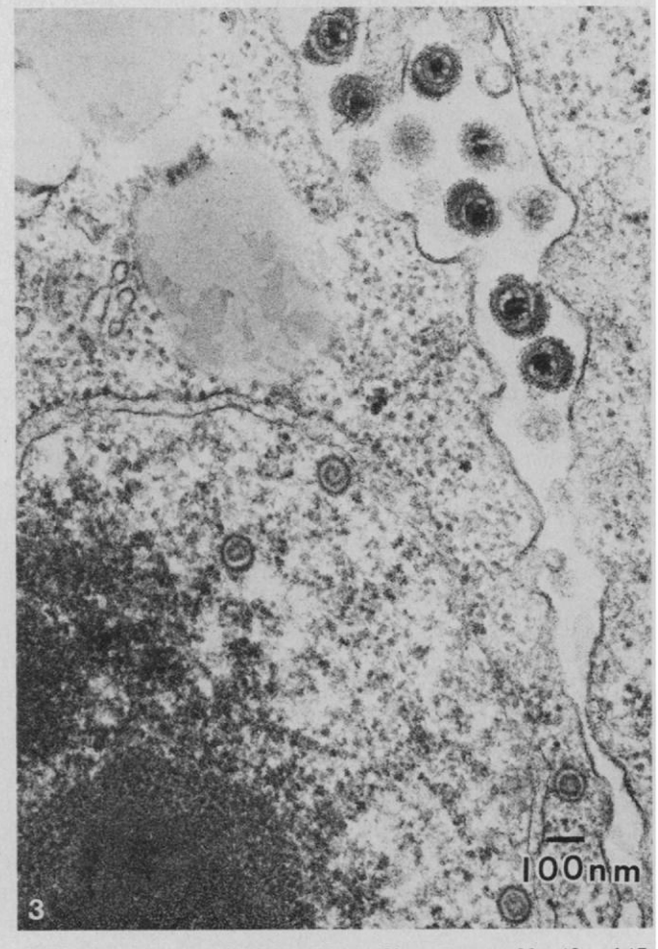


Fig. 1. (a) Karyotype of CP-81 cell (♀). Chromosomes No. 12 and 17, monosomic (double arrows). One homolog of No. 22 has a deletion in the short arm, 22 p⁻ (curved arrow), and one X chromosome is minus a portion of its long arm, X q− (short arrow). Additional nondefined chromosomes are placed in bottom row. (b) The aberrant No. 22 and X chromosomes from four additional cells of CP-81. Fig. 2. Karyotype of CP-81 female skin cell of the same orangutan as in Fig. 1, a and b, showing only a deleted No. 22 chromosome 22 p⁻ (curved arrow) and a minute chromosome. Fig. 3. CP-81, EM micrograph showing extracellular herpesvirus particles (×75,000).