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Effect of sugars and amino acids on membrane potential in two clones of sugarcane.

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EFFECT OF SUGARS AND AMINO ACIDS ON MEMBRANE POTENTIAL IN TWO CLONES OF SUGARCANE

A Thesis Presented

By

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Plant Pathology

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ABSTRACT

Sugarcane leaf parenchyma cells bathed in 1X solution maintained an average membrane potential (PD) of -135 mV in the dark. An electrogenic pump appears to contribute to PD in these cells. Sugars (25mM) added externally caused the following PD depolarizations, in mV, in clone 51 NG 97: glucose 18 ± 4, galactose 24 ± 7, 3-O-methyl-glucose 10 ± 4, sucrose 22 ± 3, fructose 21 ± 7, raffinose 9 ± 3, mannitol 0, lactose 0, melibiose 0, and 1-O-methyl-α-galactose 0. Glycine (25mM) and serine (10mM) caused depolarizations of 47 ± 7 mV and 23 ± 2 mV, respectively. Depolarization shows saturation kinetics with respect to glucose concentration, with a Km of 3-6mM. The metabolic inhibitors KCN and SHAM together greatly inhibited depolarization by 25mM glucose and 25mM raffinose. Glucose (25mM) caused almost total inhibition of depolarization by raffinose, sucrose and 3-O-methyl-glucose (all 25mM), but only partial inhibition of ΔPD to 25mM glycine. Glycine (25mM), also, only partially inhibited depolarization by 25mM glucose. Total depolarization to 25mM glycine and 25mM glucose was comparable to the active portion of PD as measured by 1mM KCN plus 1mM SHAM. No difference was found between clones 51 NG 97 and H50 7209. The results are consistent with a cotransport mechanism of membrane transport, with sugars and amino acids being transported by separate carrier systems.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

C H A P T E R I

LITERATURE REVIEW

Plant Electrophysiology

Plant cells maintain an electrical potential difference (PD) across the cell membrane with the inside about 100 mV negative to the outside (20). Higher and lower values have been reported for different tissues under various conditions. Because of the large vacuole in higher plant cells, PD is measured across both the plasma membrane and the tonoplast. The measured PD, in the vacuole, is about 10 mV more positive than the cytoplasmic PD (52). The total potential difference can arise from Donnan systems, the passive diffusion of ions across the membrane and electrogenic ion transport.

Diffusion potentials occur because the two ions of a salt normally have different mobilities. If the cell membrane is more permeable to one ion, a concentration cell is set up (1), generating an electrical potential across the membrane. Donnan potentials, which arise due to fixed charges in the cell walls, can be considered to be a special case of diffusion potentials (41). For most plant cells, the diffusion potential, when K^+ is in equilibrium with the electrochemical potential gradient, can be predicted from the Nernst equation for K^+,

$$E_k = \frac{RT}{F} \ln \frac{[K]_o}{[K]_i} ,$$

where E_k is the diffusion potential, R is the gas constant, T is absolute temperature, F is Faraday's constant and the subscripts o and i refer to the K^+ concentrations outside and inside the cell, respectively. If Na^+ and Cl^- are also significantly permeable, the Goldman equation more accurately predicts the diffusion potential,

$$E = \frac{RT}{F} \ln \frac{P_k[K]_o + P_{Na}[Na]_o + P_{Cl}[Cl]_i}{P_K[K]_i + P_{Na}[Na]_i + P_{Cl}[Cl]_o} ,$$

where P represents the permeability of the ion designated by the subscript (21).

In the 1950's and early 1960's, PD was assumed to be entirely due to passive ion fluxes across the plasma membrane (6,21). There was evidence even at this time, however, that diffusion potentials were inadequate to explain certain observations, specifically, the depolarization of PD induced by metabolic inhibitors (7). This was also the first time PD measurements were reported in higher plant cells. As this method became more widely used, certain results were obtained which were inconsistant with the diffusion theory. These included measured electropotentials too high to be accounted for by the Nernst potentials of any of the diffusible ions, hyperpolarizations upon

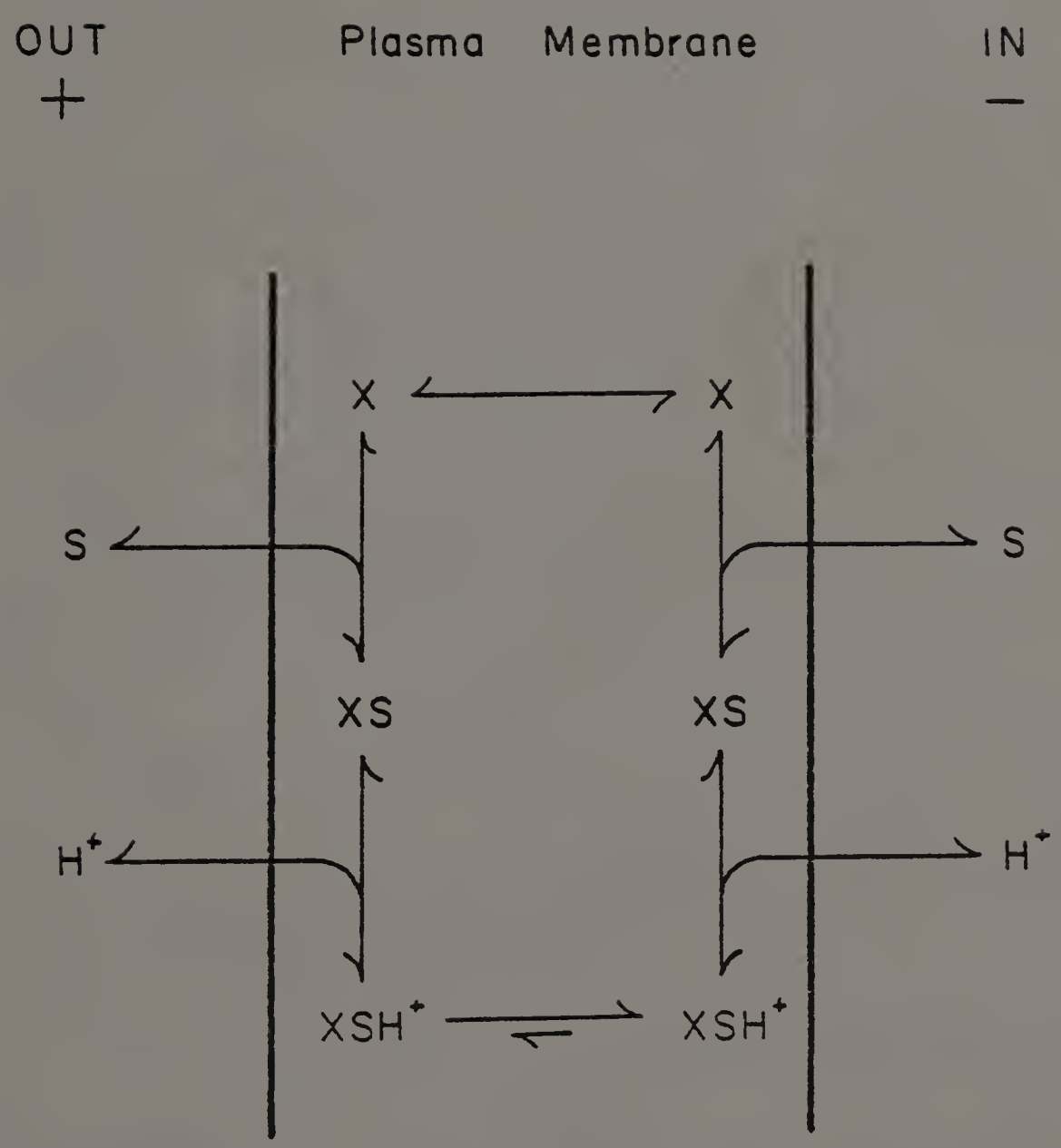
changing from dark to light in green cells, and the effect of metabolic inhibitors, which caused increased depolarization of PD with increasing concentration, up to a maximum point, which corresponded to the calculated diffusion potential (21). All of these results are consistent with a membrane potential generated by both the passive diffusion of ions and an electrogenic pump, which uses metabolic energy to drive the transport of an ion across a membrane, usually against its electrochemical potential gradient (52), resulting in a net transfer of charge (20).

There is now overwhelming evidence for an electrogenic pump in bacteria, fungi, algae, bryophytes and higher plants (19,20,50,55,59). In all of these groups, the most important and universal electrogenic system appears to be a H^+-efflux pump operating at the plasma membrane (50, 52, 56). This is an example of electrogenic uniport, where a single species is transported in one direction. In animals an antiport system is present, with three Na^+ ions pumped out of the cell for every two K^+ ions pumped in (27). Most current evidence indicates that the proton efflux pump at the plasma membrane is driven by ATP hydrolysis, with an apparent stoichiometry of two H^+ ions transported for each mole of ATP consumed (50,52). The exception is bacterial cell membranes, which, like mitochondrial and chloroplast membranes, also use redox systems to drive the pump.

The proton efflux pump serves two important functions for the cell. The most obvious of these is the regulation of cytoplasmic pH, since excess H^+ ions are produced as byproducts of cellular metabolism (52,58). The other main function of the pump is the coupling of metabolic energy to the active transport of solutes, which is predicted by Mitchell's chemiosmotic hypothesis (39,40). According to this theory, the energy produced from redox reactions and the hydrolysis of high energy bonds is coupled via the electrogenic pump to a downhill electrochemical gradient for the ion being pumped, which in turn provides the driving force for solute transport. At every step the system is reversible, the direction depending on the prevailing downhill energy gradient. The alternative to this system is one in which ATP or redox energy is supplied directly to promote uphill transport of a substance, that is, against an electrochemical gradient (50).

The chemiosmotic hypothesis has lead to the development of a cotransport theory for membrane transport in plant cells. Slayman (56) has proposed a model for proton-dependent cotransport of an uncharged substrate (Figure 1). In this model, protons and substrate at the outer membrane surface combine sequentially with a membrane carrier, forming a positively charged complex which is driven across the membrane by the electrochemical gradient for H^+. On the inner membrane surface the complex dissociates, and the

Figure 1. Slayman's cyclic carrier model for cotransport of H^+ ions and an uncharged substrate (56).



X = membrane carrier; S = substrate

H^+ and substrate are released while the carrier is recycled, The sequence is theoretically reversible at all steps, but the prevailing electrochemical gradient in plant cells tends to drive the charged complex across the membrane in only one direction, from outside to inside.

The electrochemical gradient produced by the H^+-efflux pump tends to drive this process in two ways. The pH gradient affects the rates of proton association and dissociation with the carrier, while the membrane potential acts electrophoretically on the charged proton-substrate-carrier complex (11). The difference in the chemical potential of H^+ on both sides of the membrane, $\mu^{o}_{H^+} - \mu^{i}_{H^+}$, is given by

$$\mu^{o}_{H^+} - \mu^{i}_{H^+} = RT \ln \frac{[H^+]_o}{[H^+]_i} - zFE_m ,$$

where z is the valence of H^+ and E_m is the membrane potential (41). The total energy available for solute cotransport, the proton motive force (pmf), is

$$\text{pmf} = \frac{\Delta\mu H^+}{F} = -59\Delta\text{pH} + E_m$$

at room temperature (50).

The cotransport model makes several predictions about substrate transport (11). They are the following:

1) transport should behave like a carrier-mediated process,

2) transport should cause membrane potential depolariza-

tion, and the depolarization should be related to substrate transport rates,

3) transport should be affected by factors which control pmf, either by changing membrane potential or by changing the relative internal to external H^+ concentrations, and

4) transport should be accompanied by alkalinization of the bathing medium due to proton influx.

Evidence for Cotransport in Higher Plants

The existence of H^+-cotransport systems has been well documented for bacteria, fungi and algae (see 50). Studies by Slayman and Slayman (57) on Neurospora and Komor and Tanner (29) on Chlorella have been especially important in providing the groundwork for later studies in higher plants.

Evidence has also accumulated for cotransport in a number of higher plant tissues. These include oat coleoptiles (9,10,11,28,61), leaves (25,53), and protoplasts (53), Ricinus cotyledons (23,24,30,31) and petioles (34,35), pea stems (5) and protoplasts (17), maize leaves (25), coleoptiles (5), roots (5), and scutellum (22), cotton leaves (49), cowpea leaves (67), sugarbeet leaves (14,15), soybean cotyledons (33), tomato internodes (68,69,70,71), Samanea pulvini (51), Lemna fronds (47,48), and nematode-induced transfer cells of Impatiens balsamina (26).

Several types of evidence for cotransport have been presented. Transient alkalinization of the bathing medium

occurs when sugars and amino acids are added externally (5, 23,30,31,34,51,69). Presumably this is due to proton influxes during transport of these substances. Factors which control pmf have been shown to affect labelled transport. Transport is increased by IAA (5,10,11,35) and fusicoccin (5,15,35,53), both of which stimulate proton extrusion. Exogenous ATP also increases transport (17,68) by providing extra energy to the extrusion pump. High external H^+ concentrations, which increase pmf, also promote increased transport (10,11,23,34,48,53,68). Conversely, those factors which decrease pmf decrease transport. Diethylstilbestrol, an inhibitor of the proton pump, inhibits transport (5). Abscissic acid (35), respiratory poisons (10,11,22,53) and high external pH (14,17,22,23,48) also inhibit labelled transport of solutes.

According to the cotransport model, the addition of substrates externally should cause depolarization of the membrane potential. Furthermore, the extent of depolarization should be related to substrate transport rates. Etherton and coworkers (9,10) were the first to test these predictions of the theory. They found that the addition of glucose, sucrose, glutamine, glutamic acid, histidine and alanine to oat coleoptiles caused a transient depolarization of PD followed by a partial repolarization. When the substrate was removed membrane potential hyperpolarized beyond the original resting PD. Further studies with amino

acids revealed that the amount of depolarization increased with decreasing external pH. Depolarization with respect to increasing alanine concentration showed saturation kinetics, indicating a carrier-mediated process.

Jones, Novacky and Dropkin (26) observed membrane potential depolarizations in nematode-induced transfer cells of Impatiens roots when sugars were added. Fifty mM concentrations of sorbitol, glucose, 3-O-methyl-glucose, 2-deoxyglucose, sucrose and fructose were used. Depolarization was saturated at this concentration.

Similar results were reported by Racusen and Galston (51) for Samanea pulvini bathed in sucrose solution. In addition, they found that depolarization was accompanied by proton influxes, measured by alkalinization of the bathing solution. At high concentrations (above 10mM) the cations K^+, Na^+ and Ca^{++} totally inhibited depolarization by sucrose. Lictner and Spanswick (33) found that pH also affected depolarization to 100mM sucrose in soybean cotyledons.

Several studies have used α-aminoisobutyric acid (AIB), a nonmetabolized amino acid analog, to test for depolarization during transport. Etherton and Rubenstein (10,11) found, for oat coleoptiles, a close correlation between labelled AIB uptake and membrane potential depolarization. Depolarization showed saturation kinetics with increasing AIB concentration. Changing external pH did not affect

uptake except when high concentrations of K^+ (above 10mM) or Na^+ (25mM) were present. This was due to an inhibition of uptake by these cations which was more pronounced at high pH. Membrane potential depolarization was affected by pH, with increased depolarization at low pH. Both IAA and fusicoccin stimulated uptake, and IAA also increased the amount of depolarization by AIB. Azide strongly inhibited AIB transport.

Rubenstein and Tattar (53), in experiments with oat leaves and protoplasts, found that lowering the pH of the medium caused a linear increase in AIB uptake. Increasing K^+ concentration caused corresponding decreases in both transport and membrane potential depolarization. Both white light and fusicoccin, which hyperpolarized PD, stimulated AIB transport. This was presumably due to an enhancement of the activity of the proton efflux pump.

Novacky, Ullrich-Eberius and Lüttge (48) studied depolarization by sugars in Lemna fronds. They also found a saturation effect of increasing sugar concentration on the amount of depolarization, and increased depolarization at low pH. Amino acids also caused depolarization in Lemna (47).

In recent work by Kinraide and Etherton (28), pH-dependent repolarization of oat coleoptile cells depolarized by amino acids was strongly influenced by the acidic or basic nature of the molecule. Based on these results, they

have proposed a model in which positively charged substrates are transported without a proton, uncharged substrates are transported with one proton, and negatively charged substrates are transported with one proton plus another cation.

Electrophysiology and Plant Pathology

Tattar and Blanchard (66) have reviewed the use of electrophysiological methods in plant pathology research. As electrical potential measurements in plants have become more fully understood, plant pathologists have begun to study the effect of disease on PD.

One avenue of study has concentrated on the effects of host-specific phytotoxins on PD. Mertz and Arntzen (38) first studied this type of effect using Helminthosporium maydis race T-toxin. They observed rapid partial depolarization of corn root potentials upon addition of T-toxin. Novacky and Karr (46) found that T-toxin also changed PD responses to light and dark in corn leaves.

Helminthosporoside, a toxin produced by Helminthosporium sacchari, caused an irreversible depolarization of PD in susceptible but not resistant clones of sugarcane (45, 46,72). Helminthosporoside also inhibited the light-dependent recovery of PD in the presence of cyanide.

Victorin, produced by Helminthosporium victoriae, caused reversible partial depolarization in susceptible oat

roots (43) and coleoptiles (12), but not in resistant tissues. The effect of Periconia circinata toxin on PD of sorghum coleoptiles was similar to that of victorin but slower.

Only one toxin studied did not depolarize PD. Helminthosporium carbonum toxin, instead, caused transient hyperpolarization of membrane potentials in maize coleoptiles (12).

In a series of studies by Novacky and coworkers (42, 44,45,46,49) the hypersensitive response to nonpathogenic organisms was examined. Cotton cotyledons were inoculated with either a virulent strain of Xanthomonas malvacearum or the nonpathogenic Pseudomonas pisi, both of which caused a hypersensitive response. In both cases PD depolarized due to changes in the passive diffusion permeability of the membrane. Both compatible (disease) and incompatible (hypersensitive) responses resulted in a loss of recovery of PD to cyanide depolarization.

Depolarization due to glycine was also studied in this system (49). Incompatible reactions caused lowered PD and a loss of depolarization under both light and dark conditions, as did disease in the dark condition. But in the light, the amount of depolarization did not change. Instead, a lack of recovery occurred after 20 hr.

Jones and coworkers (26) compared normal root cells to nematode-induced transfer cells of Impatiens. Both showed

similar responses of PD to different ion concentrations, respiratory and protein synthesis inhibitors, and sugars.

Stack and Tattar (60) examined the effects of virus infection on membrane potentials. Cells of cowpeas infected with tobacco ringspot virus had both higher and lower potentials than control cells. Infection caused a variable change in the response of PD to azide. Tattar (67) studied the effects of cowpea chlorotic mottle virus on PD in cowpea leaves. Infected leaves depolarized to lower PD values in the dark. Depolarization caused by asparagine, glycine and sucrose was the same in infected and control tissue.

Kota and Stelzig (32) have reported, for potato leaf petioles, that commercial pectins and fungal polysaccharides which elicited phytoalexin synthesis also caused a depolarization of membrane potential.

Jennings and Tattar (25) observed the effects of chilling on PD in both chilling-sensitive (maize) and non-chilling-sensitive (oat) leaves. Low temperatures (8°C) affected maize response to light, glycine and sucrose, compared to oats which showed similar responses at 21°C and 8°C. There was no difference in the effect of metabolic inhibitors on the two tissues.

Statement of Purpose

If cotransport occurs in higher plants, the results of studies using toxins and fungal elicitors will need to

be reevaluated, since sugars and amino acids in these preparations could have caused the observed depolarizations. It is therefore important to fully understand the response of PD to sugars and amino acids before attempting electrophysiological studies of plant disease.

The objectives of this study are:

1) to examine the effects of sugars and amino acids on PD in sugarcane leaves in light of a possible cotransport mechanism for membrane transport of these substances, and

2) to observe the effects of sugars and α-galactosides on PD in two clones of sugarcane to obtain a better understanding of observed effects of helminthosporoside on PD.

C H A P T E R II

MATERIALS AND METHODS

Plant Material

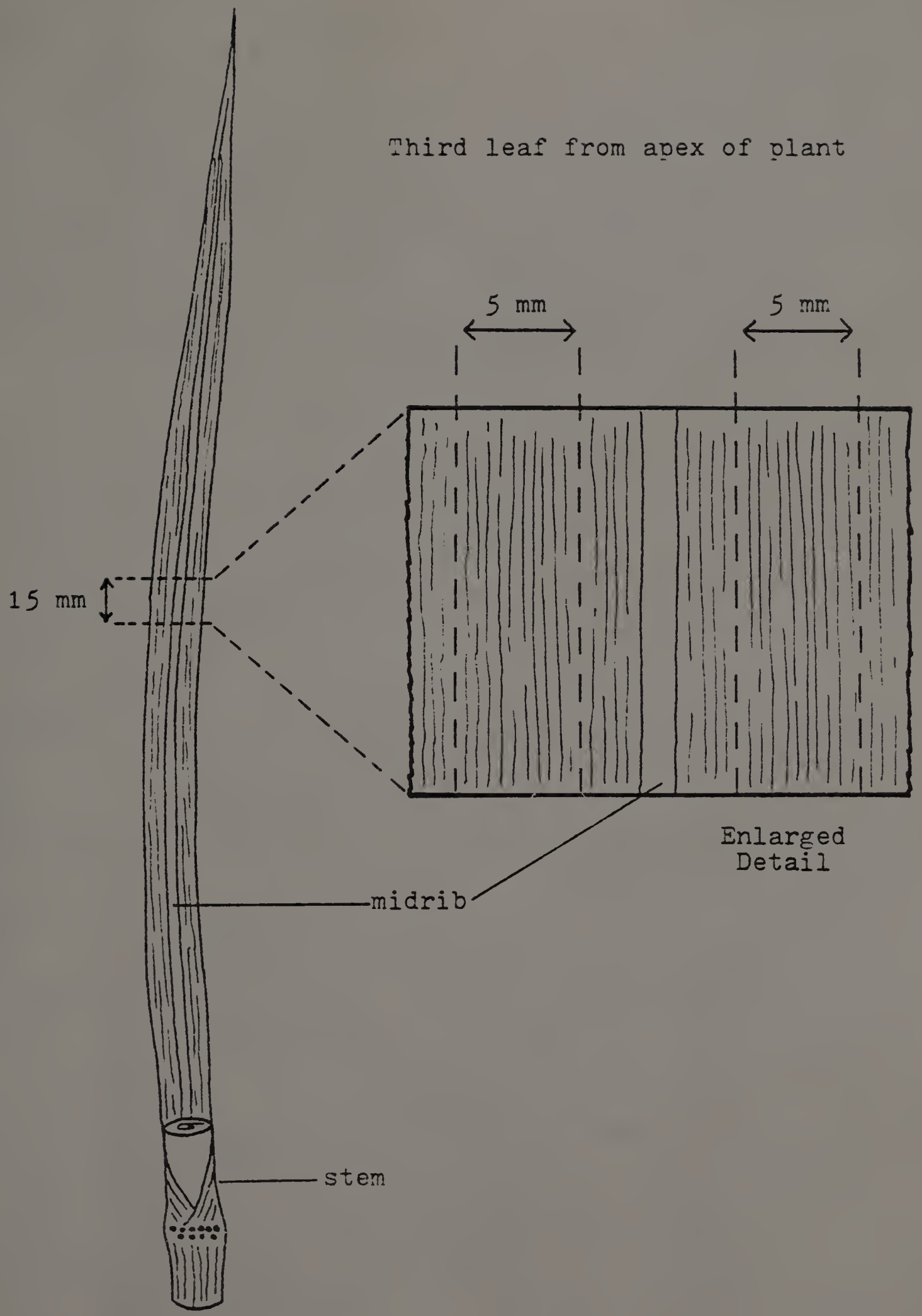
Sugarcane clones 51 NG 97 and H50 7209 were provided by Dr. G.A. Strobel. Plants were maintained in the greenhouse in ten inch plastic pots which contained a 2:1:1 mixture of loam, peat and sand. Each plant was fertilized weekly with 2-3 g formulation 16-32-16 fertilizer (Start N Gro, Agway Inc., Syracuse, NY) in about 300 ml water.

Tissue sections were cut from the third youngest leaf of a stalk using a new razor blade. Rectangular sections were cut from the area halfway between the stem and the tip of the leaf, and contained only the green tissue on either side of the midrib (Figure 2). Each section was 5 x 15 mm, with the vascular bundles parallel to the long axis. After cutting, tissue sections were aged by floating in 1X solution (see below) in the dark for at least 16 hr but not more than 24 hr.

Perfusion Solutions

The bathing solution (8) was designated 1X and contained 1.0mM KCl, 1.0mM $Ca(NO_3)_2$, 0.25mM $MgSO_4$, 0.90mM NaH_2PO_4, and 0.05mM Na_2HPO_4. The pH was 5.5 to 5.7. The following sugars, amino acids, and inhibitors were dissolved in 1X solution: glucose, raffinose, galactose,

Figure 2. Method for cutting sugarcane tissue sections.


Third leaf from apex of plant
5 mm
5 mm
15 mm
Enlarged
Detail
midrib
stem

melibiose, lactose, sucrose, fructose, mannitol, 3-O-methyl-glucose, 1-O-methyl-α-galactose, glycine, alanine, serine, N-ethyl-maleimide (NEM), carbonyl cyanide-M-chlorophenyl hydrazone (CCCP), and 2,4-dinitrophenol (DNP). Other solutions were modified from 1X to keep the final concentrations of H^+, K^+ and Na^+ the same as 1X. For 1mM sodium azide solution, NaH_2PO_4 and Na_2HPO_4 were omitted. When 1mM KCN plus 1mM salicyl hydroxamic acid (SHAM) were used, KCl was omitted. In all cases the pH of the final solution was adjusted to 5.5-5.7 with either 1mM Tris buffer or 1mM HCl.

Electrophysiological Measurements

Micropipettes (tip diameter less than 1 μm) were pulled from fiber-filled glass capillary tubes (1.2 mm OD) with a micropipette puller (Industrial Science Associates). Microelectrodes were made by filling these with 3M KCl. Only electrodes with resistances less than 20 MΩ were used because of the rigidity of the sugarcane tissue. Reference electrodes were 3 mm plugs of 3M KCl in 2% agar. Both electrodes were connected via Ag/AgCl half cells to a high input impedance electrometer ($2 \times 10^{10}\Omega$; WPI Instruments, model 725). Output was recorded on an Esterline Angus strip chart recorder.

For PD measurements, aged tissue sections, with the vascular bundles oriented vertically, were fitted into a

plexiglass holder and mounted in a plexiglass perfusion chamber (volume 2.3 ml; Figure 3). There was a continuous flow of bathing solution to the perfusion chamber at an average rate of 8 ml/min. The bathing solution delivery system contained a valve which allowed other solutions to be introduced during PD measurements. Solutions reached the chamber 30-60 s after addition (arrows in Figures 5 and 8-12). The microelectrode was lowered into the parenchyma tissue between vascular bundles with a Brinkman micro-manipulator. This process was viewed through a microscope at 200X magnification.

Parenchyma cells were penetrated in the light in 1X. After a PD measurement was obtained, the light was switched off, and cells were allowed to stabilize in the dark until a steady PD was maintained for at least 5 min. Test solutions were added to the perfusion chamber at this time, and resting PD was measured at the point of addition.

Figure 3. Schematic diagram of system used for measuring membrane potential in sugarcane leaves.

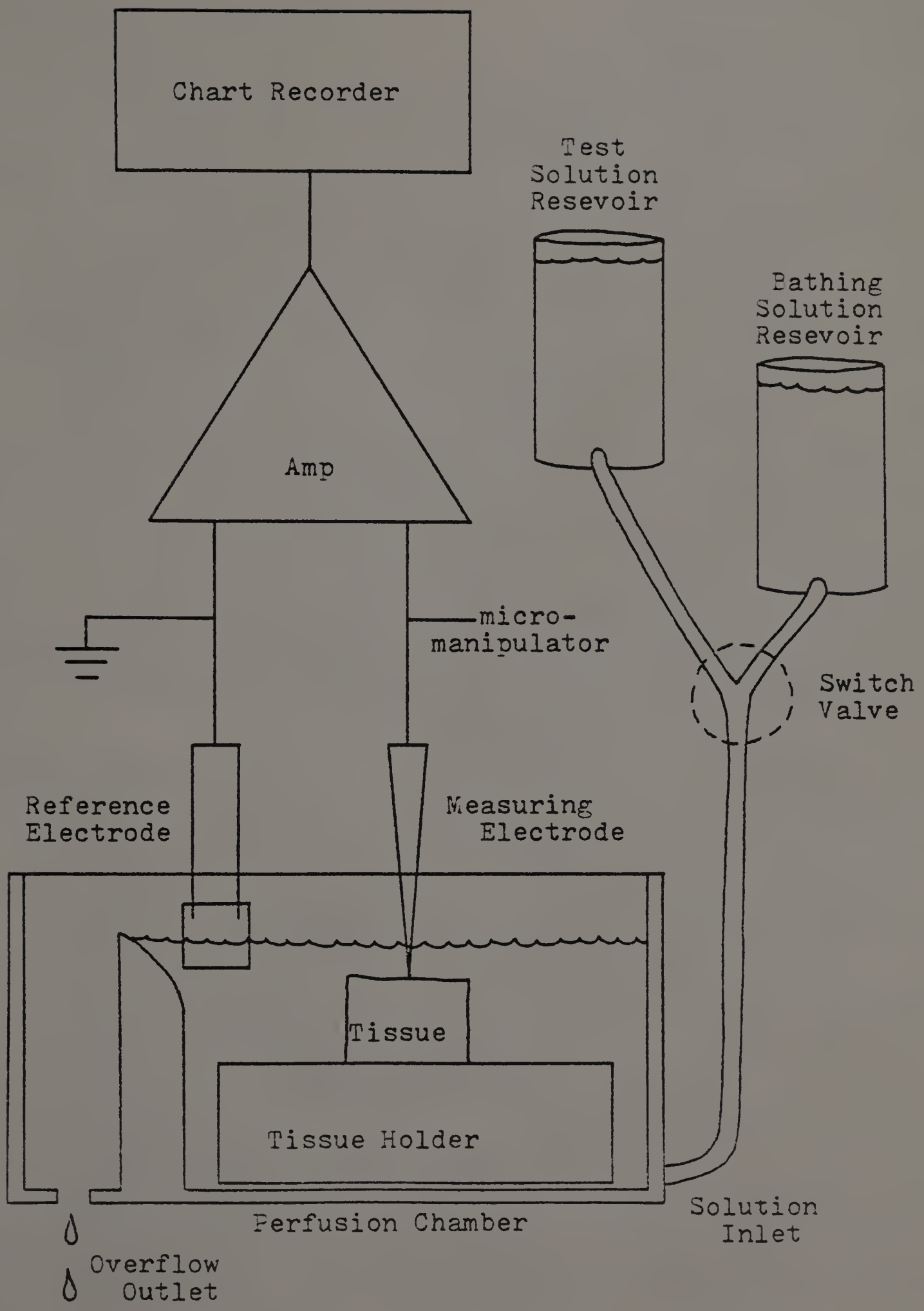


Chart Recorder
Amp
Test
Solution
Resevoir
Bathing
Solution
Resevoir
micro-
manipulator
Switch
Valve
Reference
Electrode
Measuring
Electrode
Tissue
Tissue Holder
Perfusion Chamber
Solution
Inlet
Overflow
Outlet

C H A P T E R III

RESULTS

Resting Potentials

The average resting PD in the dark for both clones of sugarcane studied was -135 mV (Table 1). PD values for individual cells ranged from -114 to -162 mV for clone 51 NG 97 and from -108 to -163 mV for clone H50 7209.

Effect of Light and Dark on PD

Cells bathed in 1X in the dark exhibited transient depolarizations of 40-45 mV when the light was switched on (Figure 4). Recovery occurred within 30 min in the light. When the light was switched off, transient hyperpolarizations of about 10 mV were observed. In most cells, the same resting potential was maintained in both the light and the dark. Both clones responded similarly to light and dark. All subsequent experiments were performed in the dark to eliminate energy input from photosynthesis.

Effect of Sugars on PD

Glucose was added externally to tissue in the dark while PD was monitored. The addition of glucose to the bathing solution resulted in a rapid depolarization of PD (Figure 5). Maximal depolarization occurred within 2-4 min, and was followed by a slow partial repolarization. If

Table 1. Resting PD and range of PD measurements in leaf parenchyma cells of Saccharum clones 51 NG 97 and H50 7209.[a]

	51 NG 97	H50 7209
PD (mV)[b]	-135 ± 10 (135)	-135 ± 11 (128)
PD range (mV)	-114 to -162	-108 to -163

[a] Data for cells bathed in 1X solution in the dark.
[b] Expressed as mean ± standard deviation, number of observations in parentheses. Clones not significantly different, P=0.05, according to the t-test.

Figure 4. Effect of switching light on and off on membrane potential (PD) of sugarcane leaf parenchyma cells bathed in 1X solution.

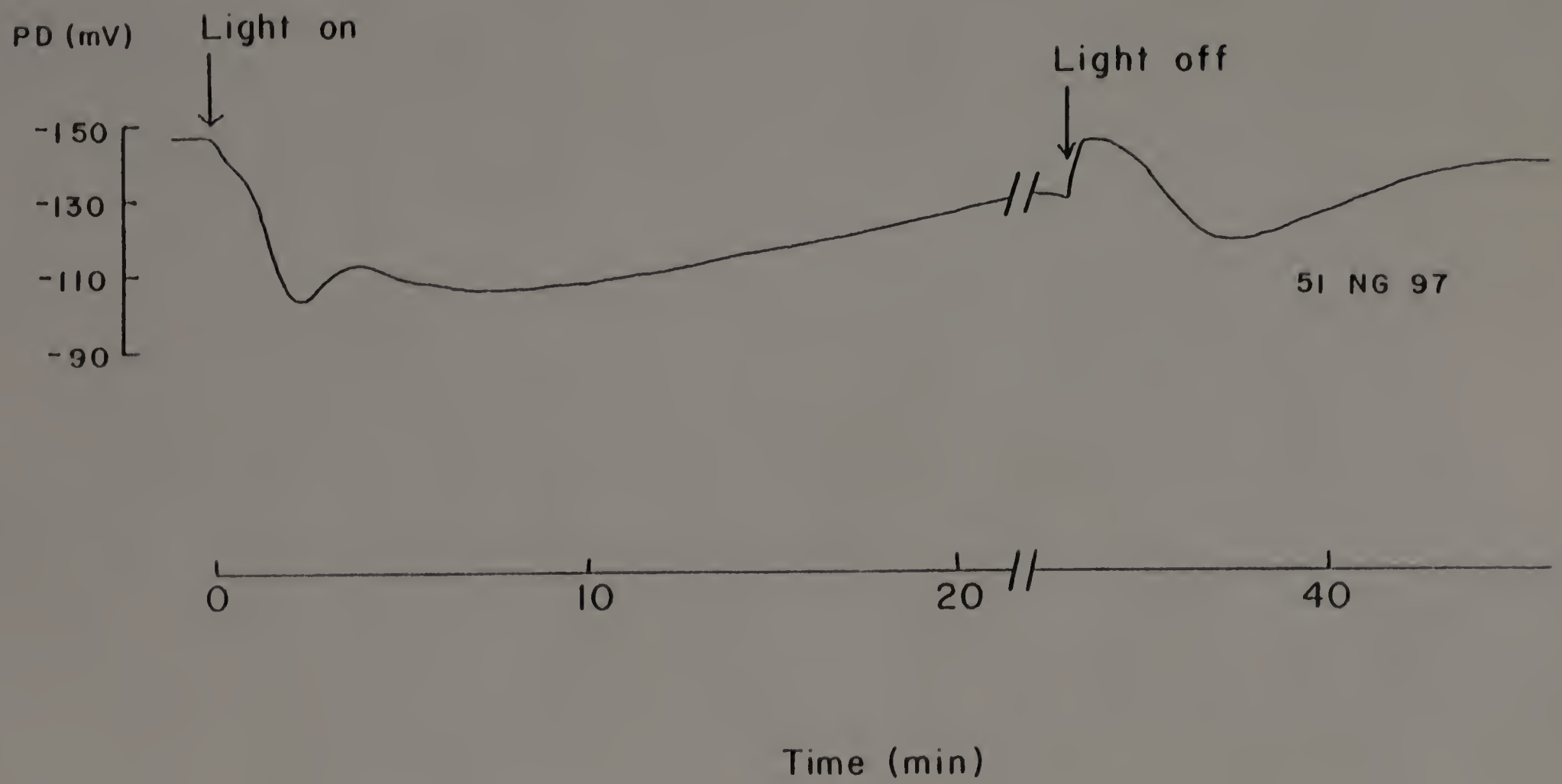
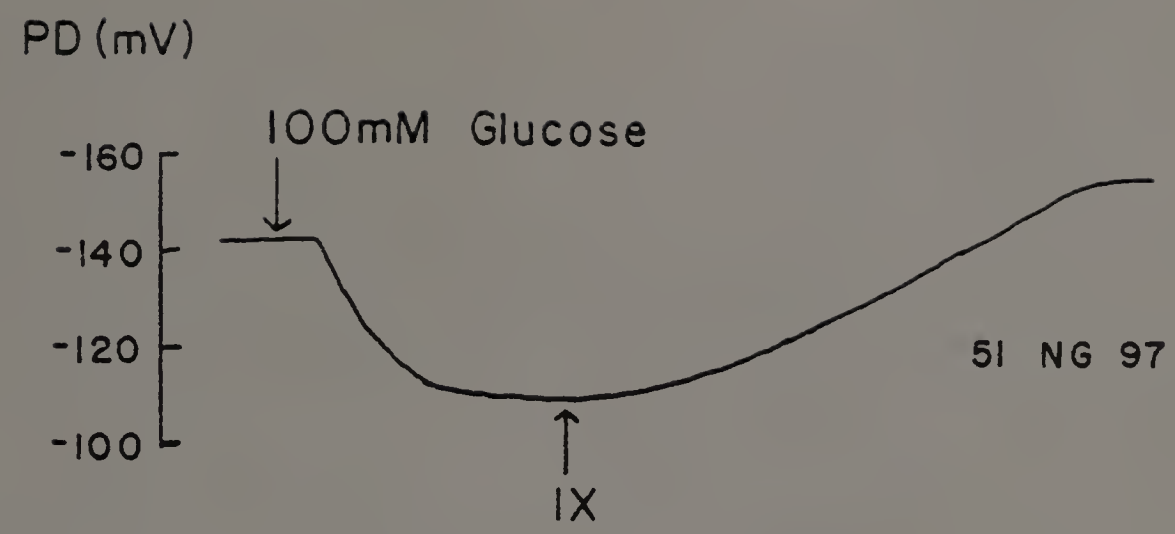
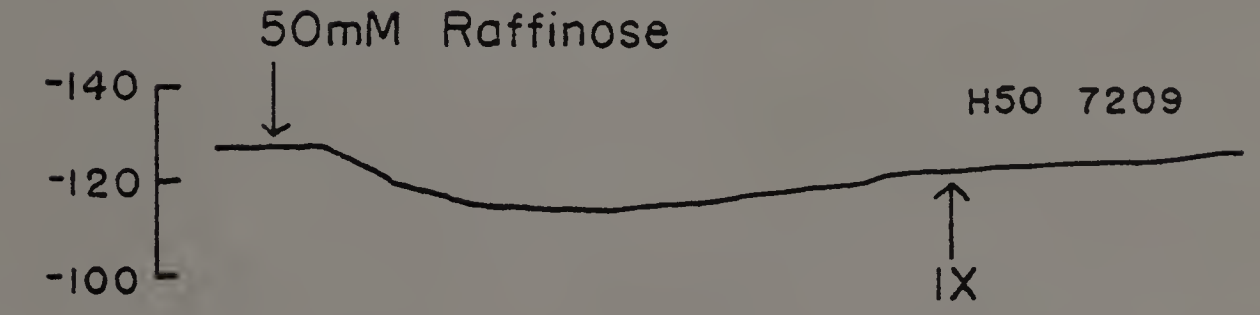
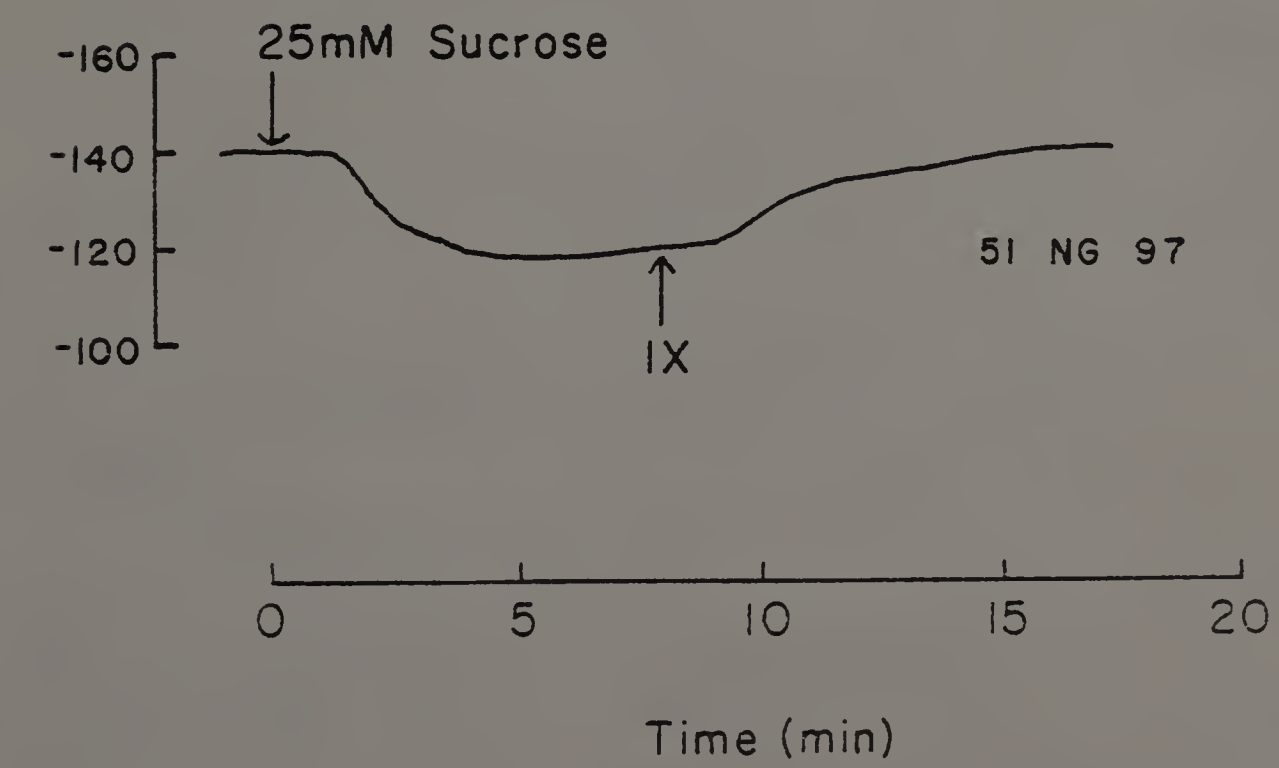

PD (mV)
Light on
Light off
-150
-130
-110
-90
51 NG 97
0
10
20
40
Time (min)

Figure 5. Effect of glucose, raffinose and sucrose on membrane potential (PD) of sugarcane leaf parenchyma cells in the dark. Solutions reached the chamber 30-60 s after addition (arrow).


PD (mV)
100mM Glucose
-160
-140
-120
-100
51 NG 97
1X


50mM Raffinose
-140
-120
-100
H50 7209
1X


25mM Sucrose
-160
-140
-120
-100
51 NG 97
1X
0
5
10
15
20
Time (min)

glucose was removed from the bathing solution, repolarization was more rapid, and PD hyperpolarized beyond the resting potential for 10-20 min.

Glucose concentrations of 100.0, 50.0, 25.0, 10.0, 5.0, 1.0 and 0.5mM were added to the bathing solution. The time course of depolarization, recovery and hyperpolarization was the same for all concentrations and did not differ between clones. The magnitude of the depolarization increased with increasing glucose concentration (Table 2). The average amount of depolarization was not significantly different between clones at the 0.05 level, except for 25mM glucose. Figure 6 shows that the relationship between concentration and depolarization was hyperbolic, approximating the kinetics of an enzyme-catalysed reaction. Concentrations greater than 15mM caused maximal depolarization. Since the depolarization response was near saturation at 25mM glucose, this was the concentration used in later experiments.

Strobel (64) reported that sugarcane leaf protoplasts of clone 51 NG 97 took up labelled raffinose, an α-galactosidic sugar, while protoplasts from H50 7209 did not. If raffinose is cotransported, it should cause depolarization in 51 NG 97 but not in H50 7209. When raffinose was added to the bathing solution there was a depolarization of PD in both clones, followed by repolarization (Figure 5). The time course of the response was similar to that caused

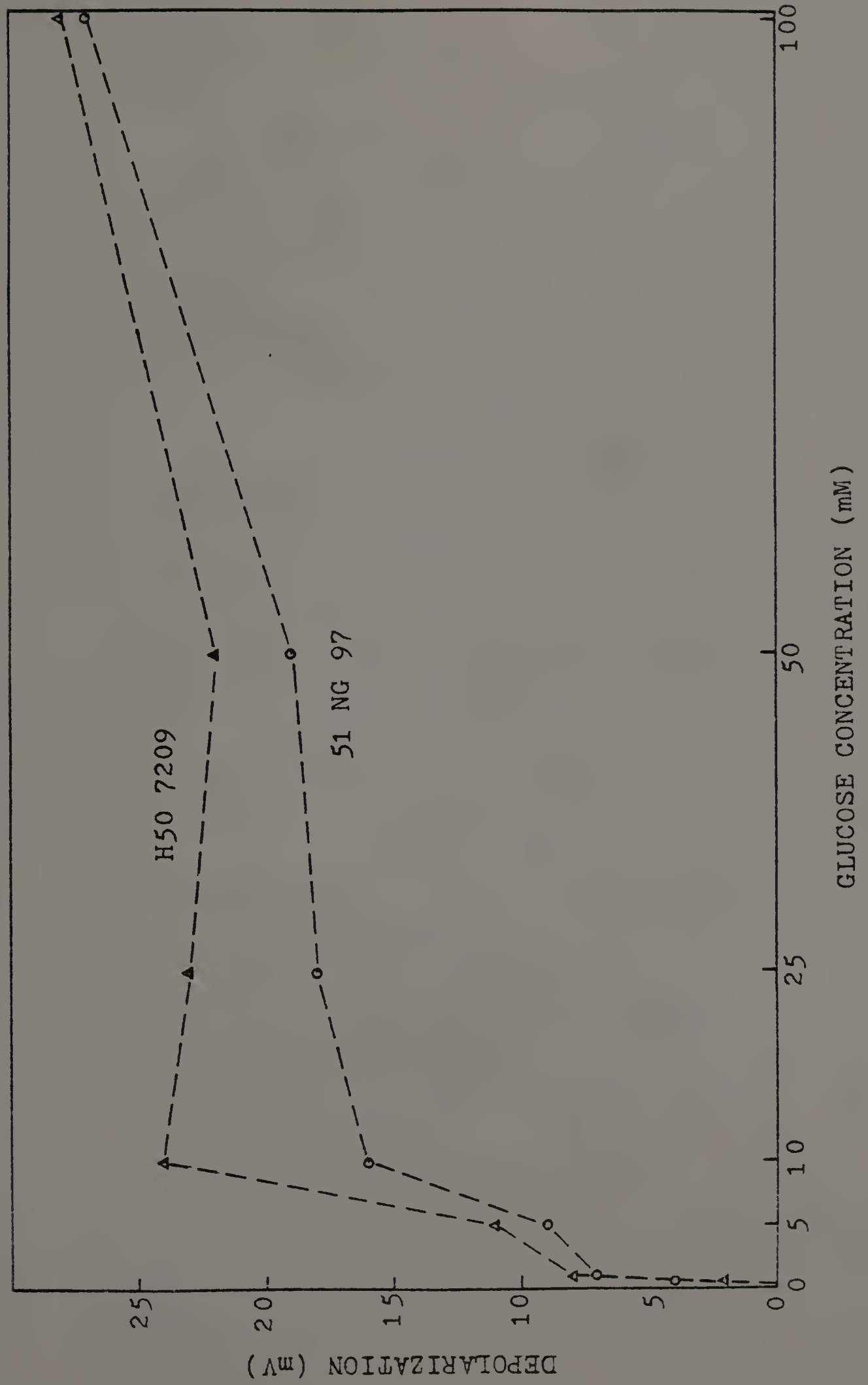
Table 2. Effect of external glucose concentration on PD of leaf parenchyma cells of Saccharum clones 51 NG 97 and H50 7209.[a]

Glucose concentration (mM)	Depolarization (mV)	
	51 NG 97	H50 7209
100.0	27 ± 8 (5)	28 ± 13 (4)
50.0	19 ± 3 (6)	22 ± 8 (5)
25.0[b]	18 ± 4 (14)	23 ± 7 (15)
10.0	16 ± 4 (5)	24 ± 8 (6)
5.0	9 ± 1 (5)	11 ± 3 (5)
1.0	7 ± 3 (5)	8 ± 3 (4)
0.5	4 ± 1 (5)	2 ± 1 (4)

[a] Experiments performed in the dark. Data expressed as mean ± standard deviation, number of observations in parentheses.

[b] Clones significantly different, P=0.05, according to the t-test.

Figure 6. Maximum depolarization of membrane potential in relation to external glucose concentration in the dark in sugarcane clones 51 NG 97 and H50 7209. Data points represent mean depolarization (see Table 2).


H50 7209
51 NG 97
DEPOLARIZATION (mV)
GLUCOSE CONCENTRATION (mM)
0
5
10
25
50
100
5
10
15
20
25

by glucose, but the amount of depolarization was much less.

The effect of 100.0, 50.0, 25.0 and 10.0mM raffinose on PD was studied. As with glucose, ΔPD increased with increasing concentration (Table 3). There was no significant difference between the two clones at the 0.05 level. A plot of depolarization versus raffinose concentration (Figure 7) is roughly hyperbolic in shape, indicating saturation kinetics for this process. Saturation occurred at about 50mM raffinose.

Two other α-galactosides, melibiose and 1-O-methyl-α-galactose, were reported to bind to membrane proteins of 51 NG 97 but not H50 7209 (64). At 25mM concentrations neither sugar affected PD in either clone.

The effects of sucrose, fructose, galactose and 3-O-methyl-glucose on PD were studied using 25mM concentrations. Each caused rapid partial depolarization of PD within 2-4 min (Figures 5 and 8). The average ΔPD caused by each sugar is given in Table 4. There was no significant difference between clones at the 0.05 level. Sucrose-, galactose- and fructose-treated cells exhibited slow recovery after depolarization, and more rapid recovery in 1X. Generally, the response of PD to these sugars was similar to that of glucose. Cells treated with 3-O-methyl-glucose showed no recovery of PD after depolarization. When methyl-glucose was removed, there was a slow recovery in

Table 3. Effect of external raffinose concentration on PD of leaf parenchyma cells of Saccharum clones 51 NG 97 and H50 7209.[a]

Raffinose concentration (mM)	Depolarization (mV)	
	51 NG 97	H50 7209
100.0	13 ± 6 (5)	12 ± 5 (5)
50.0	15 ± 6 (5)	11 ± 4 (5)
25.0	9 ± 3 (5)	6 ± 3 (5)
10.0	3 ± 1 (3)	4 ± 2 (3)

[a] Experiments performed in the dark. Data expressed as mean ± standard deviation, number of observations in parentheses. For each concentration, clones not significantly different, P=0.05, according to the t-test.

Figure 7. Maximum depolarization of membrane potential in relation to external raffinose concentration in the dark in sugarcane clones 51 NG 97 and H50 7209. Data points represent mean depolarization (see Table 3).

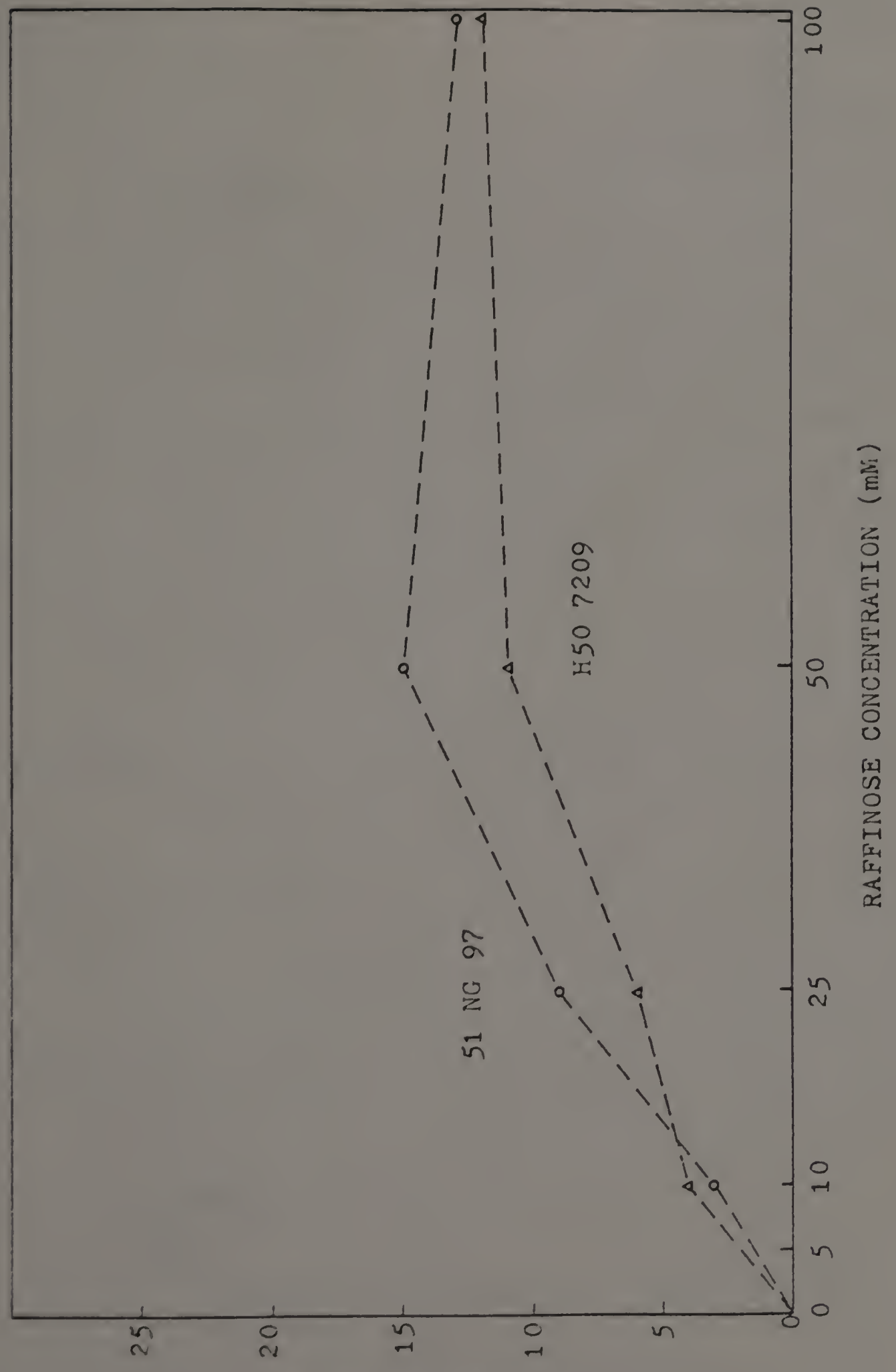

DEPOLARIZATION (mV)
25
20
15
10
5
0
0
5
10
25
50
100
RAFFINOSE CONCENTRATION (mM)
51 NG 97
H50 7209

Figure 8. Effect of external galactose, fructose and 3-O-methyl-glucose (MeGlu) on membrane potential (PD) of sugarcane leaf parenchyma cells in the dark. Solutions reached the chamber 30-60 s after addition (arrow).

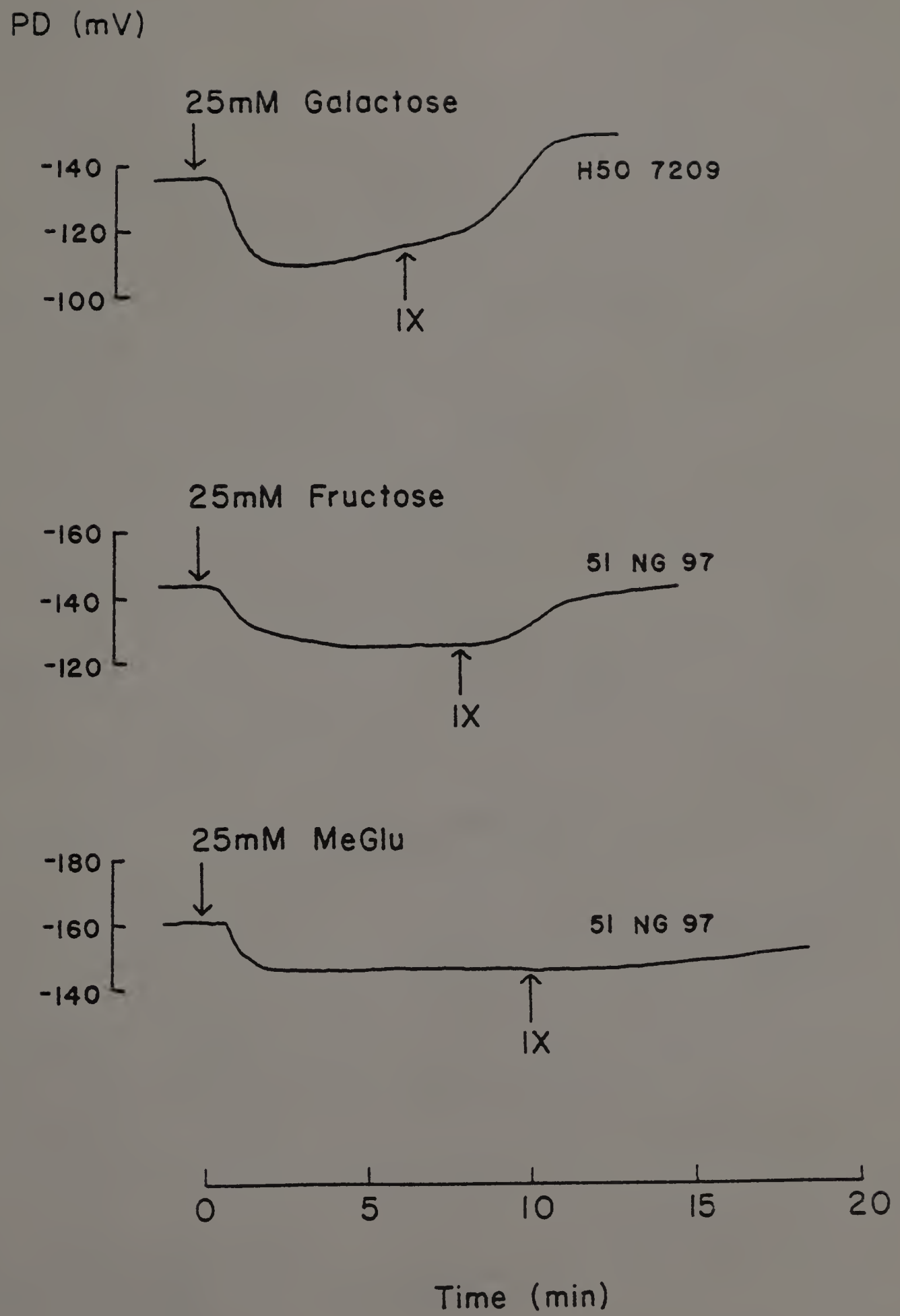

PD (mV)
25mM Galactose
-140
-120
-100
H50 7209
IX
25mM Fructose
-160
-140
-120
51 NG 97
IX
25mM MeGlu
-180
-160
-140
51 NG 97
IX
0
5
10
15
20
Time (min)

Table 4. Effect of external sugars on PD of leaf parenchyma cells of Saccharum clones 51 NG 97 and H50 7209.[a]

Sugar	Depolarization (mV)			
	51 NG 97		H50 7209	
Galactose	24 ± 7	(5)	27 ± 1	(5)
3-O-Methyl-glucose	10 ± 4	(4)	13 ± 9	(4)
Sucrose	22 ± 3	(4)	22 ± 5	(4)
Fructose	21 ± 7	(4)	14 ± 2	(4)
Mannitol	0	(2)	0	(2)
Lactose	0	(3)	0	(3)
Melibiose	0	(3)	0	(3)
1-O-Methyl-α-galactose	0	(2)	0	(2)

[a] All sugars at 25mM concentration. Experiments performed in the dark. Data expressed as mean ± standard deviation, number of observations in parentheses. Clones not significantly different, P=0.05, according to the t-test.

1X but no hyperpolarization.

Neither mannitol nor lactose had any effect on PD in either clone at 25mM concentrations.

Effect of Amino Acids on PD

The addition of the amino acids glycine, serine and alanine had an effect on PD that was similar to that of glucose and other sugars (Figure 9). The time course of depolarization, recovery and hyperpolarization was roughly the same for all these compounds. In general, for both sugars and amino acids, the greater ΔPD was, the faster recovery occurred. Average ΔPD for 10mM serine and 25mM glycine is shown in Table 5. The amount of depolarization was greater than for the sugars studied at the same concentrations. There was no significant difference between clones, $P=0.05$. Results for alanine were not included because only one trial was performed for each clone.

Metabolic Inhibitor Effects

Metabolic inhibitor studies were performed to see if sugar-induced depolarization occurred when the active component of PD was depolarized. Cyanide is a respiratory inhibitor which causes depolarization of the active portion of PD. Some plants exhibit cyanide-resistant respiration and can recover PD in the presence of cyanide (44). Preliminary studies, in which 1mM KCN was added to sugarcane

Figure 9. Effect of external glycine, alanine and serine on membrane potential (PD) of sugarcane leaf parenchyma cells in the dark. Solutions reached the chamber 30-60 s after addition (arrow).

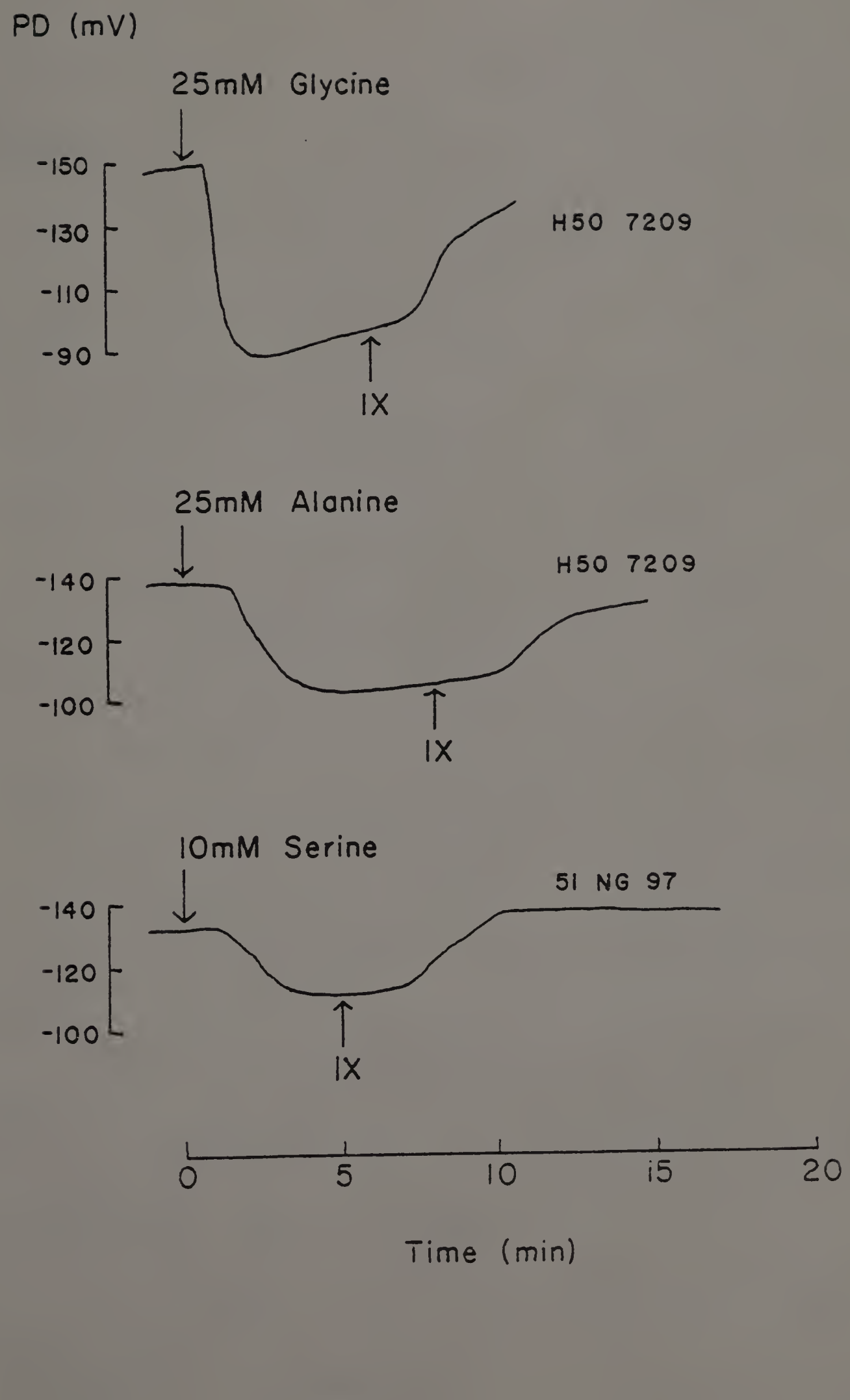

PD (mV)
25mM Glycine
-150
-130
-110
-90
H50 7209
IX
25mM Alanine
-140
-120
-100
H50 7209
IX
10mM Serine
-140
-120
-100
51 NG 97
IX
0
5
10
15
20
Time (min)

Table 5. Effect of external serine and glycine on PD of leaf parenchyma cells of Saccharum clones 51 NG 97 and H50 7209.[a]

	Depolarization (mV)	
Amino acid	51 NG 97	H50 7209
10mM Serine	23 ± 2 (3)	20 ± 1 (2)
25mM Glycine	47 ± 7 (7)	52 ± 10 (7)

[a] Experiments performed in the dark. Data expressed as mean ± standard deviation, number of observations in parentheses. Clones not significantly different, P=0.05, according to the t-test.

tissue, resulted in a depolarization of PD followed by partial recovery in cyanide (Figure 10). When 25mM raffinose or glucose was added to depolarized cells in the presence of 1mM cyanide, a second depolarization of up to 17 mV occurred. These results indicated that KCN was not completely inhibiting respiration.

To obtain maximum inhibition of the active portion of PD, 1mM SHAM was added to cells along with 1mM KCN. When both inhibitors were added to the perfusion chamber, there was a ΔPD of about 50 mV (Table 6). There was no recovery of PD when both inhibitors were present. Instead, cells maintained a steady PD at the new lower value. The addition of either 25mM glucose or 25mM raffinose to these cells, while maintaining inhibitor concentrations at 1mM each, caused only slight additional depolarizations. Membrane potential depolarization by glucose and raffinose was severely inhibited by depolarization of the active portion of PD.

Preliminary experiments were performed to study the effects of several other inhibitors on PD. Sodium azide, a respiratory inhibitor, DNP and CCCP, uncouplers of oxidative phosphorylation, and NEM, a sulfhydryl inhibitor, were studied. When added to tissue in the dark, 1mM azide caused a gradual depolarization of up to 68 mV. PD continued to depolarize slowly for over 30 min. Similar results were obtained using 0.1mM DNP, 0.1mM CCCP and

Figure 10. Effect of KCN, KCN followed by raffinose, and KCN + SHAM followed by glucose on membrane potential (PD) of sugarcane leaf parenchyma cells in the dark. Inhibitor concentrations were maintained during addition of sugars. Solutions reached the chamber 30-60 s after addition (arrow).

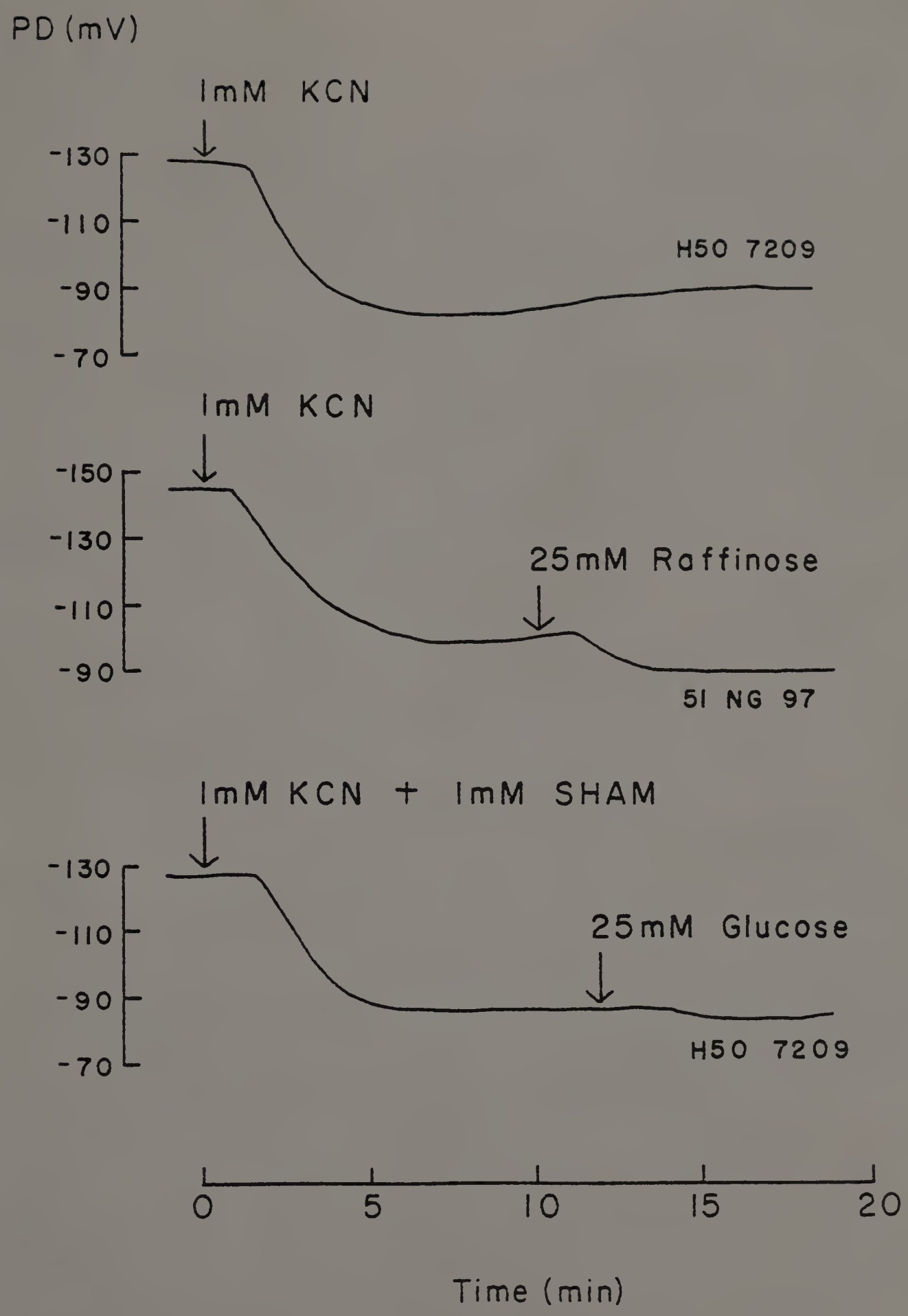

PD (mV)
ImM KCN
-130
-110
-90
-70
H50 7209
ImM KCN
-150
-130
-110
-90
25mM Raffinose
51 NG 97
ImM KCN + ImM SHAM
-130
-110
-90
-70
25mM Glucose
H50 7209
0
5
10
15
20
Time (min)

Table 6. Effect of 1mM KCN + 1mM SHAM on PD and on PD depolarization by 25mM glucose and 25mM raffinose in leaf parenchyma cells of Saccharum clones 51 NG 97 and H50 7209.[a]

	Depolarization (mV)	
	51 NG 97	H50 7209
1mM KCN + 1mM SHAM	52 ± 6 (6)	48 ± 9 (9)
+25mM Glucose[b]	3 ± 3 (3)	5 ± 2 (4)
+25mM Raffinose[b]	1 ± 0 (3)	1 ± 0 (3)

[a] Experiments performed in the dark. Data expressed as mean ± standard deviation, number of observations in parentheses. Clones not significantly different, P=0.05, according to the t-test.

[b] Depolarization by either glucose or raffinose in cells which had been maximally depolarized by 1mM KCN + 1mM SHAM.

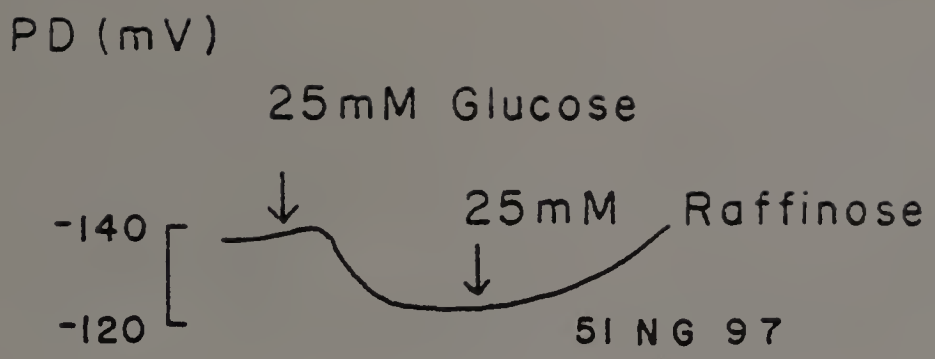
0.5mM NEM. Increasing the concentration of any of these inhibitors caused more rapid depolarization and cell death. Lower concentrations caused only partial depolarization of the energy-dependent PD. Because cells were unable to maintain a stable diffusion potential in the presence of these inhibitors, no experiments with sugars were attempted.

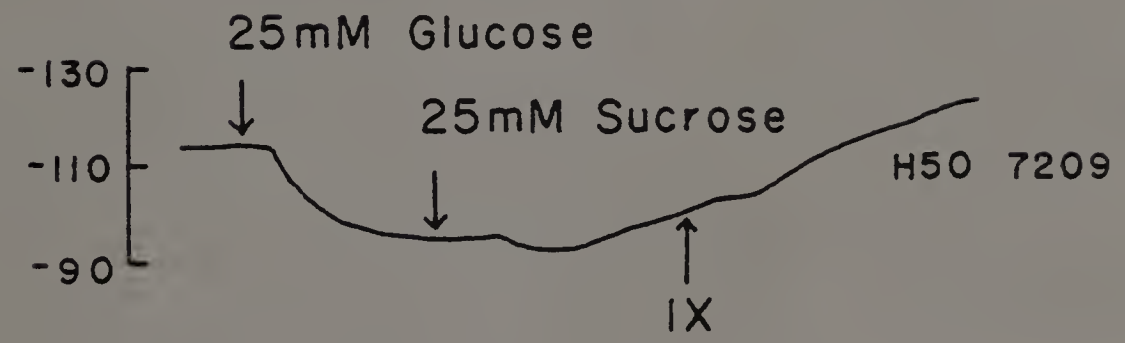
Sugar and Amino Acid Interactions

Glucose, sucrose and 3-O-methyl-glucose are transported via the same carrier sites in sugarcane, and compete with each other for uptake (2,3,4). A set of experiments was designed to see if this could be correlated with a competitive inhibition by glucose of membrane potential depolarization by sucrose, raffinose and 3-O-methyl-glucose. Glucose (25mM) was added externally to cells until maximal depolarization occurred. At this concentration glucose depolarization was saturated. About 4-6 min after the addition of glucose, before cells began to repolarize, glucose was removed, and either 25mM sucrose, raffinose or 3-O-methyl-glucose was immediately added to see if further depolarization would occur.

The results of this set of experiments are shown in Figure 11 and Table 7. Glucose strongly inhibited depolarization by the other sugars tested. Only sucrose caused an additional depolarization of PD. This was small, though, ranging from 1 to 4 mV beyond the depolarization caused by

Figure 11. Effect of glucose followed by raffinose, sucrose or 3-O-methyl-glucose (MeGlu) on membrane potential (PD) of sugarcane leaf parenchyma cells in the dark. Glucose was removed upon addition of other sugars. Solutions reached the chamber 30-60 s after addition (arrow).


PD (mV)
25mM Glucose
25mM Raffinose
-140
-120
51 NG 97


25mM Glucose
25mM Sucrose
-130
-110
-90
H50 7209
IX

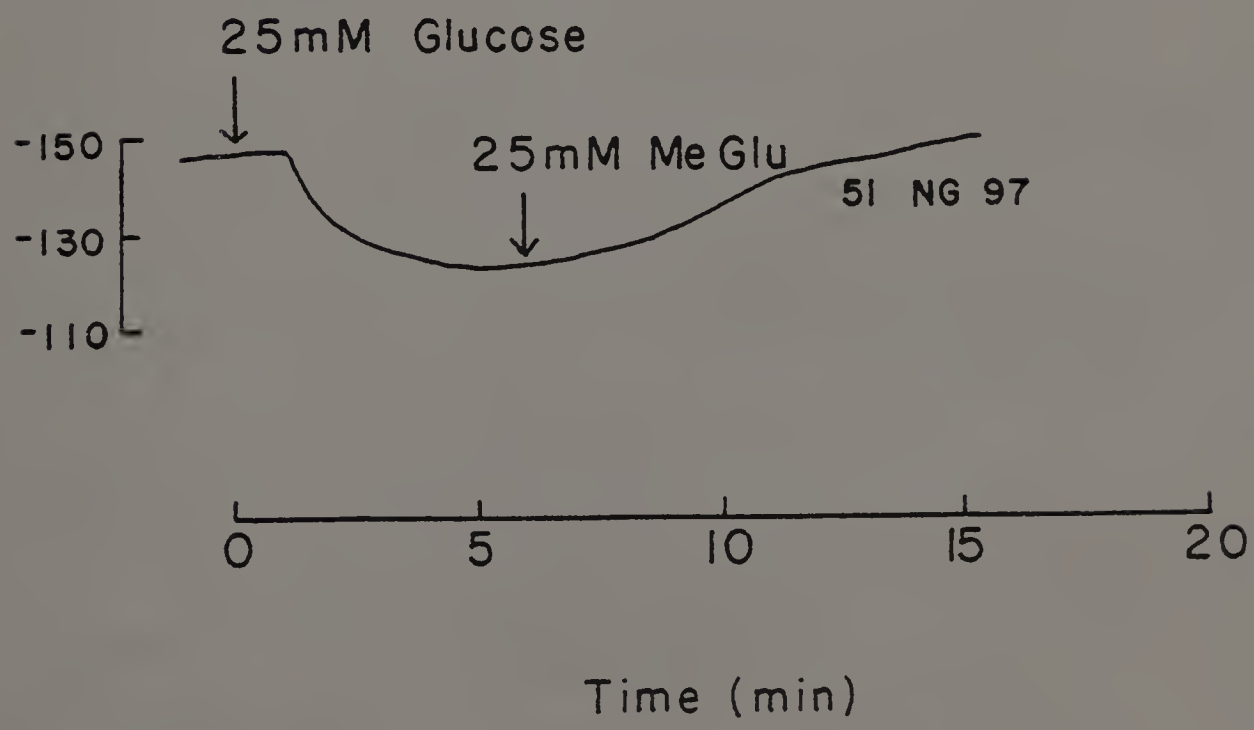

25mM Glucose
25mM Me Glu
-150
-130
-110
51 NG 97
0
5
10
15
20
Time (min)

Table 7. Effect of initial depolarization of PD by glucose on subsequent depolarization by sucrose, raffinose and 3-O-methyl-glucose in leaf parenchyma cells of Saccharum clones 51 NG 97 and H50 7209.[a]

	Depolarization (mV)					
	51 NG 97			H50 7209		
X	+Glucose	+X	Total	+Glucose	+X	Total
Sucrose	18	1	18	37	4	41
	15	2	17	16	2	17
	14	2	16	19	2	21
Raffinose	11	0	11	18	0	18
	16	0	16	17	0	17
	16	0	16	19	1	20
3-O-Methyl-glucose	13	0	13	18	0	18
	24	0	24	18	0	18
	22	0	22	24	0	24

[a] Glucose was added externally until cells were maximally depolarized. At this point, glucose was removed and replaced by another sugar (X). All sugars were at 25mM concentration. Presented data is results from single experiments.

glucose. Raffinose caused a further slight depolarization in one case.

A similar pair of experiments was carried out using glucose and glycine. In one, 25mM glucose was added to cells until depolarization occurred, then glucose was removed and 25mM glycine was added. Glycine caused a second, substantial depolarization (Figure 12 and Table 8). This was smaller, though, than the depolarization caused by glycine alone. It appears that glucose only partially inhibits glycine depolarization.

To understand this interaction more fully, the reverse of this experiment was performed. Glycine (25mM) was added to cells, removed after 4 min, and replaced by 25mM glucose. Glucose depolarization was partially inhibited, but some additional depolarization did occur. The total depolarization, by glucose and glycine together, in both of these experiments, was comparable to the depolarization caused by 1mM KCN and 1mM SHAM (Table 6).

Figure 12. Effect of glycine followed by glucose and of glucose followed by glycine on membrane potential (PD) of sugarcane leaf parenchyma cells in the dark. The first compound was removed upon addition of the second in both cases. Solutions reached the chamber 30-60 s after addition (arrow).

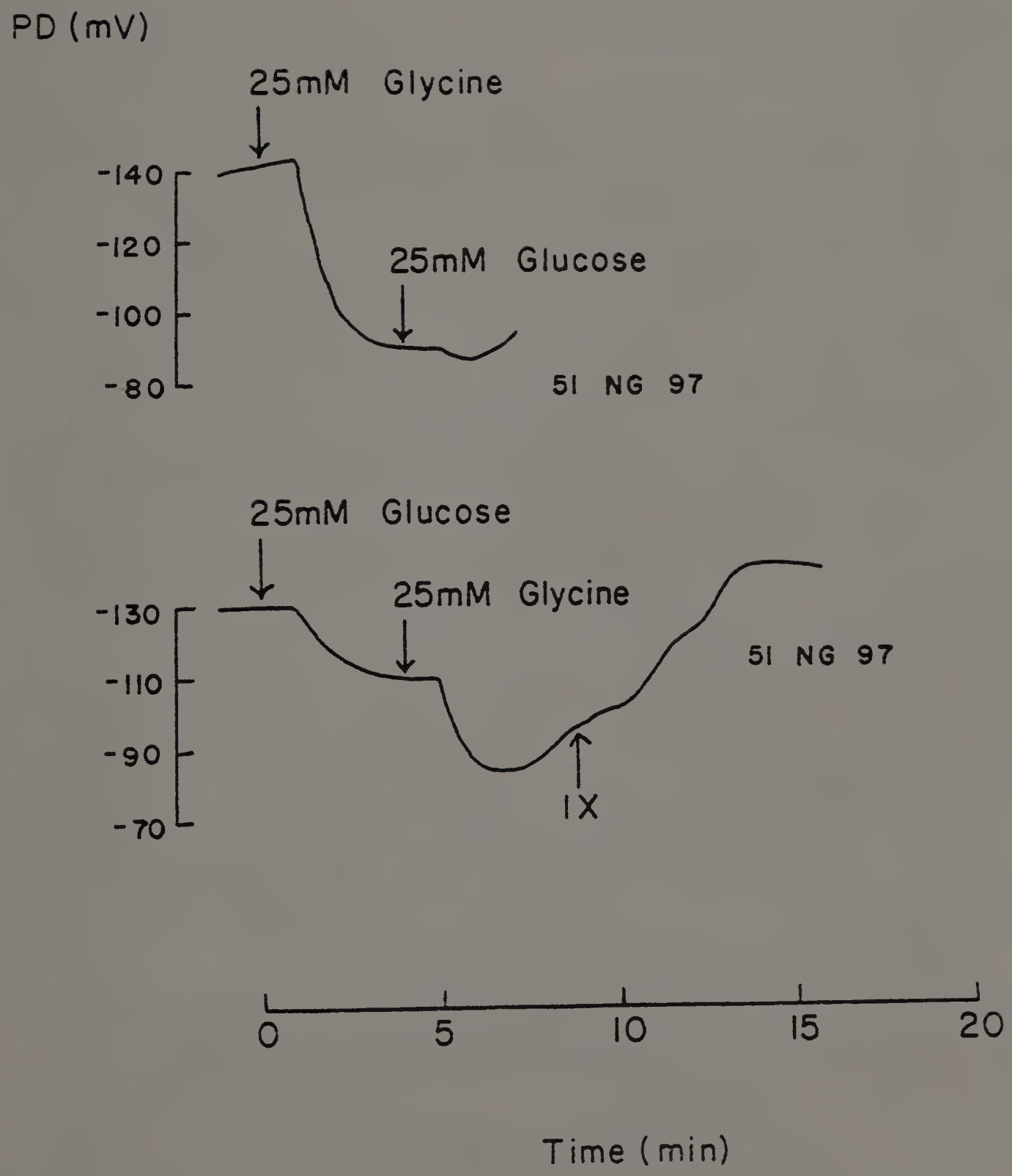

PD (mV)
25mM Glycine
-140
-120
-100
-80
25mM Glucose
51 NG 97
25mM Glucose
25mM Glycine
-130
-110
-90
-70
51 NG 97
1X
0
5
10
15
20
Time (min)

Table 8. Effect of depolarization of PD by glucose or glycine on depolarization by glycine or glucose, respectively, in leaf parenchyma cells of Saccharum clones 51 NG 97 and H50 7209.[a]

	Depolarization (mV)					
	51 NG 97			H50 7209		
A/B	+A	+B	Total	+A	+B	Total
Glucose/Glycine	19	40	59	30	32	62
	16	29	45	27	38	65
	19	22	41	17	24	41
Glycine/Glucose	56	12	68	52	5	57
	53	3	56	40	2	42
Mean Total ΔPD			54			53

[a] Either glucose or glycine (A) was added externally until maximal depolarization occurred. At this point, the original (A) solution was removed and replaced by either glycine or glucose (B), respectively. Concentrations were 25mM. Experiments were performed in the dark. Presented data is results from single experiments.

C H A P T E R IV

DISCUSSION

Pd in sugarcane leaf cells is due, in part, to an electrogenic pump. The evidence for this includes resting potentials too large to be diffussion potentials, transient changes in PD upon switching from dark to light and vice versa, and depolarization by metabolic inhibitors. Novacky and Karr (46) have provided similar evidence for an electrogenic pump in sugarcane. Electrogenesis is a prerequisite for cotransport of sugars and amino acids.

Of the sugars which caused depolarization, glucose, galactose, 3-O-methyl-glucose, sucrose and fructose are all actively transported into sugarcane cells (2,3,13,54). There is no evidence for raffinose being transported, but it is present in sugarcane cells as a metabolic intermediate (2,64). Therefore, it is likely that cells do have the ability to transport it across the plasma membrane. Of the sugars which did not cause depolarization, mannitol is not transported in higher plants (41), and the others have not been studied but are not likely to be transported. Lactose is of very limited occurrence in plants (16), melibiose is not important in cellular metabolism, and 1-O-methyl-d-galactose is an unnatural sugar with no important metabolic analog. Novacky, Karr and Van Sambeek (45) also observed a lack of depolarization of sugarcane cells

to methyl-α-galactoside. It appears from these results that depolarization is due to transport across the plasma membrane.

Depolarization increased with increasing glucose concentration until the response was saturated. This indicates a carrier-mediated process with a Km between 3 and 6mM glucose (Figure 6). Bowen (3) studied labelled glucose uptake as a function of concentration in immature storage tissue of sugarcane. He obtained a Km of 6.7mM for active uptake of glucose. Bieleski (2) reported a Km of 1.7mM for glucose uptake in mature storage tissue of sugarcane. This is good evidence that active transport of glucose is accompanied by membrane potential depolarization, and that the amount of depolarization is directly proportional to the rate of transport.

Glucose, sucrose, fructose, 3-O-methyl-glucose and galactose compete with each other for uptake, and, therefore, are probably transported via the same carrier sites (3,4,13,37). A similar competitive inhibition occurred here when sucrose, 3-O-methyl-glucose and raffinose were added to glucose-depolarized cells. When the depolarization due to glucose was saturated, no further depolarization occurred when 3-O-methyl-glucose or raffinose was added. Sucrose caused a slight additional depolarization in all cases. This is probably due to the fact that sucrose is hydrolysed by a cell wall invertase in sugarcane,

and is transported as glucose and fructose (4,54). This has the effect of increasing the total sugar concentration at the plasma membrane and causing increased depolarization if the carrier sites are not already saturated by 25mM glucose. Novacky and coworkers (48) observed a similar inhibition of depolarization in Lemna. At 20mM glucose the depolarization response was saturated. Adding 20mM glucose plus 20mM fructose did not increase the amount of depolarization.

The lack of susequent depolarization was not due to a lack of energy. Depolarization by KCN and SHAM showed that about 50 mV of PD is actively maintained and theoretically available for transport. Furthermore, even after depolarization with KCN and SHAM, both glucose and raffinose caused slight additional depolarizations. Bowen (3) found that cyanide and other metabolic inhibitors caused a strong inhibition of sugar transport in sugarcane. This shows that energy is necessary for sugar-induced depolarization, and the amount of energy available for this process is equal to the active portion of PD. The lack of further depolarization by 3-O-methyl-glucose and raffinose, and the small additional ΔPD caused by sucrose after glucose depolarization, show that the depolarization by all these sugars occurs by the same mechanism. Presumably this is the single carrier site involved in sugar transport.

The amount of depolarization caused by glycine, alanine

and serine was much greater than that caused by the same concentrations of sugars. Several studies (9,10,11,47, 53,67) have provided evidence for the cotransport of amino acids. Furthermore, Maretzki and Thom (36) found that arginine and lysine transport in sugarcane is an energy-dependent, carrier-mediated process. It appears that glycine, alanine and serine are cotransported in sugarcane, and that they are taken up to a greater extent than the sugars studied. The larger depolarization due to these amino acids was not due to additional charges on the molecules themselves because all three are neutral at pH 5.7. These results do not provide evidence either for or against the model for amino acid transport proposed by Kinraide and Etherton (28).

The depolarizations produced by glucose-glycine combinations can best be explained by a cotransport mechanism with two separate carriers involved. When the addition of glycine followed maximal depolarization by glucose, an additional ΔPD occurred which was less than that caused by glycine alone. The reverse, glycine depolarization followed by glucose, caused a similar result. At first glance it appears that each partially inhibits depolarization by the other. But it is more likely that two separate active sites for sugars and amino acids are involved. The explanation which is most consistent with all the data is that the cell continues to depolarize to the second substrate

until the diffusion potential is reached. This is supported by a comparison of the average ▵PD caused by glucose plus glycine, 53 to 54 mV (Table 8), with the average ▵PD caused by KCN plus SHAM, 48 to 52 mV (Table 6). If the active portion of the potential was greater, depolarization to glucose and glycine would be expected to increase until both carriers were saturated. This same explanation of the limiting effect of the diffussion potential cannot be applied to the lack of depolarization to a second sugar after glucose, because PD is still well above the diffussion potential in this case.

The time course of response of sugars and amino acids was generally similar. Depolarization occurred rapidly after the addition of substrate to the bathing medium. Time studies of accumulation of labelled glucose, fructose, sucrose, galactose and 3-O-methyl-glucose have shown that all are taken up immediately by sugarcane cells (3,13,37), so the time during which depolarization occurs corresponds to active transport.

After maximal depolarization had occurred, cells were able to repolarize in most cases. Full recovery and hyperpolarization occurred after the substrate was removed from the bathing solution. No recovery occurred with 3-O-methyl-glucose, and repolarization occurred slowly upon removal of this sugar from the bathing solution. There was no subsequent hyperpolarization in this case. 3-O-methyl-

glucose is a glucose analog which is transported like glucose but is not metabolized (37). Novacky et al. (48) reported, for Lemna, that recovery of PD to depolarization by 3-O-methyl-glucose was considerably slower than for other sugars.

These results may be explained in terms of the cotransport model by a shift in cell metabolism after depolarization. The electrogenic pump becomes more active in order to return PD to its original value, but it is unable to completely repolarize the cell while H^+ influx due to cotransport is occurring. When the substrate is removed, the increased activity of the pump is no longer balanced by a H^+ influx, and hyperpolarization occurs. After a short time the pump activity is again adjusted to the resting potential. Because of the long aging period in the dark, the tissues used in this study had extremely low energy reserves. Repolarization was dependent on ATP energy supplied by metabolism of the transported substrate. The nonmetabolized 3-O-methyl-glucose could not supply this energy,

The amount of depolarization was different for different sugars and amino acids. In both clones the amount of depolarization by 3-O-methyl-glucose was about 55% of the ΔPD due to glucose. In storage tissue of sugarcane, the rate of transport of 3-O-methyl-glucose is 60-90% that of glucose (3,13). Furthermore, the extent of depolariza-

tion by glucose is correlated to its rate of transport at different concentrations. From this, it appears that raffinose and 3-O-methyl-glucose have a lower affinity for the membrane carrier than the other sugars studied, and that the amino acids glycine, alanine and serine are transported to a greater extent than sugars.

An alternate explanation for the observed ΔPD is stimulation of cation extrusion by mitochondria, caused by the metabolism of these substrates (48). This is not likely, since the nonmetabolized 3-O-methyl-glucose caused depolarization of PD. Another explanation which can be ruled out is an osmotic effect on PD (26). If this were an osmotic phenomenon, all 25mM sugars would have produced the same amount of depolarization.

Bowen (3) reported a pH effect on active transport which appears inconsistent with a cotransport mechanism. He found that the optimum pH for sorbose uptake in immature internodal parenchyma of sugarcane was 6.5. Uptake dropped rapidly at higher pH values, and more slowly as pH was lowered below 6.5. Hawker and Hatch (18), though, found that the uptake of glucose was unaffected by pH between 4.0 and 7.0 in mature sugarcane tissue. A similar lack of effect of changing pH has been reported for other tissues (5,22) in studies where there has been otherwise strong evidence for cotransport. One explanation for this is that pH may not affect transport unless the concentra-

tion of H^+ becomes limiting.

In light of the previous transport studies in sugarcane, the evidence presented here strongly supports a cotransport mechanism of membrane transport of sugars and amino acids. All of the data presented is consistent with, and best explained by, the cotransport model. Most evidence so far presented for cotransport in higher plants has been circumstantial. The use of fusicoccin and diethylstilbestrol, specific inhibitors of the H^+-pump, may provide more conclusive evidence for a specific mechanism of membrane transport.

The two clones of sugarcane studied here differ in their susceptibility to the fungal pathogen Helminthosporium sacchari, causal agent of the eyespot disease of sugarcane. Clone 51 NG 97 is resistant to the fungus and its host-specific toxin, helminthosporoside; clone H50 7209 is susceptible. Helminthosporoside appears to contain two (62) or three (Karr, personal communication) separate toxins, one of which has the proposed structure 2-hydroxycyclopropyl-α-D-galactopyranoside. Strobel (63) found that the application of one of the α-galactosides raffinose, melibiose or methyl-α-galactopyranoside prevented symptom development due to this toxic compound, while other sugars did not block toxicity. The binding site of the toxin was reported to be a plasma membrane protein whose normal physiological role was α-galactoside transport (64). This

transport function was not present in resistant clones of sugarcane.

There was no significant difference between the two clones in their PD responses to sugars, α-galactosides and amino acids. Furthermore, raffinose appears to be cotransported by the same carrier site as other sugars, while melibiose and 1-O-methyl-α-galactoside are probably not cotransported. Therefore, the binding site reported for the toxin by Strobel does not seem to be involved in cotransport of α-galactosides. Cotransport does not appear to be related to resistance or susceptibility to the toxin.

Whether the toxin enters the cell has not been determined. It may act at the plasma membrane by affecting permeability (65), or it may enter the cell and alter chloroplast function (45), or it may have some other mode of action. Van Sambeek, Novacky and Karr (72) reported that helminthosporoside causes a rapid, reversible depolarization in both resistant and susceptible sugarcane leaves, followed by a second, irreversible depolarization in susceptible tissue and recovery in resistant tissue. Novacky (49) has speculated that the first depolarization is due to cotransport of the toxin into the cell, while the second depolarization is due to a toxic affect on a cellular organelle.

No conclusions con be drawn from these studies (45, 46, 72), however, because the toxin preparation used contained

high concentrations of other sugars which may have affected PD (Novacky, personal communication). Studies are now being conducted by Karr and Darus (Karr, personal communication) aimed at obtaining a pure preparation of helminthosporoside. With a purified toxin preparation and an understanding of cotransport in sugarcane, membrane potential studies should yield more conclusive evidence for the mode of action of helminthosporoside.

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