Plant Physiol. (1974) 54, 712–716

The Effect of Cyanide and Carbon Monoxide on the Electrical Potential and Resistance of Cell Membranes[1, 2]

Received for publication June 6, 1974 and in revised form July 23, 1974

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ABSTRACT

The rapid reduction in cell electropotentials induced by metabolic inhibitors is strong evidence for an electrogenic ion pump. According to Ohm's law, such a depolarization might be explained by a reduction in electric current, I, with unidirectional transport of a given ion, or an increase in permeability (decrease in resistance). With cells of etiolated seedlings of *Pisum sativum* L. cv. Alaska and *Zea mays* cv. Golden Bantam, carbon monoxide inhibition, which occurs only in the dark and is readily reversed by light, allows repeated cycling of depolarization and repolarization; there is no effect on cell membrane resistance. In contrast, cyanide inhibition results in a marked increase in membrane electrical resistance; with cyanide following repeated pulses of current used in measuring cell membrane resistance, the resistance eventually (about 10 minutes) shows an abrupt drop as in the "punch-through" effect reported by H. G. L. Coster (1965. Biophys. J. 5: 669–686).

Earlier work with etiolated seedling tissues has shown that metabolic inhibitors cause a rapid depolarization of cells which is reversible (9). According to Ohm's law (presumed to hold in this case) E = IR, such a reduction in electropotential might be caused by a blockage of ion transport (I) or an increase in membrane permeability (decrease in R). The present report is concerned with the effects of carbon monoxide and cyanide on cell electropotential and membrane resistance.

Cyanide at 10^{-5} to 10^{-3} M, within about 5 min, reduces the cell electropotential by as much as 50% (9); recovery requires approximately 30 min, a longer period presumably because of the time required for CN^- to be removed from the tissue. The rapid depolarizing effect of respiratory poisons on electropotential is probably the most critical evidence for the presence of electrogenic ion pumps at cell membranes; when a metabolically driven electrogenic pump is shut off, as upon interruption of respiratory electron flow or ATP supply, that component of the observed electropotential which is produced by the separation of charge at the pump will immediately become zero, and depolarization of the membrane by that amount will be observed.

Although the cyanide effect in higher plants is relatively rapid, it is very much longer than the ATP turnover time in the cell, which is less than 20 sec (2). Slayman *et al.* (21) have shown spectrophotometrically that after poisoning, ATP concentration in *Neurospora crassa* decays with a time constant of 5.4 sec.

In an attempt, therefore, to find an inhibitor with a poisoning effect which is not rate controlled by diffusion into the tissue, as CN^- almost certainly is, we have used solutions saturated with CO to bathe the tissue and have induced inhibition by switching off the light. Since the tissue can be allowed to saturate with CO in the light, there is no diffusional lag at the onset of inhibition which is, therefore, essentially instantaneous. Hopefully, resolution times of the order of milliseconds can be achieved and the effects occurring within the ATP turnover time can be monitored.

Carbon monoxide reacts with both Fe and Cu to form carbonyl compounds (11, 24, 25) but only the iron carbonyl is light-reversible; light at 430 nm appears to be the most active in causing the disassociation of the iron carbonyl (19). Early work showed that CO inhibits respiration by competing with O_2 for Cyt oxidase, the degree of inhibition being dependent upon the ratio of CO to O_2 (14). Weeks and Robertson (26) demonstrated that CO inhibition of respiration in carrot tissue is abolished in the light. The respiration decrease caused by 1 mM CN^- (another inhibitor of Cyt oxidase) was greater than that caused by saturating the incubation medium with 95% CO; this difference was attributed to the noncompetitive inhibition exhibited by CN^- *versus* the competitive type assumed for CO (11, 14). Robertson *et al.* (18) found that CO in the dark strongly inhibited the enhanced respiration induced in carrot discs by 2,4-dinitrophenol.

Carbon monoxide has also been shown to inhibit the accumulation of K^+, Cl^-, and Br^- in the dark but not in the light, and again these inhibitions depend upon the O_2:CO ratio (14). When the ratio was 1:9, it was found that Br^- uptake in barley roots was 48% of controls, but when the ratio was 1:19, Br^- uptake was only 2% of controls. In the same tissue, 95% CO (1:19) decreased respiration to 52% of controls in the dark and was only partially reversed in the light; 90% CO (1:9) gave less respiratory inhibition but was completely light reversible.

Slayman (19) found that CO produced a rapid depolarization, readily reversible in the light, of cell electropotential in *Neurospora crassa*. He also showed that anoxia produced a very similar degree of depolarization, while azide, cyanide, and 2,4-dinitrophenol produced larger depolarizations. Similar

[1] The research was supported by National Science Foundation Grant GB 19201, Amendment 1, and in part by funds provided for biological and medical research by the State of Washington Measure No. 171.

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electropotential depolarizations were found by Blinks *et al.* (3) in *Halicystis* during periods of anoxia. Interestingly, Slayman (19) found that the membrane resistance of *Neurospora* cells increased when the cells were depolarized with respiratory inhibitors, but the increase in membrane resistance appeared after the decrease in membrane potential.

In this paper, we described the effects of CO and CN^- poisoning on the cells of pea and corn roots and of pea shoots. Electropotentials were recorded and membrane resistances measured, using a technique recently described by us (1). The implications of these observations are discussed and are thought to provide further strong evidence that a large component of the higher plant cell electropotential is due to an electrogenic pump.

MATERIALS AND METHODS

Plant Material. Etiolated epicotyl tissue was obtained from pea seedlings (*Pisum sativum* L. cv. Alaska) grown from surface-sterilized seeds (10 min washing in 6% Clorox solution) for 7 days in total darkness at 25 C in vermiculite watered with nutrient solution. Root tissue was obtained from seedlings of peas (10 min washing in 6% Clorox solution) for 7 days in total darkness at 25 C in vermiculite watered with nutrient solution and of corn (*Zea mays* L. cv. Golden Bantam). Seeds were surface-sterilized, allowed to germinate on moist paper, and then grown hydroponically in nutrient solution at 20 C in light for 4 to 5 days. The nutrient solution used throughout all the studies described in this paper, previously designated 1X, has the following composition in mM: KCl, 1.0; NaH_2PO_4, 0.904; Na_2HPO_4, 0.048; $Ca(NO_3)_2$, 1.0; $MgSO_4$, 0.25. The pH of this solution is 5.8.

Preparation of Carbon Monoxide Solutions. Carbon monoxide solutions were prepared by vigorously bubbling either C.P.-grade CO or a 1:19 mixture of O_2 and CO (6, 11) into 1X nutrient solution while the solution was rapidly stirred with a magnetic stirring device. No difference in cell potential was found with the 1:19 mixture and those solutions prepared by bubbling only CO. At room temperature, the amount of CO in this salt solution was determined to be approximately 0.8 mM by gas chromatography on the vapor above aliquots of boiling solution (6).

Electrical Measurements. The potential differences between the external solution and the vacuoles of cells under investigation were measured essentially as described elsewhere (6, 9). Microcapillary salt bridges filled with 3 M KCl were connected through silver-silver chloride electrodes and through a Bioelectric NF 1 recording amplifier to either a Keithley Model 610B electrometer and a Heath Model EUW-20A servorecorder or to a Tektronix Model 532 oscilloscope fitted with a type 1A1 dual-trace unit and a Polaroid camera. Membrane-resistance measurements were made by a novel technique recently described by us (1). Very briefly, calibrated unipolar square-wave current pulses were injected at the intracellular microelectrode via a series resistor (Victoreen Hi Meg 10^9 Ω) from an Exact 500B waveform generator, and the resulting voltage signals from the cell were used to evaluate membrane resistance. Positive or negative pulses gave the same results, *i.e.* no rectification was observed (1).

Experimental Procedure. Pea stem tissue was excised and the segments were incubated for at least 10 hr in aerated 1X solution at 20 C before being used for measurements, to avoid the initial transient responses which follow excision (13, 16). Observations on root tissue were made with intact seedlings. During electrical measurement, the stem-tissue segments were mounted vertically in a 2-ml chamber which fitted to the vertical stage of a microscope for viewing both the cells and the intracellular microelectrode; the root tissue was mounted horizontally in an elongated chamber designated to accommodate intact seedlings, which also could be fitted to the microscope stage (13). Electrical recording of normal cells was carried out with a stream of 1X solution flowing through the specimen chambers. Carbon monoxide solutions were admitted to the chambers by stopping the inflow of 1X solution and injecting into the chamber a volume of CO-saturated solution equal to ten times the chamber volume. The tissue was illuminated by the microscope lamp and by a spotlight (150-w General Electric Cool Ray). Both these lamps were external to the Faraday cage surrounding the electrical recording circuits. Cyanide solutions at pH 7.0, buffered with tris, were applied to the tissue by switching the inflow from the 1X-solution reservoir to one containing 1X solution plus the required concentration of NaCN.

RESULTS

Effect of Carbon Monoxide on Cell Electropotential. In the light, CO solutions have no effect on electropotential in any of the tissue types studied (pea shoot and root and corn root), but in the dark they induce a rapid and reversible depolarization. There is no effect of light-dark switching when the tissues are bathed in 1X solution without CO. A typical response is given in Figure 1 for a cell in a pea epicotyl segment aged for several hours in 1X solution; note that the cell potential can be taken through a complete cycle of inhibition and recovery in 10 min, although most of the cycle is over in 3 to 4 min. The inhibition is complete in 90 sec, the first 50% of the effect being achieved in 40 sec; recovery is extremely fast initially, the time taken for 50% of recovery being approximately 10 sec. The implication of this difference in initial response times for inhibition and recovery will be discussed later. Although it is not shown in Figure 1, a cell can be taken through a succession of as many as 10 to 15 such cycles. The effect is reduced over many cycles, probably because of CO loss to the atmosphere by diffusion from the open specimen chamber. Replenishment with fresh CO-saturated solution restores the full effect.

Cells from pea roots give a similar though smaller inhibitory response. In this case, the fast time course was measured and it shows that again initial recovery of electropotential on releasing respiratory inhibition by switching on the light is faster than the initial inhibitory response (Fig. 2). In Figure 3 we

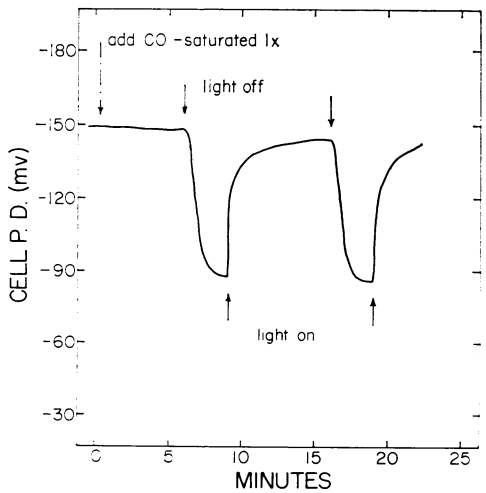

FIG. 1. Depolarization of cells from etiolated pea stem by CO and reversal under light.

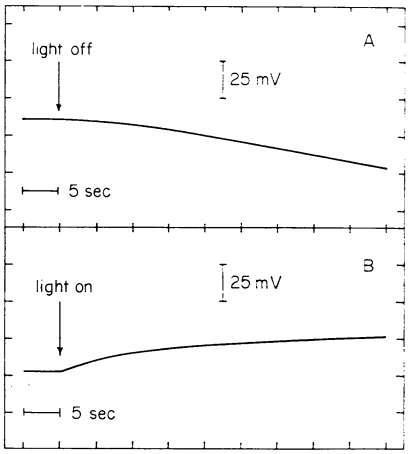

FIG. 2. Fast response of depolarization by CO of a pea root cortex cell and repolarization in light. A: depolarization in the dark; B: repolarization in the light. The figure represents a photographic record of an oscilloscope tracing.

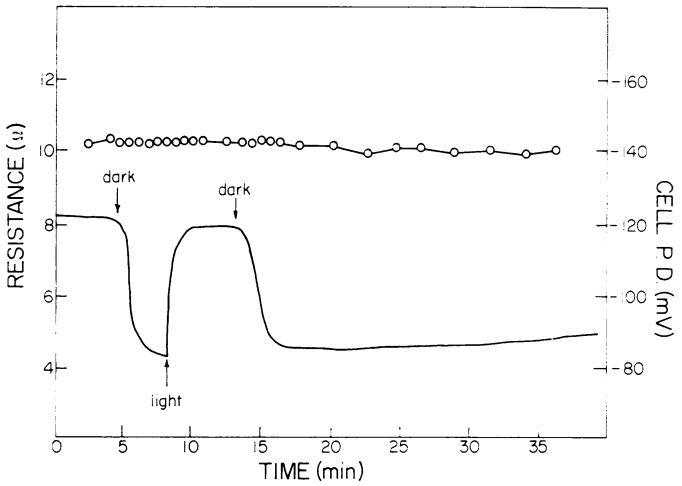

FIG. 3. CO effect on pea root cortical cell. The electrical resistance (○) of a pea root cell is not changed by CO during a depolarization-repolarization cycle (——, cell PD [potential difference]) or with prolonged inhibition in the dark.

show that the inhibition can be held for long periods of time by keeping the tissue dark. The slow, slight recovery is again probably due to diffusional loss of CO to the atmosphere. The second line (open circles) on Figure 3 shows the membrane-resistance values obtained on the same cell. Note that there is essentially no change in resistance although the cell potential is responding dramatically to the respiratory inhibition.

The effect noted above should be contrasted with the effect of CN^- poisoning shown in Figure 4. Within about 10 min after applying 10^{-3} M NaCN in 1X solution, the cell electropotential decreases to about 40% of its normal value. In approximately half of the cases, CN^- initially induces a transient hyperpolarization (Fig. 4) (9, 15). During this time course membrane resistance rises rapidly; it increases approximately 3-fold during the initial hyperpolarizing response and then continues to increase as the cell depolarizes. The dramatic decrease in membrane resistance which was observed at about 10 min after applying CN^- is thought to be the result of membrane breakdown, a "punch-through" induced by the electric current used to measure resistance, as has been described in *Chara australis* (= *C. corallina*) and *Nitella* sp. (4, 15). This interpretation is reinforced by the nonrecovery of the electropotential of this particular cell after removal of CN^-. We have observed in many cells where either resistance has not been measured, or where care has been taken to avoid electrical punch-through, that cell electropotential did recover upon removal of CN^-.

Finally, it should be stated that the maximal depolarizations caused by CO were always less than those induced by either CN^- or by carbonyl cyanide *m*-chlorophenylhydrazone (6). The depolarizations caused by cyanide overshadowed those caused by CO; when added in either order, the resultant depolarization in the cells of any of the tissues, was equal to that caused by CN^- alone (Fig. 5), and in the presence of CN no repolarization could be shown in the light.

DISCUSSION

The rapidity of cell depolarization and repolarization with CO inhibition provides us with information on the nature of electrogenesis in several respects: (*a*) combined with the fact that measured cell electropotentials exceed those predicted

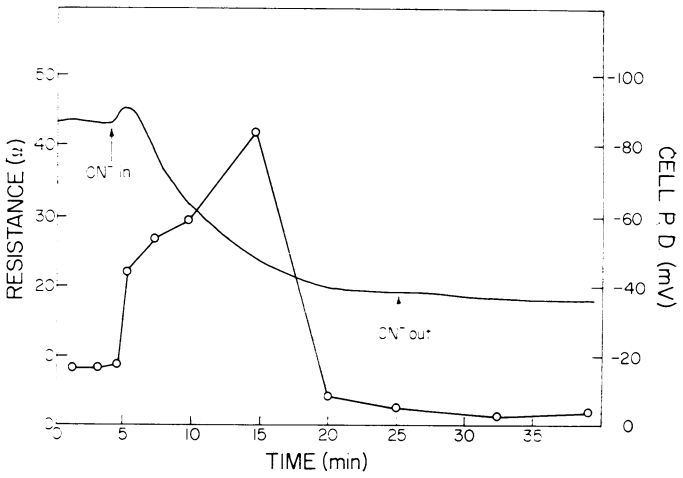

FIG. 4. Cyanide effect on pea root cortical cell. The electrical resistance (○) of a pea root cell as modified by CN^- during depolarization (——, cell PD). The drop in resistance at 15 min is believed to be a "punch-through" effect induced by the current pulses used for measuring R.

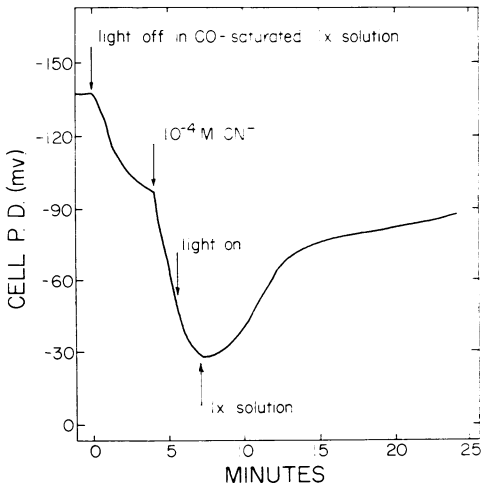

FIG. 5. Added effect of CN^- on CO depolarization of a pea stem cell; light fails to reverse CN^- depolarization.

by the Goldman equation, it provides strong evidence for the existence of an electrogenic pump in higher plants; (*b*) it indicates that this electrogenic mechanism is directly or indirectly dependent upon an iron-containing compound, most likely Cyt a,a_3; and (*c*) short time responses to CO inhibition suggest the requirement for a metabolic pool by the electrogenic mechanism.

Under most conditions reported here, there seems to be no alternative to the conclusion that an electrogenic pump is operating, since the electropotential is appreciably greater than that predictable from ionic diffusion. For example, in the pea stem experiment of Figure 1 the membrane potential, E_M, is -149 mv whereas the K^+_{in}/K^+_{out} ratio is about 40, giving an E_K ($= 58 \log K^+_{in}/K^+_{out}$) value of -77 mv, far below the actual voltage. E_M, predicted from the Goldman voltage equation would be appreciably below E_K, since anion permeability is significant.

Under conditions in which E_K (-98) exceeds E_M (-86), *e.g.*, in pea root (Fig. 4), the argument might be made that passive diffusion and induced changes in cation/anion permeability could account for the results without invoking an electrogenic pump. However, the time course of depolarization is far too rapid for such a large change in ion content. Further, an increase in membrane permeability ordinarily would be detected by a decrease in the electrical resistance of the membrane which was not observed when CO was used. It is conceivable that the cation/anion permeability ratio could change in a manner not affecting the over-all membrane resistance to current. To detect this, the chord conductance of ions, requiring voltage clamping, would be required; this has not been accomplished. We think that such a fortuitous change is highly unlikely.

It is much more satisfactory to explain the observations in terms of there being an electrogenic pump component of the measured cell potential, which is rapidly inhibited by respiratory poisons. Such electrogenic pumps have been demonstrated in many plant tissues (3, 6, 9, 13, 19, 22, 23) and have been discussed at several theoretical levels (7, 8, 22, 23). All that needs to be stated here is that an electrogenic pump, while operating, will contribute directly to the observed cell potential, and that an energy supply is necessary for the pump's operation. A highly satisfactory explanation of the rapidity of CO inhibition is that shortly after respiration is inhibited, the cell membrane depolarizes because the electrogenic pump stops. In the light respiration resumes, the pump starts, and is observed as a repolarization of membrane potential.

Initial rates of repolarization are approximately four times as fast as are the initial depolarization rates (Fig. 2). This should not be true if there were a direct coupling of the pump to respiratory electron flow, *i.e.*, via the Cyt system; in this case the rates of depolarization and repolarization should be the same. On the other hand, if the formation of a metabolite was blocked by CO, this pool might gradually diminish and a pump powered by this pool would also slow down. The same pump might have a faster initial rate if (*a*) the chemical reaction of the pump had zero-order kinetics with respect to substrate during normal operation but first-order kinetics after CO blockage depleted the substrate pool size or (*b*) if the rate of diminution of the pump depended upon the decay of a metabolite pool, the time course of which exceeded the rate of its diffusion to the pump. For the latter case, if we use ATP as an example, the ATP turnover rate in higher plant cells has a half-time of between 2 and 20 sec (2), but the time required for the diffusion of a metabolite the size of ATP over the distances involved is under one sec.

The choice of ATP as the metabolite involved is attractive since a good correlation between ATP pool decay and the cell potential in *Neurospora* has been shown by Slayman *et al.* under CN^- inhibition (20, 21). The time course of ATP and electropotential decay in *Neurospora* was found to have a half-time of about 5 sec, which is approximately equal to our data for depolarization and repolarization. Also, the presence of an ATPase in higher plant plasma membranes whose activity correlates closely with K^+ accumulation in intact cells now appears to be firmly established (5, 10).

We have observed no dramatic change in measured membrane resistance upon poisoning of the pump with CO. This is in marked contrast to the data on light effects obtained by Spanswick in *Nitella translucens* (22), where the membrane resistance increases 9-fold upon presumed cessation of pump activity. In these experiments with *Nitella,* the pump was not switched on and off by respiratory poisons but by light-dark switching of photosynthetic activity. This may provide a partial explanation of the difference; the electrogenic mechanism which is coupled to photosynthesis may differ from that in our tissues which are not photosynthetic.

Cyanide poisoning, which causes a very strong membrane depolarization in all tissues studied, also induces a very rapid increase in membrane resistance (Fig. 4). This suggests that there is indeed a more direct coupling of iron-containing redox compounds to the facilitated passage of ions across the membrane and reinforces the idea that the first effect is at the surface, while the second requires diffusive permeation of CN^- to the mitochondria. Perhaps the old controversy of the existence of Fe-containing redox compounds directly involved in carrying ions across the plasmalemma, as suggested independently by Lundegårdh (12) and Robertson and Wilkins (see 17), remains unresolved.

In passing, it may be noted that the initial hyperpolarization often found with CN^- poisoning (9) (Fig. 4) can be explained by this increase in initial membrane resistance. At short times the electrogenic pump rate is not affected by CN^- but the resistance rises so that, by Ohm's law, the potential contribution of the pump increases and the membrane hyperpolarizes. The argument has been developed more fully elsewhere (1); similar observations have recently been reported by Pickard (15), but their significance apparently escaped the author. The increase in membrane resistance induced by cyanide might reflect the blocking of Fe-containing compounds which might act as ion transporters. CO apparently does not cause such blocking, and the implication may be taken that they are not O_2 receptors but possibly Fe compounds associated with transport sites, which are inhibited by association with CN^-. These may be the ion-transporter recognition sites which one of us (7) has speculated upon in the past.

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