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Responses of corn root protoplasts to exogenous reduced nicotinamide adenine dinucleotide: Oxygen consumption, ion uptake, and membrane potential

(NADH oxidation/ion transport)

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ABSTRACT **Addition of 1.5 mM NADH tripled the O_2 consumption in corn root protoplasts. The stimulation was temperature and pH dependent, specific to NADH, and accompanied by a 2- to 3-fold increase in K^+ and P_i uptake into protoplasts. The increase in ion uptake was not due to the accumulation of NADH into protoplasts. The effect of exogenous NADH on O_2 consumption and ion uptake was also evident in corn root segments but to a lesser extent. A 20-mV hyperpolarization of protoplast membrane potential occurred on addition of NADH and was abolished by the uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone. Increases in cell volume of 30% and 40% were detected in response to NADH/H^+ and NADH/H^+/K^+, respectively. The data are discussed in terms of a transmembrane redox reaction and the possibility that some part of the energy-linked ion transport may be driven by a NADH → O_2 electron-transport system in the plasmalemma.**

In plant cells, ion transport is believed to be driven by an electrochemical proton gradient established by a plasmalemma ATPase and the hydrolysis of ATP (1–3). A transmembrane electrochemical gradient of protons can also be established by a redox loop consisting of an alternating sequence of hydrogen and electron carriers (4) without direct involvement of ATP. Although such a redox system in the plasmalemma has been suggested in animal (5, 6) and plant (7, 8) cells, the direct linkage of this redox system to ion transport in plant cells has yet to be demonstrated. This study demonstrates the existence of a plasmalemma NADH → O_2 redox chain that is involved in ion transport in corn root cells.

MATERIALS AND METHODS

Root protoplasts were isolated from 3-day-old etiolated corn (*Zea mays L.* Pioneer Hybrid 3320) seedlings as described (9, 10) and suspended for all experiments in a basic medium of 0.65 M mannitol/0.2 mM $CaCl_2$/1 mM Hepes buffer, pH 6.0. Additions for measuring K^+ or P_i uptake were 1 mM KCl or K phosphate labeled with carrier-free $^{86}Rb^+$ or $^{32}P_i$, respectively. After 15 min of incubation at 30°C in the presence of radiolabeled ions, protoplasts were isolated by rapid centrifugation through silicone oil (10).

For determination of NADH uptake, [*adenosine*-^{14}C(U)]-NADH was freshly prepared by mixing $Na_2S_2O_3$ and [*adenosine*-^{14}C(U)]NAD^+ (New England Nuclear) (2:1, mol/mol) at pH 7.0 for 5 min and then adding this mixture to the protoplast suspension in basic medium to give 1.5 mM NADH. Preliminary experiments showed >95% conversion to NADH.

Membrane potential was determined by accumulation of methyltriphenylphosphonium ($MePh_3P$) ion; protoplasts were incubated with and without 1.5 mM NADH for 10 min and then with and without 10 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP). [*methyl*-^{3}H]$MePh_3PBr$ (New England Nuclear) was then added to a final concentration of 1 mM (10 μCi; 1 Ci = 3.7×10^{10} becquerels). At each time point, protoplasts were isolated by centrifugation from 300-μl aliquots (≈10^6 protoplasts) of the mixture. The tip of the Microfuge tube containing the protoplasts was cut off, the protoplasts were lysed with 0.5 ml of boiling water, and radioactivity and protein were determined (protein was used to estimate protoplast number and hence volume). Calculations of membrane potential were based on steady-state internal/external $MePh_3P^+$ concentrations (after 1 hr of incubation) as described by Rubinstein (11).

Packed protoplast volume measurements were made after centrifugation in hematocrit tubes.

Oxygen consumption was measured by the O_2 electrode. Assay mixtures consisted of 0.9 ml of basic medium plus 0.1 ml of protoplast suspension (≈10^6 protoplasts) and were incubated at 30°C (except in temperature-response experiments) with various additions of substrate. For root segment respiration, five 0.5-cm segments (≈0.1 g in 1 ml of 0.2 mM $CaCl_2$/0.2 mM K phosphate, pH 6.0) were used. Uptake of (^{86}Rb)K^+ and $^{32}P_i$ into root segments was determined as described (12). CO_2 evolution was determined after 5 and 10 min of incubation of protoplasts or segments in the same solution used for determining O_2 consumption, except that the solution was first equilibrated with CO_2-free air for 5 min. Actual measurement was by IR absorption (13).

RESULTS

Recent work (9, 10, 14) has shown that the transport of inorganic ions (H^+, K^+, $H_2PO_4^-$, Cl^-, and SO_4^{2-}) and the respiration of isolated corn root protoplasts closely approximates that in the tissue from which they were derived. Addition of 1.5 mM NADH to protoplasts in basic medium (pH 6.0) produced an immediate 3-fold increase in O_2 consumption (Fig. 1, trace 1) without altering the rate of CO_2 evolution [e.g., 0.26 μmol of CO_2/hr per 10^6 protoplasts and 23.89 (μmol of CO_2/hr)/g (fresh weight) without NADH and 0.25 μmol of CO_2/hr per 10^6 protoplasts and 22.50 (μmol of CO_2/hr)/g (fresh weight) with 1.5 mM NADH]. NADH disappearance from the solution was calculated from the decrease in A_{340} ($\varepsilon = 6.22 \times 10^6$ cm^2). Its molar ratio to the extra O_2 consumed was 2.3, slightly larger

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Abbreviations: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; $MePh_3P$, methyltriphenylphosphonium.

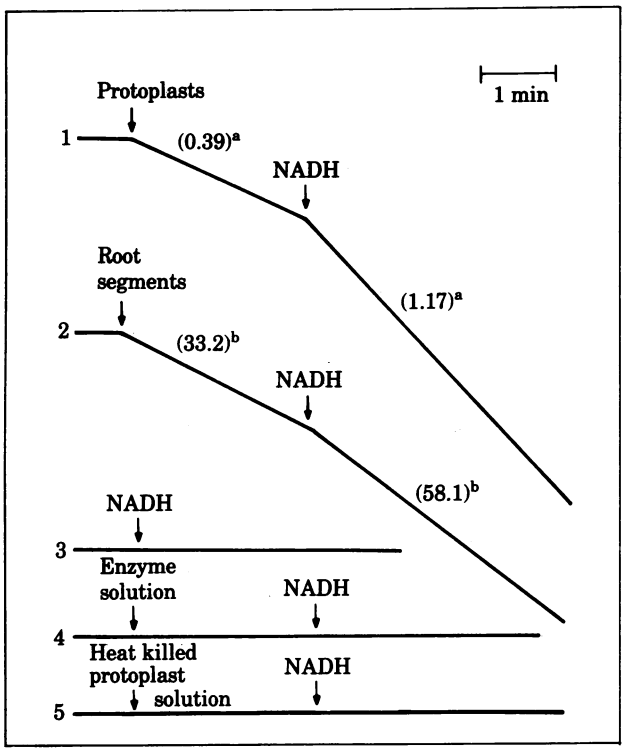

FIG. 1. O_2 consumption in corn root tissue. Traces: 1, 1×10^6 protoplasts and 1.5 mM NADH were added as indicated; 2, 0.12 g of five 0.5-cm corn root segments was used; 3, mannitol buffer only (no tissue) was used; 4, 100 μl of cell wall digestion enzyme solution was used; 5, 100 μl of 10-min heat-killed protoplast solution was used. [a], In units of μmol of O_2/hr per 10^6 protoplasts; [b], in units of (μmol of O_2/hr)/g (fresh weight).

than the expected value of 2.0. To show that this oxidation is not an artifact of the isolation procedure, several control experiments were carried out. Neither mannitol buffer alone (Fig. 1, trace 3) nor the enzyme digestion mixture used to isolate the protoplasts (Fig. 1, trace 4) showed any response to the addition of NADH. Heat-killed protoplast suspensions were also inactive (Fig. 1, trace 5). Qualitatively, the same NADH oxidation is seen in root segments (Fig. 1, trace 2); the lower increase may be due to the fact that only superficial layers of root cells were exposed to the added NADH.

The stimulation of O_2 consumption in protoplasts was specific for NADH. There was no response to added NAD^+ and thus no evidence that the NADH stimulated O_2 consumption was an indirect consequence of supplying a respiratory cofactor. Other reductants such as dithiothreitol, ferridoxin, and methyl viologen at the same or comparable concentrations did not stimulate O_2 uptake, while 1.5 mM NADPH gave <10% of the activity of NADH. Typical substrates of mitochondrial respiration including malate, pyruvate, succinate, and citrate were also ineffective. This suggests that mitochondria are not involved and demonstrates that the protoplast preparation was free of mitochondrial contamination. Ascorbate oxidase and poly- or diphenol oxidases, common oxygenases in plant cells (15), are capable of oxidizing NADH. Ascorbate or catechol at 1.5 mM caused no detectable change in O_2 consumption, nor did they alter the NADH stimulation. These data suggest that NADH may stimulate or activate a nonmitochondrial NADH–O_2 redox chain (7) or serve as a substrate for the system and result in increased O_2 consumption.

Kinetics of the stimulated O_2 consumption showed the reaction to be saturated at 2 mM NADH; a K_m of 0.8 mM (range, 0.1–2.5 mM) for NADH was calculated. Most NADH dehydrogenases have far lower K_m values—i.e., in the micromolar range (16). However, it has recently been reported that ascites tumor cells can oxidize exogenous NADH (as our cells appear to do) and that the reaction has an apparent K_m of 0.15–0.5 mM (17).

As expected, temperature affected O_2 consumption under both basal and NADH-stimulated conditions (Fig. 2). A Q_{10} of >1.5 was found between 15°C and 35°C for NADH-stimulated O_2 consumption. The nonlinear response of O_2 consumption in the lower part of the range (<15°C) (Fig. 2 *Inset*) suggests the involvement of a membrane–enzyme interaction. There is a discontinuity in the Arrhenius plot at ≈9°C in both basal and NADH-stimulated O_2 consumption consistent with a phase transition of phospholipids inside the plasma membrane that in turn may affect both the permeability of the substrate and the microenvironment of the bound enzyme. The transition point at 9°C and the 8–10 kcal/(mol/K) (1 cal = 4.18 J) activation energy observed here are similar to the reported figures for corn root tissue (18, 19). It appears that exogenous NADH oxidation, like normal respiration, is a membrane-linked phenomenon.

The pH sensitivity of NADH oxidation by protoplasts is shown in Fig. 3. Beyond pH 6.5, there is a rapid decrease in NADH oxidation that does not occur in basal respiration. The NADH oxidation appears to have a broad pH optimum in the acid range. A confounding factor may arise from the need for H^+ as a substrate [$NADH + H^+ + (1/2)O_2 \rightarrow NAD^+ + H_2O$].

Novak and Ivankina (7) have postulated that a NADH to O_2 redox chain is localized in the plasmalemma of *Elodea* and *Vallisneria* cells. The operation of this redox chain produces a spatial separation of H^+ and OH^- on different sides of the plasmalemma, resulting in the potential and the pH gradients that drive other ions across the membrane. Recently, stimulation by exogenous NADH of amino acid uptake was demonstrated in Ehrlich ascites tumor cells (20). When NADH was used as the

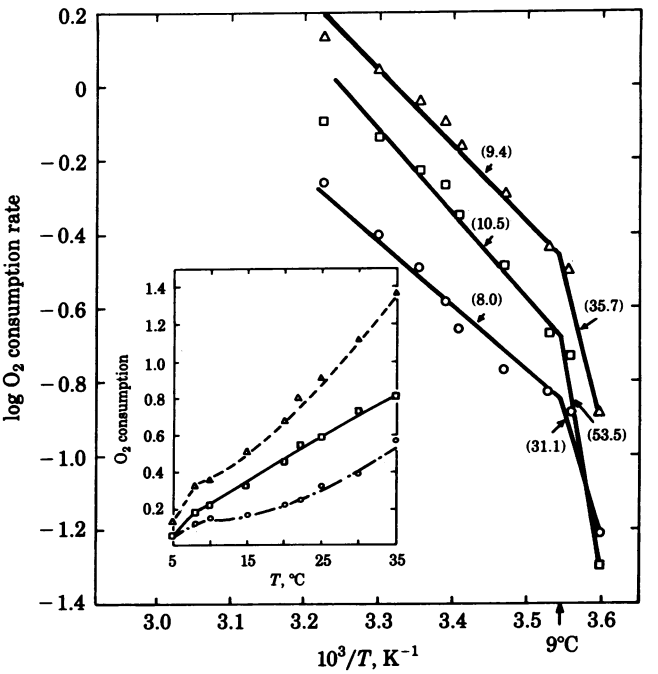

FIG. 2. Arrhenius plot of oxygen consumption (μmol of O_2/hr per 10^6 protoplasts) in corn root protoplasts. (*Inset*) Relationship between temperature (T) and O_2 consumption. ○, Basal rate; △, NADH-stimulated rate; □, difference between basal and NADH-stimulated rates. NADH was used at 1.5 mM. Values in parentheses are activation energies in units of (kcal/mol)/K.

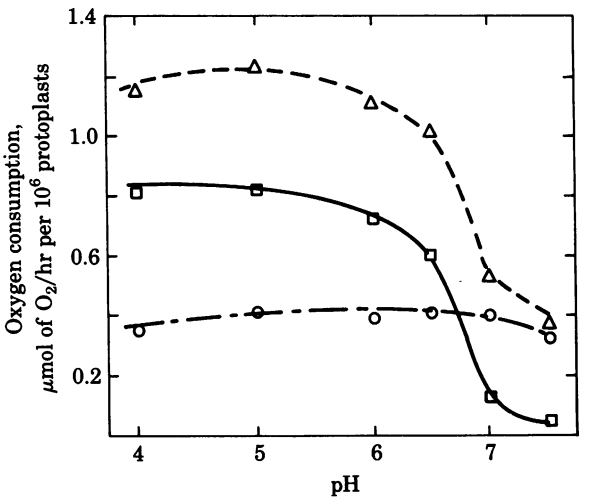

FIG. 3. Relationship between external pH and oxygen consumption of corn root protoplasts. ○, Basal rate; △, NADH-stimulated rate; □, difference between basal and NADH-stimulated rates. NADH was used at 1.5 mM.

hydrogen donor and phenazine methosulfate was used as the electron acceptor, there was a 1.5- to 2-fold increase in the uptake of several amino acids.

The stimulation of O_2 consumption of corn root protoplasts by exogenous NADH could result from transfer of H^+ (and/or e^-) to O_2, which would then generate an electrochemical gradient across the plasmalemma. Accordingly, tests were made to determine whether the extra O_2 consumption with NADH was energy linked. As shown in Table 1, the NADH oxidation by both protoplasts and root segments produced greater K^+ and P_i influx, which was uncoupled by FCCP. Addition of ATP had no effect on O_2 uptake of protoplasts but inhibited uptake in root segments. Quinacrine, a suggested specific inhibitor for a transmembrane oxidoreductase (6), inhibited both basal and NADH-stimulated uptake in root segments but inhibited only the NADH-stimulated uptake in protoplasts, a result similar to that obtained with amino acid uptake in tumor cells (20). The reason for the discrepancy between protoplasts and segments is not known.

Accumulation of $[^3H]MePh_3P^+$ was used to estimate cell membrane potential. As shown in Fig. 4, there was increased energy-linked accumulation of this freely permeating organic cation with NADH oxidation. The calculated membrane potential at 60 min in the control was −65 mV, which was reduced to −20 mV with FCCP, for an electrogenic component of 45 mV. In the presence of 1.5 mM NADH, the corresponding values were −85 mV and −25 mV, for an electrogenic component of −65 mV.

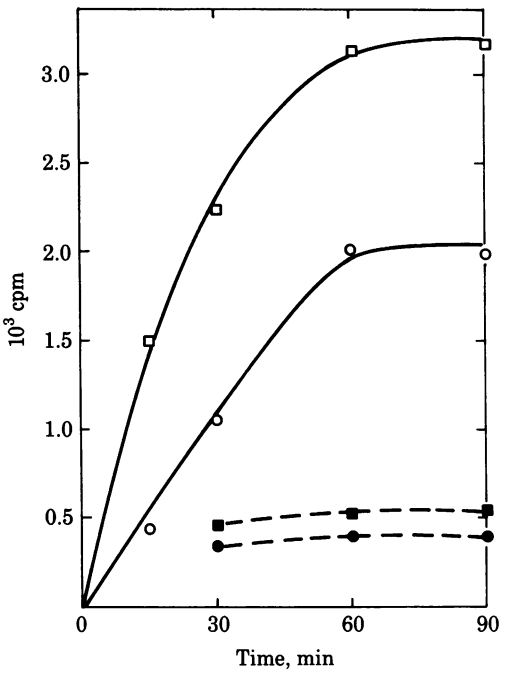

FIG. 4. Time course of $[^3H]MePh_3P^+$ accumulation in corn root protoplasts in the presence of various additions. ○, Control (no additions); □, 1.5 mM NADH; ●, 10 μM FCCP; ■, 1.5 mM NADH/10 μM FCCP.

Removal of the cell wall enabled the observation of cell volume changes in response to the osmotic pressure changes across the cell membrane. As expected, NADH increased the packed protoplast volume—from 33.64 ± 2.10 to 43.73 ± 1.60 μl per 10^6 protoplasts at the steady state. Addition of 1.0 mM KCl further increased the volume to 47.10 ± 2.05. Addition of NADH alone increased the cell volume 30% while NADH/K^+ increased it 40%.

There is a basic question as to whether NADH is oxidized at the outer surface of the protoplast or first taken up into the protoplast. The instantaneous response to added NADH (Fig. 1) makes the latter possibility unlikely but, on the other hand, it is difficult to visualize the apoplast normally supplying NADH to the protoplast *in vivo*. Use of labeled NADH showed that, in 15 min of incubation, 444 nmol of NADH was oxidized per 10^6 protoplasts while only 1.3 nmol was accumulated. When labeled NAD^+ was supplied in place of NADH, only 0.1 nmol was accumulated. Thus, there is no evidence that NADH must be accumulated before oxidation or, if transport into the protoplast is required, the NAD^+ formed must be removed rapidly without significant mixing with internal NAD^+.

Table 1. Effect of exogenous NADH and ATP on K^+ and P_i uptake in corn roots

	Uptake in protoplasts, nmol/hr per 10^6 protoplasts		Uptake in root segments, (μmol/hr)/g (fresh weight)	
	$K^+(^{86}Rb^+)$	$P_i(H_2{}^{32}PO_4^-)$	$K^+(^{86}Rb^+)$	$P_i(H_2{}^{32}PO_4^-)$
None (control)	25.30 (100)	5.24 (100)	2.52 (100)	0.44 (100)
NADH	70.84 (280)	10.38 (198)	3.40 (135)	0.48 (110)
NADH/FCCP (10 μM)	1.25 (5)	1.05 (20)	0.11 (4)	0.05 (11)
Quinacrine (0.5 mM)	24.50 (97)	5.30 (101)	0.36 (14)	0.27 (61)
NADH/quinacrine	30.42 (120)	6.23 (119)	0.37 (15)	0.26 (59)
ATP	25.72 (102)	4.98 (95)	1.94 (77)	0.31 (70)

NADH and ATP concentrations were 1.5 mM and 2.0 mM for protoplasts and root segments, respectively. Values in parentheses represent percentage of control.

DISCUSSION

It does not seem rational that root cells and isolated protoplasts should possess an energy-linked system for oxidizing exogenous NADH, but the empirical fact is that they do, as do ascites tumor cells (20). One possibility is that protoplast isolation has altered the plasmalemma such that NADH can penetrate to the oxidase system. However, intact root cells also oxidize exogenous NADH (Fig. 1) in a fashion that increases K^+ influx (Table 1), which indicates that the external cell layers behave like protoplasts. What may be acting here is the reversal of some system that normally transfers reducing equivalents from inside to outside. That is, there may be a NADH–NAD^+ exchange system or a transmembrane redox system in the plasmalemma, thus increasing the redox potential at the inner surface of the membrane. Due to the very limited amount of labeled NAD passing into the protoplasts, the former alternative is unlikely.

One system in roots requiring a transmembrane redox reaction is the reduction of Fe^{3+} at the cell surface. Chaney *et al.* (21) suggest that a flavin or cytochrome might carry the electron. Craig and Crane (8) have recently described a transplasmalemma electron transport system in cultured carrot cells that reduces external and impermeant ferricyanide. A fraction of corn coleoptile microsomes that has properties suggestive of plasmalemma contains flavoprotein and cytochrome *b*, the latter reduced by blue light (22, 23). It may be that flavoprotein and cytochrome *b* are implicated in a transmembrane redox reaction required to pass reducing equivalents across the membrane. If this pathway is reversible, the energy-linked oxidation of NADH can be physiologically rationalized. In this respect, it may be relevant that the inner membrane of plant mitochondria also oxidizes exogenous NADH, and a transmembrane transhydrogenation has been suggested from internal NADH (24).

A transmembrane redox reaction might furnish the energy linkage through oxidative phosphorylation (i.e., $NADH_{out} \rightarrow NADH_{in} \rightarrow$ mitochondria $\rightarrow$ ATP $\rightarrow$ membrane ATPase). However, mitochondrial respiration is under control of the phosphate potential, and there is no reason to believe that increases in the cytosol NADH/NAD^+ ratio would bypass this control to give 3-fold greater O_2 consumption. In their review, Low and Crane (5) concluded that a NADH dehydrogenase system in the plasmalemma might drive transport of ions and amino acids. In ascites tumor cells, the establishment of a Na^+ gradient appears to be responsible for the amino acid uptake (20). For plant cells, there is still some evidence that a component of respiration may transport ions (7). Novak and Ivankina (7) have postulated a NADH $\rightarrow$ O_2 redox chain in plant plasmalemma. In recent studies (25, 26), questions have been raised as to whether a membrane ATPase can be exclusively responsible for ion transport and hyperpolarization. Studies of NADH oxidation by isolated protoplasts may be of great service in dealing with these problems.

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