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NADH OXIDASE ACTIVITIES OF INTACT LEAF DISCS OF SPINACH

BY

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ABSTRACT

NADH oxidase activities of intact leaf discs of spinach. - Leaf disks, 1 cm in diameter, were punched from leaves of market spinach and used for assay of NADH oxidase. The disks exhibited a steady state rate of NADH oxidation of about 1 nmole/min/disk. The activity was proportional to time of incubation and to the number of disks. The activity was stimulated by the synthetic auxin 2,4-D to a maximum near 1 μM 2,4-D. The disks provide a convenient system to measure the cell surface NADH oxidase in leaves.

Abbreviations: NADH, reduced nicotinamide adenine dinucleotide; NOX, plasma membrane-associated NADH oxidase; 2,4-D, 2,4-dichlorophenoxyacetic acid; IAA, indole-3-acetic acid.

INTRODUCTION

Ongoing work in the laboratory has identified an external cell surface NADH oxidase of soybean hypocotyls (HICKS and MORRE, 1998) that can be readily assayed using intact hypocotyl sections (BARR *et al.*, 2001).

The activity correlated well with findings from right side-out isolated plasma membrane vesicles (DEHAHN *et al.*, 1997). The cell surface NADH oxidase of soybean assayed either with intact sections or with isolated right side-out vesicles was stimulated by active auxins 2,4-dichlorophenoxyacetic acid (2,4-D) or indole-3-acetic acid (IAA), but was unaffected by inactive or weak auxins 2,3-dichlorophenoxyacetic acid (2,3-D) or β-naphthaleneacetic acid (β-NAA) (MORRÉ *et al.*, 1988). Additionally the auxin-stimulated components of the surface NADH oxidase and of growth were blocked by thiol reagents such as N-ethylmaleimide (BARR *et al.*, 2001). Similar results were obtained with plasma membrane vesicles where the auxin-stimulated component of the activity was blocked by thiol reagents (MORRÉ *et al.*, 1995b).

In this report, the stimulation of NADH oxidase activity by auxin already reported for plasma membrane vesicles and intact soybean stem sections was extended to leaf disks of spinach. The system provides a convenient method for the study of the surface NADH oxidase of leaves.

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MATERIALS AND METHODS

Plant material

Commercial spinach (*Spinacia oleracea* L.) was used. One cm diam leaf disks were punched using a sharp cork borer. Disks from 10 to 20 different spinach leaves were collected directly into 0.1 M sucrose, 5 mM $CaCl_2$, 10 mM NaCl and 10 mM KCl (sucrose-salts). The sections were maintained at room temperature before and during all experiments.

NADH oxidase activity

The conditions selected were to incubate 10 to 20 of the 1 cm diam. disks in 30 ml of sucrose-salts containing 100 μM NADH with mild initial shaking. The rate of NADH oxidation was determined on a 6 ml sample from the decrease in A_{340} using a Sequoia-Turner Model 340 spectrophotometer. After determining the optical density, the solution was returned to the flask and the incubation continued. The blank rate in the absence of disks was negligible (0.1 O.D. units per 180 min).

RESULTS

Based on the decrease in absorbance at 340 nm, 1-cm leaf disks cut from leaves of spinach oxidize NADH (Fig 1A). The rate in the absence of 2,4-D or KCN calculated from the disappearance of NADH (decrease in absorbance at 340 nm) was 9 nmol/ min/10 1-cm disks (Fig. 1). Following addition of 2,4-D, the rate of NADH disappearance was increased by about 50% to 13 nmol/min/10 1 cm diam. leaf disks (Fig. 1). The blank rate in the presence of NADH but in the absence of tissue (<0.5 nmol/min) was substracted. In the presence of tissue but in the absence of NADH, the absorbance increased slightly. No rates were observed with tissue segments boiled for 10 min prior to assay. Distilled water or 50 mM Tris-Mes, pH 7.0, gave results equivalent to sucrose-salts.

The rate of NADH oxidation was proportional to number of disks (Figs. 2 and 3). The 2,4-D-stimulated component of the cell surface NADH oxidase was resistant to 1 mM potassium cyanide (Fig. 1B). This resistance was seen only with the auxin-stimulated component of the activity. Both in the presence or absence of 2,4-D, the rate of NADH oxidation by the leaf disks was greatly reduced by the addition of 1 mM cyanide. However in the presence of 2,4-D, a 2,4-D-induced increment in activity in the presence of cyanide (Fig. 1B) similar to that observed in the absence of cyanide (Fig. 1A) was observed. The cyanide-resistant component was proportional to the number of leaf disks in the cuvette (Fig. 3). The degree of auxin stimulation (proportion of total) was enhanced considerably over that in the absence of cyanide because the cyanide-susceptible component of NADH oxidation was reduced.

The response of NADH oxidation to the logarithm of 2,4-D concentration revealed an optimum at 1 μM with the 1-cm disks (Fig. 4). The weak auxin 2,3-dichlorophenoxy-acetic acid, at 1 μM, elicited no auxin-stimulated rate (not shown). With 2,4-D, the total

2,4-D-induced increment of activity was increased by about 10 nmoles/min/30 sections both in the absence and presence of cyanide and the ratio of plus 1 μM 2,4-D to no 2,4-D was greatly augmented due to the reduced background in the presence of cyanide (Fig. 4).

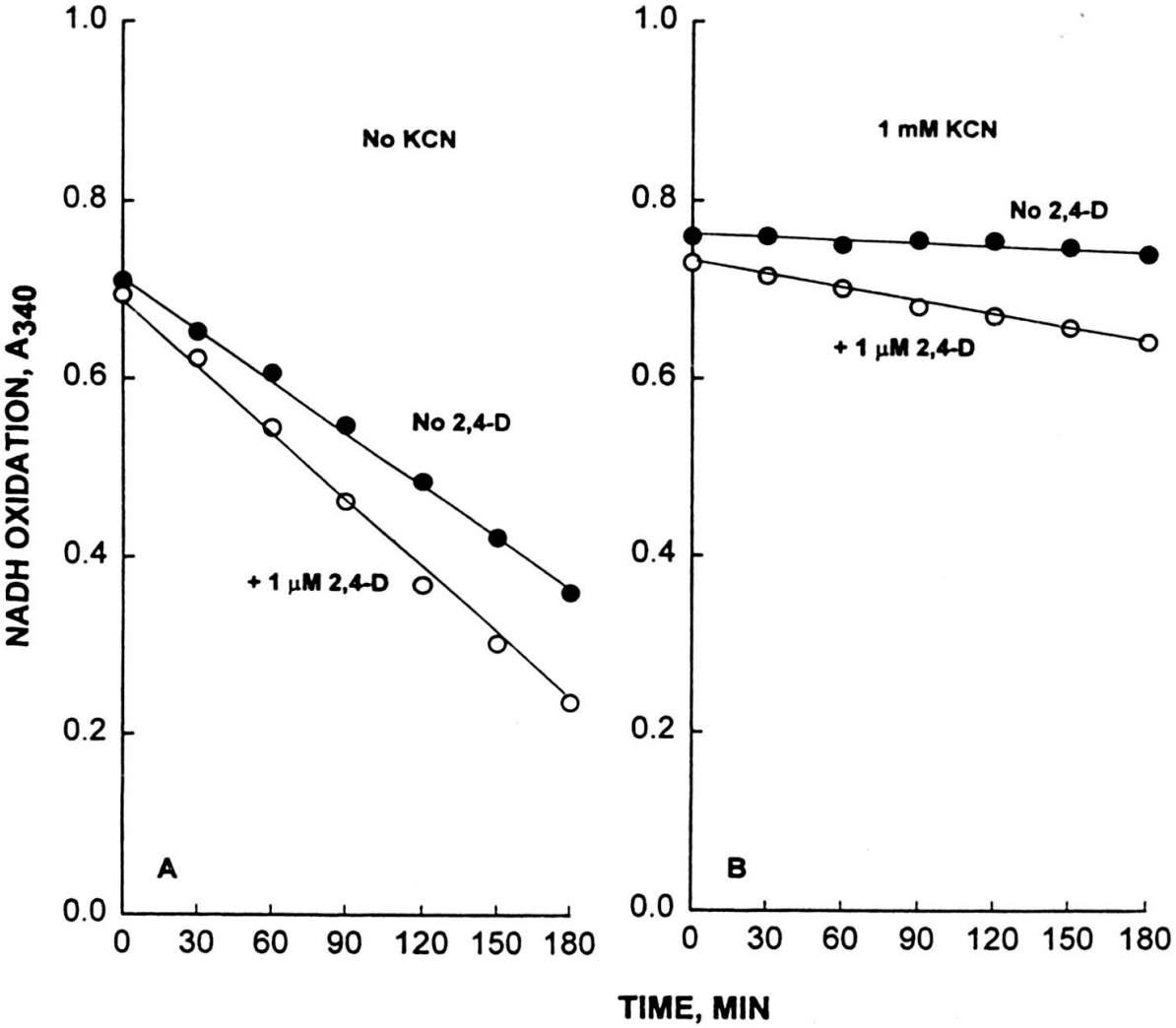

FIG. 1.

Time course of NADH oxidation catalyzed by 10 1-cm diameter spinach leaf disks as determined by the decrease in absorbance at 340 nm in the absence (solid symbols) or presence (open symbols) of 1 μM 2,4-D. A: no KCN. B: 1 mM KCN.

DISCUSSION

Since an extracellular source of NADH in plants sufficient to reach concentrations at or near the Km for the surface NADH oxidase is highly unlikely, some constituent endogenous to the plant plasma membrane, such as hydroquinones or protein thiols, has been assumed to be the physiological electron donor for the 2,4-D-stimulated cell surface NADH oxidase activity. Both protein thiols (CHUEH *et al.*, 1997) and reduced phylloquinone (BRIDGE *et al.*, 2000) have been implicated. In addition to oxygen (MORRÉ, 1994), protein disulfides appear to serve as acceptors of protons and electrons coming

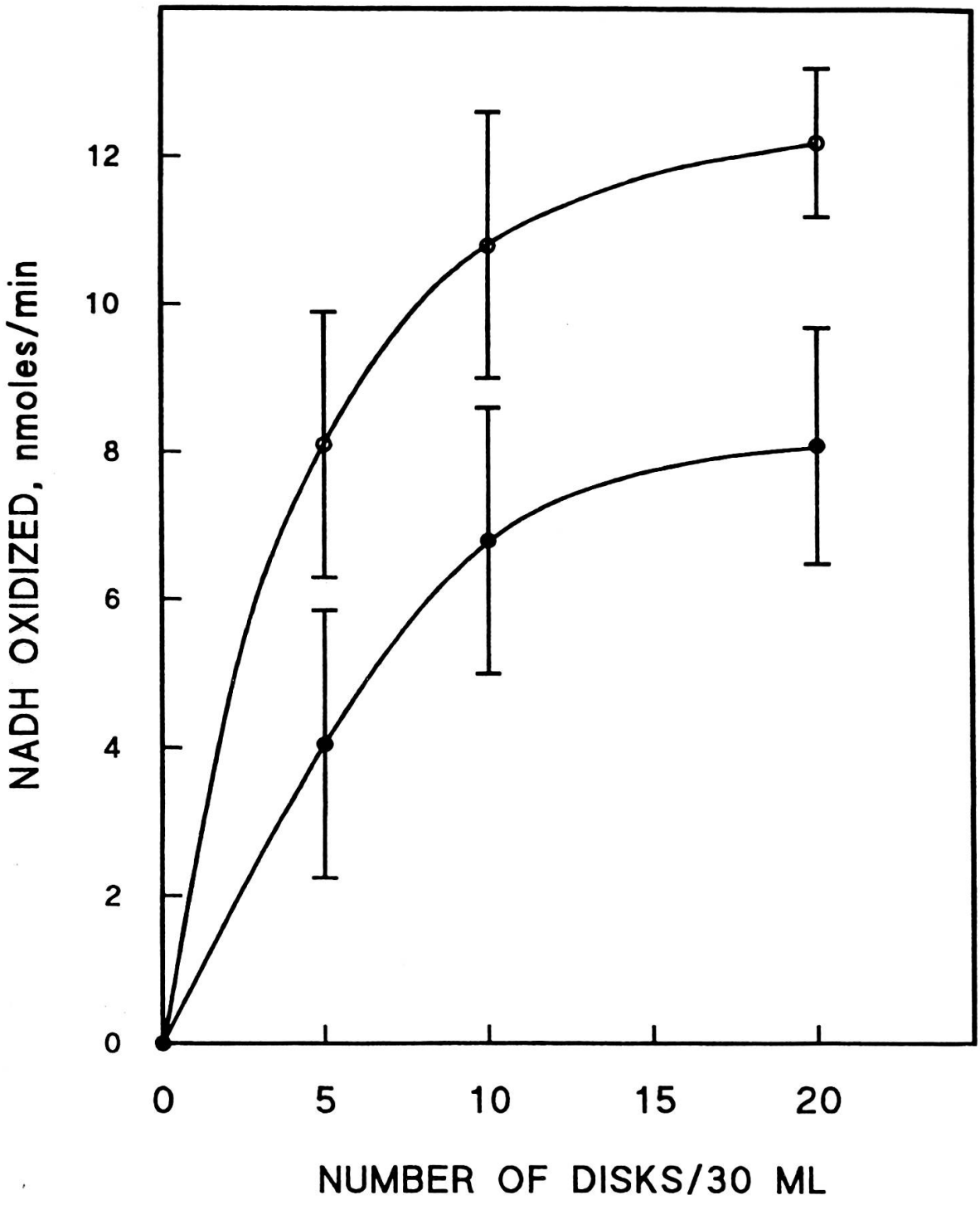

FIG. 2.

Cell surface plasma membrane NADH oxidase is proportional to number of 1-cm diameter spinach leaf disks in the absence (solid symbols) or presence (open symbols) of 1 μM 2,4-D and in the absence of KCN. Values are averages of 3 experiments ± standard deviations among experiments.

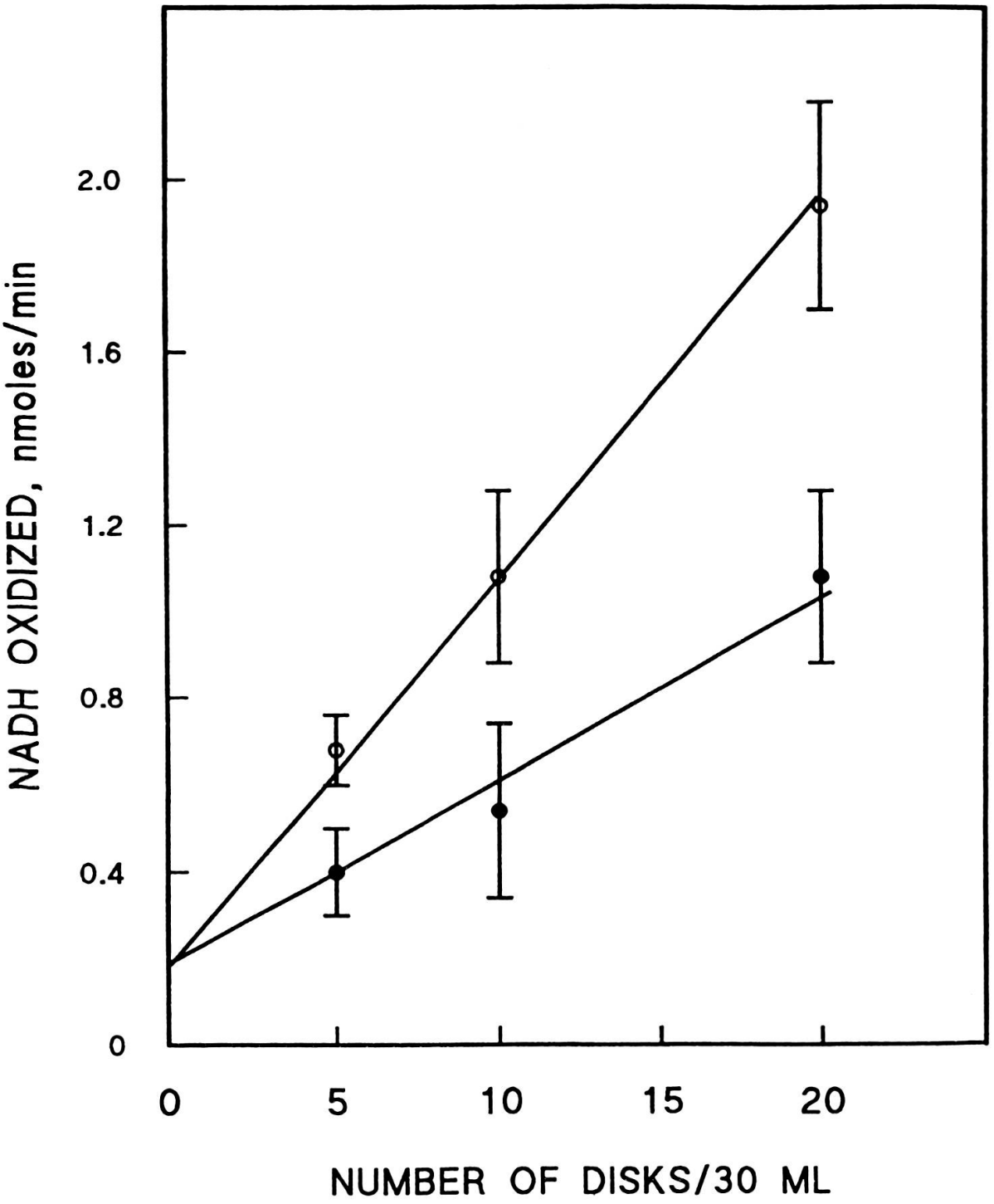

FIG. 3.

Cell surface plasma membrane NADH oxidase is proportional to number of 1-cm diameter spinach leaf disks in the absence (solid symbols) or presence (open symbols) of 1 μM 2,4-D but in the presence of 1 mM KCN. Values are averages of 3 experiments ± standard deviations among experiments.

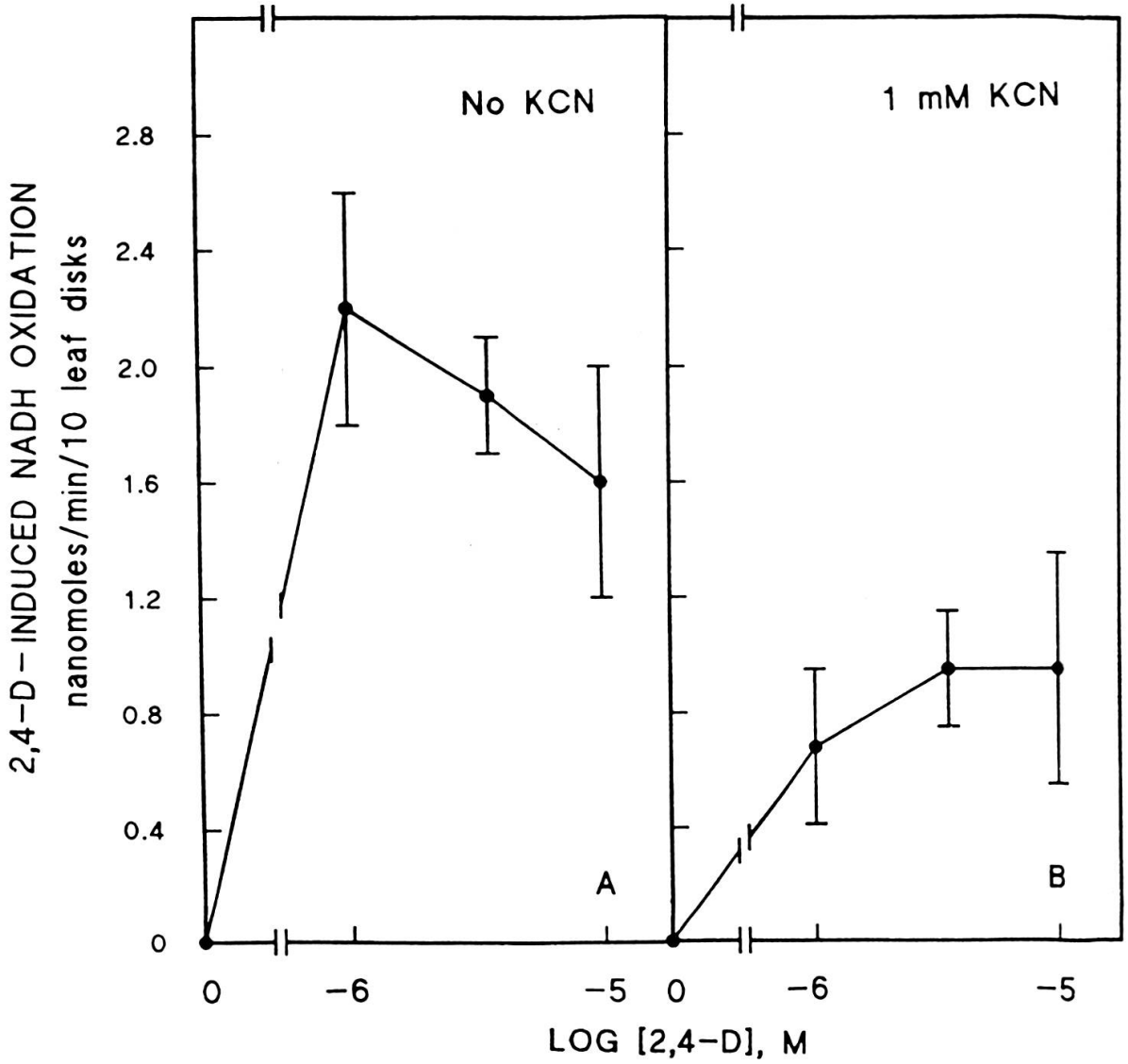

FIG. 4.

Response of NADH oxidase of 10 1-cm diameter leaf disks of spinach to the logarithm of 2,4-D concentration in the absence and presence of 1 mM KCN. The 2,4-D-responsive cell surface plasma membrane NADH oxidase is cyanide resistant. Values are averages of 2 or 3 experiments ± standard deviations among experiments. A: no KCN. B: 1 mM KCN.

from NADH (CHUEH *et al.*, 1997) or from the physiological electron donor, reduced phylloquinone (BRIDGE *et al.*, in press). A model has been developed where the protein disulfide-thiol interchange portion of the activity (MORRÉ *et al.*, 1995) could function in physical membrane displacements important to the enlargement growth of plant cells (MORRÉ, 1994).

An external location of the growth-related NADH oxidase was first raised for plants from studies comparing the 2,4-D-responsive oxidation of NADH by right side-out and inside-out plasma membrane vesicles (DEHAHN *et al.*, 1997). Both populations of vesicles contained an NADH oxidase activity but the component of the activity stimulated by 2,4-D was accessible only to NADH supplied to right side-out vesicles and was, therefore,

located at the external cell surface. The possibility of an external NADH oxidase in plants was raised early by LIN (1984) for plant roots. However, until recently (MORRÉ, 1998), the functional significance of an external NADH oxidase was unclear.

A similar situation of having NADH oxidase activity at both the internal and external surfaces of the plasma membrane exists for cultured mammalian cells as well. That at least one of the NADH oxidase activities associated with the mammalian cell surface was external was indicated from studies of the antitumor sulfonylurea-inhibited NADH oxidase activity of cervical carcinoma (HeLa) derived human cell line (MORRÉ, 1995). The drug-inhibited oxidase was observed only with vesicles of right side-out orientation (MORRÉ, 1995). Intact HeLa cells subsequently were shown to exhibit the drug-responsive activity (MORRÉ *et al.*, 1997) as would be expected for a drug-inhibited NADH oxidase associated with the cell surface. That both the drug and the NADH site of the drug-responsive NADH oxidase were external was demonstrated subsequently by inhibition of NADH oxidase activity using impermeant conjugates of an antitumor sulfonylurea (KIM *et al.*, 1997).

The resistance of external NADH oxidase to inhibition by cyanide was not unexpected since the assay procedure for the auxin-responsive NADH oxidase of plants (BRIGHTMAN *et al.*, 1988, MORRÉ *et al.*, 1986) as well as that for the drug inhibited activity of mammalian membranes (MORRÉ *et al.*, 1995) has included cyanide in the assay mixture. Clearly with the intact hypocotyl sections of the soybean, the 2,4-D responsive activity was cyanide resistant as was the total NADH oxidase activity of right-side-out plasma membrane vesicles (BRIGHTMAN *et al.*, 1988). What was unexpected considering only assays of isolated plasma membrane vesicles was that the intact sections appeared to exhibit an NADH oxidase activity that was cyanide sensitive. Such an activity normally has been low or absent with plasma membrane vesicles. It has previously been attributed to mitochondrial contamination (MOYA-CAMARENA *et al.*, 1995). In the work with intact soybean sections, it was unlikely that the cyanide sensitive activity was from cut surfaces since neither washing the sections nor sealing the ends of the sections with lanolin eliminated the activity. The explanation proposed is that the cyanide-sensitive activity was the result of a peroxidase that was either loosely associated with the cell surface or was present in the apoplast and normally lost during the preparation of plasma membranes. A similar phenomenon was observed for intact soybean sections (BARR *et al.*, 2001) and this explanation may be equally applicable to leaf disks of spinach.

RÉSUMÉ

ACTIVITÉS NADH OXYDASE DE DISQUES INTACTS DE FEUILLE D'ÉPINARD

Des disques sont prélevés sur des feuilles d'épinard et utilisés pour une mesure directe de l'activité NADH oxydase. Mis en présence de NADH, les disques oxydent le NADH de façon continue au taux d'environ 1 nmole/min/disque. Cette activité est linéaire dans le temps et proportionnelle au nombre de disques de feuilles. Elle est stimulée par l'auxine synthétique 2,4-D, avec un maximum à 1 µM. L'utilisation de disques de feuilles permet l'étude de la NADH oxydase présente sur la surface des cellules.

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