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Hormone- and Growth Factor-Stimulated NADH Oxidase

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An NADH oxidase activity of animal and plant plasma membrane is described that is stimulated by hormones and growth factors. In plasma membranes of cancer cells and tissues, the activity appears to be constitutively activated and no longer hormone responsive. With drugs that inhibit the activity, cells are unable to grow although growth inhibition may be more related to a failure of the cells to enlarge than to a direct inhibition of mitosis. The hormone-stimulated activity in plasma membranes of plants and the constitutively activated NADH oxidase in tumor cell plasma membranes is inhibited by thiol reagents whereas the basal activity is not. These findings point to a thiol involvement in the action of the activated form of the oxidase. NADH oxidase oxidation by Golgi apparatus of rat liver is inhibited by brefeldin A plus GDP. Brefeldin A is a macrolide antibiotic inhibitor of membrane trafficking. A model is presented where the NADH oxidase functions as a thiol–disulfide oxidoreductase activity involved in the formation and breakage of disulfide bonds. The thiol–disulfide interchange is postulated as being associated with physical membrane displacement as encountered in cell enlargement or in vesicle budding. The model, although speculative, does provide a basis for further experimentation to probe a potential function for this enzyme system which, under certain conditions, exhibits a hormone- and growth factor-stimulated oxidation of NADH.

KEY WORDS: NADH oxidase; plasma membrane; growth factors; growth; thiol/disulfide interchange; membranes; brefeldin A; Golgi apparatus.

INTRODUCTION

Previous studies have identified an NADH oxidase activity of the plasma membrane of rat liver (Morré *et al.*, 1988c; Morré and Brightman, 1991; Brightman *et al.*, 1992), keratinocytes (Morré *et al.*, 1992), and plant stems (Morré *et al.*, 1986b; Brightman *et al.*, 1988) that was stimulated by hormones and growth factors. In cancer, the activity was constitutively activated and no longer hormone responsive (Morré *et al.*, 1991; Bruno *et al.*, 1992). In both plants (Morré *et al.*, 1988a, b) and animal cells in culture (Morré *et al.*, 1992), stimulation or inhibition of the activity correlated closely with inhibition or stimulation of growth.

The NADH oxidase activity of liver and plant plasma membranes was unique among oxidoreductase activities not only in its response to growth factors and hormones but, also, in its response to inhibitors and activators other than growth factors and hormones (Morré and Crane, 1990; Morré *et al.*, 1988c, 1992). In this regard it differed from the more usual NADH-ferricyanide oxidoreductase activity also found with the mammalian plasma membrane (Crane *et al.*, 1985).

In this paper, evidence will be summarized that suggests that the NADH oxidase activity that is hormone- and growth factor-responsive is, in reality, a thiol–disulfide interchange activity. The physiological donor may very well be protein thiols and the physiological acceptor protein disulfide. An involvement of the activity in thiol–disulfide exchanges was first indicated from the extreme sensitivity of the hormone- and growth factor-stimulated as well as the constitutively activated NADH oxidase activity of the plasma membrane of both plant (Morré *et al.*, 1994a) and animal (Morré and Morré, 1994) cells and tissues to thiol reagents. A thiol–disulfide inter-

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change activity was subsequently confirmed from a correlation between the stimulation and inhibition of NADH oxidase activity by hormones or NADH oxidase inhibitors and corresponding changes in the ability of plasma membranes to catalyze the activation of inactive, oxidized and scrambled, ribonuclease (Morré *et al.*, in preparation).

The function of the thiol–disulfide interchange activity measured as a hormone- and growth factor-stimulated NADH oxidase remains to be established. However, recent evidence suggests that it may be involved as an essential component of membrane displacements as occur in vesicle budding and cell enlargement. The activity is inhibited by the macrolide antibiotic inhibitor brefeldin A as well as by certain antitumor sulfonylureas and is activated by ATP. The evidence in support of such a functional role will be summarized and a molecular model presented. A relationship to ascorbate is also indicted not only at the plasma membrane but also with internal membranes, and especially with the Golgi apparatus. Protein disulfide isomerase has dehydroascorbate reductase activity (Wells *et al.*, 1990) and a relationship between ascorbate and vesicle budding at the trans Golgi apparatus has been observed previously (Sun *et al.*, 1984; Morré *et al.*, 1985, 1987).

CHARACTERISTICS–DONOR AND ACCEPTOR SPECIFICITY

The hormone-stimulated NADH oxidase activity was first observed with isolated plasma membrane vesicles of soybean in response to the synthetic auxin, 2,4-dichlorophenoxyacetic acid (Table I) (Morré *et al.*, 1986b). In the initial experiments, attempts were made to use ascorbate or ascorbate radical (ascorbate plus dehydroascorbate) as an external acceptor, but no exogenous external acceptor was required for the hormone-stimulated activity. The auxin-induced reponse in the absence of ascorbate or ascorbate radical was equivalent to that observed in its presence (Table I). The acceptor subsequently was assumed to be oxygen (Morré and Brightman, 1991) but, for the hormone-stimulated activity, may in fact be thiols or some combination of thiols and oxygen.

Table I. NADH Oxidation by Soybean Plasma Membranes Is Isolated in the Presence of Ascorbate Stimulated by Auxins[a]

Additions	Concentration	nmol/min/mg protein
None		15
2,4-D	10 μM	24
Ascorbate	1.7 mM	20
Ascorbate +2,4-D	1.7 mM ± 10 μM	31
Monodehydroascorbate	1 mM	24
Monodehydroascorbate +2,4-D	1 mM + 10 μM	31

[a] The reaction mixture contained, in a total volume of 3 ml, 50 mM Tris-HCl, pH 7.4, 0.3 M sucrose, 85 μM NADH, and 30–50 μg plasma membrane protein. The monodehydroascorbate was generated from an equal mixture of ascorbate and dehydroascorbate adjusted to pH 7.4 with 10 mM imidazole. From Morré *et al.* (1986b).

The plasma membrane of both plant and animal cells contains peroxidase activities (Crane *et al.*, 1985). However, there is no evidence for hydrogen peroxide generation for the hormone-stimulated oxidation of NADH (Morré and Brightman, 1991). This is supported by several lines of evidence. The hormone-stimulated NADH oxidase was designated a type I oxidase which is normally assayed at pH 7.0–7.4 with micromolar concentrations of NADH and is cyanide insensitive. Nor is the type I activity inhibited by catalase or superoxide dismutase (Pupillo *et al.*, 1986; Brightman *et al.*, 1988). Type II NADH oxidase activity is most commonly assayed at pH 5.5–6.0 with 1 mM NADH or more. This activity is inhibited by cyanide and by catalase and exhibits characteristics of a peroxidase (Asard *et al.*, 1987; Vianello and Macri, 1989). In addition, this activity is inhibited by scavengers of free radicals such as superoxide dismutase and ascorbate. The hormone-stimulated activity is inhibited by neither superoxide dismutase nor ascorbate (see Table I). Thus, several lines of evidence distinguish the hormone-stimulated NADH oxidase activity from NADH oxidase II which has peroxidase activity.

While there is evidence that both the NADH oxidase and a transmembrane reductase capable of reducing ferricyanide as an electron acceptor are hormone-responsive (Gayda *et al.*, 1977; Goldenberg *et al.*, 1979; Crane *et al.*, 1985), the growth factor- and hormone-stimulated NADH oxidase is distinct from the transmembrane NADH-ferricyanide oxidoreductase system. The growth factor- and hormone-stimulated NADH oxidase does not utilize ferricyanide as an electron acceptor (Table II). The NADH-fericyanide oxidoreductase of the plasma membrane is not hormone-stimulated in plants under conditions where the NADH oxidase activity is stimulated by hormones. The growth factor- and hormone-stimulated NADH

Table II. NAD(P)H-Dependent Redox Activity of Soybean Plasma Membranes[a]

Electron acceptor	E^0, pH 7 (V)	Specific activity		Auxin effect (%)
		Control	Auxin	
Nitrate	−0.421	0.4	0.4	0
Dehydroascorbate	+0.058	3.1	3.3	+9
Cytochrome C	+0.240	7.8	7.0	−10
Ferricyanide	+0.360	309	273	−12
Oxygen	+0.815	0.7	1.6	+123
NADPH → oxygen		1.9	1.1	−38

[a] Activities were determined as the oxidation of NADH (decrease in absorbance at 340 nm) in the presence of electron acceptor. Auxin (1 μM 2,4-D) was added to the reaction after the initial rate had been established. Specific activity units are nmol/min/mg protein. From Morré and Brightman (1991).

oxidase can be separated from the bulk of the NADH-ferricyanide oxidoreductase by DE-52 ion exchange chromotography using purified plasma membranes from rat liver as the starting material (Fig. 2 of Morré and Brightman, 1991). Also, the NADH oxidase activity is strongly stimulated by transferrin with plasma membranes of rat liver (Table III), whereas the NADH-ferricyanide oxidoreductase activity is not (Sun *et al.*, 1987). It also appears that the proteins responsible for the two activities have very different molecular weights.

Higuchi *et al.* (1993) identified two distinct NADH oxidases of *Streptococcus mutans* corresponding to H_2O_2 and H_2O-forming activities. The H_2O_2-forming enzyme required flavin adenine dinucleotide (FAD) for full activity. The H_2O-forming activity was independent of exogenously added flavin. Neither the basal H_2O-forming or the growth factor- and hormone-induced NADH oxidase of the mammalian plasma membrane appears to require added flavin for activity.

Plant (Crane *et al.*, 1985) and animal plasma membranes (Segal, 1989; Cross and Jones, 1991) have the capacity to oxidize both NADH and NADPH (Crane *et al.*, 1985). However, the growth factor- and hormone-stimulated NADH oxidase activity appears to have specificity for NADH (cf. Table II). There appear to be both external and internal sites for NADH. Specific activities for right side-out and inside-out vesicles are nearly identical. Also, intact cells are capable of oxidizing NADH through an external site. However, as will be developed in the sections which follow, thiols, rather than oxygen, may be the normal acceptors for the activity.

The activity of the growth factor- and hormone-stimulated NADH oxidase of rat liver responded to guanine nucleotides, but in a manner that differed in important ways from that of the classic trimeric and

Table III. NADH Oxidase of Plasma Membranes of Liver and Hepatomas of the Rat (from Bruno *et al.* (1992)[a]

Addition	Concentration	NADH oxidase activity	
		Liver	Hepatoma
None		1.05 ± 0.7	2.46 ± 0.12
Insulin	6 μl/ml	2.35 ± 0.29	2.54 ± 0.15
EGF	1 mg/ml	2.23 ± 0.21	2.52 ± 0.13
Transferrin	1 μg/ml	1.96 ± 0.27	2.06 ± 0.20
Lactoferrin	1 μg/ml	1.62 ± 0.32	2.27 ± 0.14
Glucagon	0.01 μg/ml	1.27 ± 0.20	2.71 ± 0.12

[a] NADH oxidase activity was determined from the change in absorbance of NADH monitored at 340 nm with reference at 430 nm using an SLM-2000 (Aminco) dual-bean spectrophotometer in the dual-wavelength mode of operations. For calculation, an extinction coefficient of 6.2 $mmol^{-1}$ cm^{-2} was used. Units are nmol/min/mg protein.

low-molecular-mass monomeric G proteins (Morré *et al.*, 1993a). Over the range 1–100 μM, both GTP and GDP as well as guanosine 5′-[γ-thio]triphosphate (GTP[γ-S]) but not guanosine 5′-[β-thio]diphosphate (GDP[β-S]) stimulated the activity in the absence of added bivalent ions. Di- and trinucleotides other than GDP and GTP also stimulated, but only at concentrations of 100 μM or higher. Neither NADH oxidation nor guanine nucleotide stimulation required added bivalent ions. Added alone, bivalent ions ($Mg^{2+} > Mn^{2+} \geq Ca^{2+}$) stimulated NADH oxidase activity only slightly at low concentrations but inhibited at high concentrations. GDP or GTP[γ-S], but not GTP, augmented the inhibitions. In the presence of 0.5 mM $MgCl_2$ the activity was the same, or less. GTP at 1 to 100 nM and other nucleotides at 0.1 to 1 mM still stimulated. Mastoparan activated the NADH oxidase activity, but aluminum fluoride was weakly inhibitory. Only marginal responses were elicited by cholera and pertussis toxins. Higher concentrations of Mg^{2+}, GDP, and GTP[γ-S] were required to inhibit the activity when the membrane preparations were first solubilized with Triton X-100. The concentrations of GTP that were stimulatory were similar both in the presence and in the absence of Triton X-100. The results suggest a role for guanine nucleotides in the regulation of plasma membrane NADH oxidase. However, the properties of the regulation were found to differ from those normally associated with regulation by either the trimeric or the low-molecular-mass G proteins.

In plants, the activity of the auxin-stimulated NADH oxidase of the plasma membrane of hypocotyls of etiolated soybean also responded to guanine and other nucleotides but in a manner both different from the activity of rat liver plasma membrane and of the classic trimeric or monomeric G protein (Morré *et al.*, 1993b). In the absence of either the auxin growth hormones or of added divalent ions, both GTP and GDP stimulated the activity as did GTP-γ-S and other nucleotides. In the presence of added magnesium ions, GTP and GTP-γ-S stimulated at 1 nM. Other nucleotides also stimulated but usually at concentrations of 100 nM and above. Both mastoparan and aluminum fluoride stimulated, but the bacterial toxins were largely without effect.

That oxygen was the acceptor for the NADH oxidase-catalyzed oxidation of NADH by plasma membranes was based on the stoichiometry of NADH oxidized to oxygen consumed by the oxidase of plasma membranes of approximately 2 (Morré and

Table IV. Relative Oxygen Uptake by Isolated Vesicles of Soybean and Inhibition by 2,4-D and Oxidized Glutathione (from a Study with Rita Barr, Purdue University)

Plasma membrane	0.8 ± 0.1
+1 μM 2,4-D	0.6 ± 0.1
+10 μM GSSG	0.1 ± 0.05
+10 μM GSH	1.9 ± 0.05

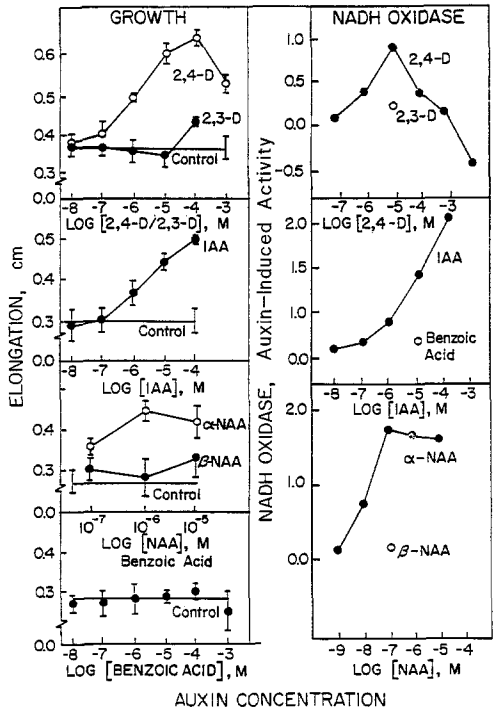

Fig. 1. Stimulation of elongation growth (left) and NADH oxidase activity (right) in soybean by auxins. Growth of soybean hypocotyls was measured as elongation of 1-cm segments. NADH oxidase activity was of isolated plasma membrane vesicles prepared from similar hypocotyl segments, prior to treatment with auxins. Active auxins, indole-3-acetic acid (IAA), 2,4-dichlorophenoxyacetic acid (2,4-D), α-naphthyleneacetic acid (α-NAA), and their inactive analogs, 2,3-D, β-NAA, and benzoic acid, were compared. The active and inactive auxins were added to the isolated vesicles for measurement of NADH oxidase activity or to the hypocotyl segments for measurement of growth and the response determined. Values are averages of three different experiments (10 segments/datum point/experiment) ± standard deviations among experiments for growth of 1-cm hypocotyl segments and from determinations with at least two different plasma membrane preparations for NADH oxidase. Note that the stimulation of NADH oxidase was specific for growth-stimulatory auxins and the concentrations curves for stimulation of growth and NADH oxidase were similar. Modified from Morré and Brightman (1991).

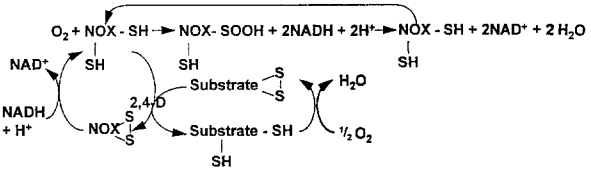

Fig. 2. Diagram to explain the response of the plasma membrane NADH oxidase of soybean to NADH and to auxin. Involvement of a vicinal thiol in the active site of the enzymes is assumed. In the absence of auxin, oxygen is consumed with an approximate stoichiometry of NADH oxidized to oxygen consumed of approximately 2 (Morré and Brightman 1991). Hydrogen peroxide does not appear as a major product. However, once auxin is added, oxygen consumption is more than halved as NADH oxidation is nearly doubled. These observations are incorporated into the diagram as a competing reaction involving disulfides and thiols. The identities of the substrate disulfides or thiols are unknown but are presumed to represent proteins endogenous to the plasma membrane. The reduction of the oxidized NADH oxidase (NOX) by NADH would then account for the auxin stimulation of NADH oxidase activity. A slow and potentially rate-limiting reoxidation of the substrate thiols by molecular oxygen may occur in parallel but not in direct stoichiometry to the NADH oxidized.

Brightman, 1991). However, these determinations were for the basal NADH oxidase activity and not the hormone-stimulated activity. That oxygen is not the natural acceptor for the hormone-stimulated activity is indicated from the data of Table IV which show relative rates of oxygen consumption in the absence and presence of the synthetic plant growth hormone 2,4-dichlorophenoxyacetic acid (2,4-D). This substance stimulated NADH oxidation by about a factor of 2 with isolated plasma membranes (Fig. 1), yet oxygen consumption was inhibited.

THIOL-DISULFIDE OXIDO-REDUCTASE

That addition of growth hormone should inhibit oxygen consumption while at the same time increase NADH oxidation is rationalized by the diagram of Fig. 2. Shown in the diagram is a mechanism whereby the hormone stimulates a second reaction to a postulated alternative electron acceptor that is not oxygen. This would leave the active-site thiols of the NADH oxidase in an oxidized form which could then provide the acceptor to account for the observed enhanced oxidation of NADH without any corresponding increase in oxygen consumption.

The growth factor- and hormone-stimulated NADH oxidase may, in fact, be a thiol oxido-reductase. This conclusion receives strong support from results with thiol reagents. In plants, the basal NADH oxidase activity (with oxygen as acceptor) and the basal growth rate (in the absence of added auxins) were little affected by thiol reagents (Fig. 3). The data with PCMB were representative of several different thiol reagents tested including DTNB, NEM, DTT, GSH, and β-mercaptoethanol.

With rat liver, the basal activity also was unresponsive to the same thiol reagents as for plants, but the constitutively activated form of the activity in hepatoma plasma membranes and in plasma membranes of HeLa cells (cervical carcinoma

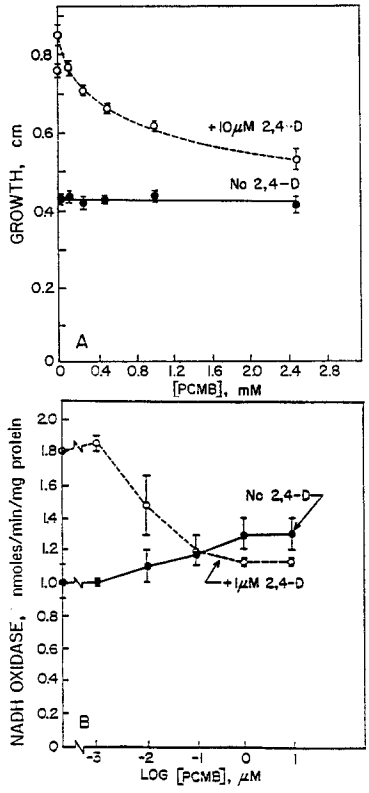

Fig. 3. Response of NADH oxidase activity of soybean plasma membranes (A) and elongation growth (B) to the thiol reagent *p*-chloromercuribenzoate (PCMB). The solid symbols and curves are for the basal level of activity whereas the open circles and dashed lines are for the NADH oxidase activity (A) and elongation growth (B) induced by the synthetic auxin growth hormone 2,4-dichlorophenoxyacetic acid (2,4-D). PCMB inhibited specifically the auxin (2,4-D)-induced component of both NADH oxidase (A) and growth (B) whereas the basal components of both NADH oxidase and growth are resistant to inhibition by PCMB. Adapted from Morré *et al.* (1994a).

derivation) is responsive to thiol reagents (stimulated or inhibited for hepatoma and inhibited for HeLa) (Fig. 4; Morré and Morré, 1994). The marked susceptibility to inhibition by thiol reagents of both 2,4-D-induced growth and the 2,4-D-induced NADH oxidase of soybean plasma membrane (Fig. 1) suggested initially involvement of essential active site thiols in the auxin growth mechanism. A similar thiol dependency was seen with the constitutively activated NADH oxidase activity of hepatoma and HeLa cell plasma membranes (Fig. 4).

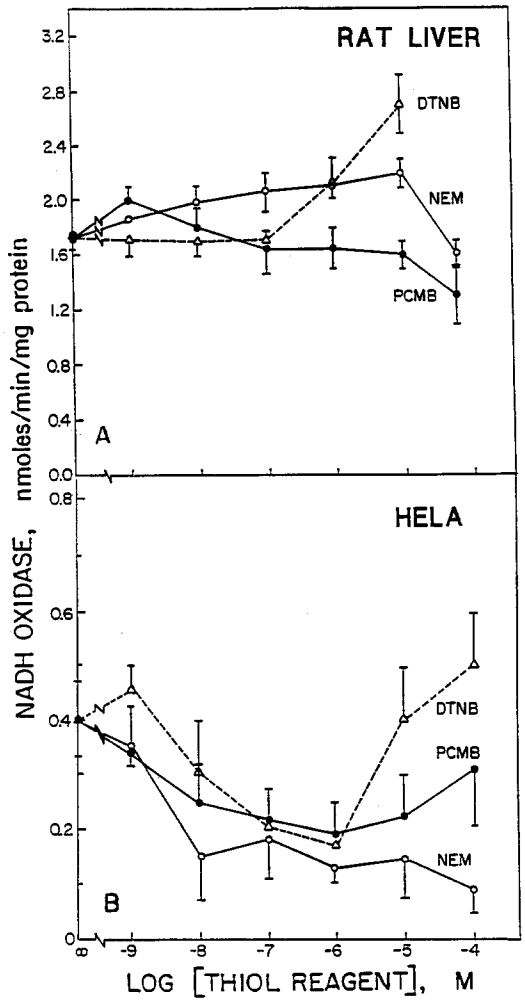

Fig. 4. NADH oxidase activity of plasma membranes in response to the logarithm of the concentration of the thiol reagents 5,5′-dithiobis(2-nitrophenylbenzoate) (DTNB), *N*-ethylmaleimide (NEM), and *p*-chloromercuribenzoate (PCMB). (A) Liver. The thiol reagents were without effect over a wide range of concentrations. (B) HeLa cells. In contrast to results with liver, all three thiol reagents inhibited the NADH oxidase activity. Adapted from Morré and Morré (1994).

Further evidence for an involvement of thiols in the action of the plasma membrane NADH oxidase of HeLa cells came from the specific inhibition of the NADH oxidase activity by the antitumor sulfonylurea, *N*-(4-methylphenylsulfonyl)-*N*′-(4-chlorophenyl) urea (LY181984). This drug inhibits the NADH oxidase of plasma membranes of HeLa cells and of other drug-susceptible cells lines but not that of liver and nontransformed cell lines that do not respond to the drug. With HeLa plasma membranes, free thiols as measured by reaction with DTNB (Ellman's reagent) decrease with time in the absence of NADH and increase in the presence of NADH, consistent with the operation of a thiol oxidoreductase in the plasma membrane. Both the disappearance and the formation of DTNB-reactive thiol groups in the membrane is blocked by the active antitumor sulfonylurea LY181984 but not by its inactive counterpart *N*-(4-methylphenylsulfonyl)-*N*′-(phenyl) urea (LY181985) (Fig. 5). LY181985 is also without effect in inhibiting the NADH oxidase of HeLa cell plasma membranes.

In plants, plasma membranes incubated with the growth hormone 2,4-D exhibited increased levels of thiols reactive with DTNB, with [^{14}C]*N*-ethylmaleimide or with biotin maleimide after removal of the 2,4-D (Morré *et al.*, 1994a). A similar result was seen with the natural auxin hormone, indole-3-acetic acid (IAA). With the use of either [^{14}C]*N*-ethylmaleimide or biotin maleimide with detection using alkaline phosphatase coupled to avidin, labeled components were observed at 72 and 36 kD in response to the 2,4-D treatment. These molecular weights of the plasma membrane components with auxin-responsive thiols (72 and 36 kD) correspond to molecular weights of two of the major peptide bands of the active NADH oxidase fraction previously purified from plasma membranes of soybean hypocotyls (Brightman *et al.*, 1988). Taken together, the results provide evidence for thiols induced during or following the auxin stimulation of NADH oxidase of soybean hypocotyl membranes and of their apparent protection by auxin presence.

Similarly, with HeLa plasma membranes treated with *N*-ethylmaleimide in the presence of the active antitumor sulfonylurea LY181984, protein bands of ca. 34 and 72 kD are subsequently found to be reactive upon removal of the sulfonylurea and incubation with [^{14}C]*N*-ethylmaleimide or with biotin maleimide. This

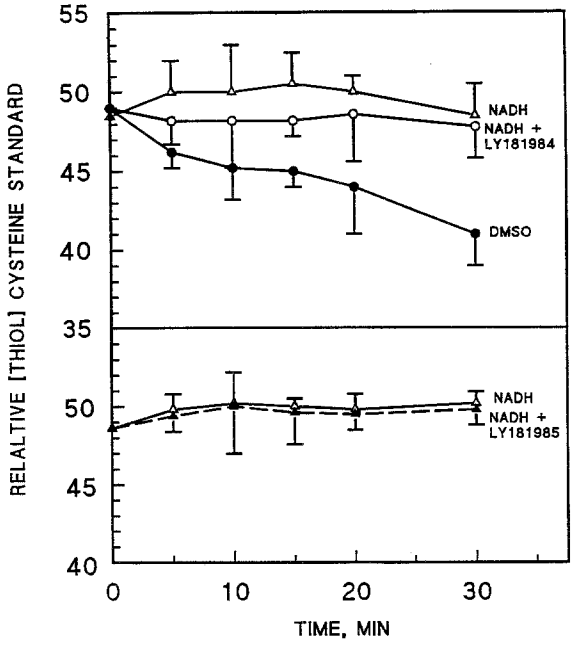

Fig. 5. Thiol–disulfide interchange activities of HeLa plasma membranes as measured by generation of thiol groups accessible to reaction with DTNB (Ellman's reagent) inhibited by 10 μM LY181984 (active) but not by 10 μM LY181985 (inactive). The sulfonylureas were added in DMSO (0.1% final concentrations) with comparisons to 0.1% DMSO alone (DMSO).

identifies the complex of a 34–36-kD and a 72-kD protein as being related to the NADH oxidase activity of both plants (see also Brightman *et al.*, 1988) and animals and a role of essential thiols in the active site of the hormone-induced and constitutively active forms of the activity but not in the basal activity.

THE GROWTH FACTOR- AND HORMONE-STIMULATED NADH OXIDASE (THIOL–DISULFIDE OXIDOREDUCTASE) IS A UNIQUE PROTEIN DIFFERENT FROM THE BASAL NADH OXIDASE OF PLASMA MEMBRANE PREPARATIONS

There are now a number of features that point to unique features of the growth factor- and hormone-stimulated NADH oxidase (thiol–disulfide oxidoreductase) of both plant and animal plasma membranes. With plants and with the constitutively activated form in transformed cells, the activity is responsive to thiol reagents but not in nontransformed cells. Also in transformed cells, the NADH oxidase appears to be unresponsive as well to low concentrations of guanine nucleotides (Morré and Morré, 1994). An altered drug sensitivity is also encountered with the response to the antitumor sulfonylureas.

In plants, polyclonal antisera were raised in rabbits to a peptide corresponding to internal amino acid sequence from a 36-kD band from a purified NADH oxidase preparation from soybean plasma membranes. These antisera, which react specifically with a 36-kD band on Western blots of soybean plasma membranes, inhibits almost completely the auxin-induced increase in NADH oxidase activity of isolated plasma membranes (Table V) or of partially purified NADH oxidase preparations. In contrast, the basal NADH oxidase activity is inhibited only sightly if at all. This further points to the uniqueness of the growth factor- and hormone-induced activity compared to the basal activity already observed with regard to sensitivity to thiol reagents and differences in utilization of oxygen versus oxidized thiols as the accepter.

THIOL–DISULFIDE INTERCHANGE ACTIVITY

In the scheme of Fig. 2, the substrate of the growth factor- and hormone-induced thiol–disulfide oxidoreductase activity is unspecified except that protein thiols of the membranes appear to be involved. The classic assay for thiol–disulfide interchange is the activation of oxidized (or reduced) and denatured (scrambled) RNase. By reduction and oxidation of thiols in the scrambled RNase under renaturing conditions, a thiol interchange activity (protein disulfide isomerase or protein disulfide isomerase-like activity) will restore the lost activity.

Table V. Effect of Peptide Antibody on NADH Oxidase from Soybean Plasma Membranes

Addition	Activity (nmol/min/mg protein)
None	1.05 ± 0.05
+Water	1.05 ± 0.05
+Antisera	1.0 ± 0.08
+Preimmune sera	1.0 ± 0.14
+10 μM 2,4-D	2.0 ± 0.18
+10 μM 2,4-D + antisera	0.9 ± 0.25
+10 μM 2,4-D + preimmune sera	1.94 ± 0.34

Both plant (Morré *et al.*, in preparation) and mammalian (Mandel *et al.*, 1993) plasma membranes contain a protein disulfide-like isomerase activity. In plants, this activity is stimulated by 2,4-D and indole-3-acetic acid, the active auxin hormones, but not by the inactive 2,3-D. Both auxin-induced growth (Schindler *et al.*, 1994; Morré, 1994) and auxin-induced NADH oxidase are eliminated by treatment with brefeldin A, a fungal macrolide antibiotic that specifically blocks membrane trafficking.

In animal plasma membranes, the protein disulfide isomerase activity responds as well to brefeldin A (Ghani *et al.*, 1993; Morré, 1994) and to the active antitumor sulfonylurea LY181984 but not to the inactive antitumor sulfonylurea LY181985. Thus, the NADH oxidase (thiol oxidoreductase) complex that is growth factor-stimulated in plants and constitutively activated in cancer cells exhibits a protein disulfide isomerase-like activity. It appears not to be related to a protein disulfide isomerase (EC 5.3.4.1) *per se* since peptide antisera directed to the consensus sequence for the active site of the 55-kD protein disulfide isomerase of mammalian endoplasmic reticulum failed to inhibit the activity. Nor do we know if the activity resides with the 34–36-kD or the 72-kD peptide component or both.

Protein disulfide isomerase (PDI) was so named because it catalyzes the *in vitro* isomerization of intramolecular disulfide bridges (Noiva and Lennarz, 1992). PDI is also the beta subunit of prolyl hydroxylase (EC 1.14.11.2) (Kivirikko and Myllylä, 1987). Procollagen peptide, α-ketoglutarate, and molecular oxygen are substrates for the reaction, while ascorbic acid and Fe^{2+} are required cofactors (Kivirikko and Myllylä, 1987). The beta subunit also contains dehydroascorbate reductase activity (Wells *et al.*, 1990). While there appears to be some relationship between the growth factor- and hormone-stimulated NADH oxidase (thiol–disulfide oxidoreductase) or thiol–disulfide interchange activity, it seems unlikely that the thiol–disulfide interchange activity is mediated through a true protein disulfide isomerase of the type encountered in the endoplasmic reticulum.

ROLE IN PHYSICAL MEMBRANE DISPLACEMENT

A role of the NADH oxidase/thiol transferase activity in vesicle budding, especially at the Golgi apparatus, was indicated from several lines of investigation. NADH oxidase activity is contained in both isolated Golgi apparatus of both rat liver (Morré *et al.*, 1994b) and of soybean hypocotyls (Morré, 1994). The activity is inhibited by brefeldin A (Morré *et al.*, 1993c; Morré, 1994; Morré *et al.*, 1994b), a fungal macrolide antibiotic that blocks trafficking, including that from the trans face of the Golgi apparatus to the plasma membrane (Miller *et al.*, 1990). Addition of GDP is required for optimal inhibition by brefeldin A. Brefeldin A also blocks the cell enlargement in plants induced by auxins (Schindler *et al.*, 1994; Morré, 1994).

Brefeldin A is normally considered to act through interference with a guanine nucleotide exchange enzyme (Donaldson *et al.*, 1992; Helms and Rothman, 1992) that results in dissociation of a 110-kD peripheral membrane protein (β-cop) from the Golgi apparatus (Donaldson *et al.*, 1990). Possibly by some interference with guanine nucleotide exchange, brefeldin A might result in inhibition of the NADH oxidase both at the plasma membrane and at the Golgi apparatus (Morré, 1994). Thus, both elongation growth induced by auxin and the production of Golgi apparatus secretory vesicles might be expected to be blocked by brefeldin A. The role proposed for the NADH oxidase in physical displacement of membranes provides one link that may be common to the two processes.

Some relationship between the oxidation of NADH and the Golgi apparatus is indicated from other lines of evidence. Rodriguez *et al.* (1992) have described a cell-free system where transfer of radiolabeled lipids or proteins from trans Golgi apparatus elements to inside-out plasma membrane vesicles immobilized on nitrocellulose was promoted by NADH. That trans elements of Golgi apparatus of rat liver have the ability to oxidize NADH was demonstrated earlier from cytochemical studies (Morré *et al.*, 1978). In the presence of a complex of copper and ferricyanide, deposits of insoluble copper ferrocyanide (Hatchett's brown) were produced when NADH was added. These deposits were electron dense and visible in the electron microscope (Morré *et al.*, 1978). Sun *et al.* (1984) showed that the oxidation of NADH by isolated Golgi apparatus was stimulated and reversibly regulated by the presence or absence of surface (membrane coat) proteins including clathrin or clathrin-associated proteins (see also Morré *et al.*, 1987).

Not only was transfer of radiolabeled lipid and proteins from trans Golgi apparatus elements to

inside-out plasma membrane vesicles stimulated by NADH but the presence of ascorbate or ascorbate radical promoted transfer (Rodriguez *et al.*, 1992). Ascorbate has been reported as a constituent present in clathrin-coated vesicles (Sun *et al.*, 1984; Morré *et al.*, 1985, 1987), Golgi apparatus (Sun *et al.*, 1985), chromaffin granules (Njus *et al.*, 1983; Srivastava *et al.*, 1984), and secretory vesicles (Van Zastrow *et al.*, 1983) and has been implicated as being important to the secretory process (Njus *et al.*, 1983; Srivastava *et al.*, 1984; Sun *et al.*, 1984; Van Zastrow *et al.*, 1984; Morré *et al.*, 1985, 1987).

The antitumor sulfonylurea LY181984 also exhibits an effect on the Golgi apparatus of sulfonylurea-responsive cells and tissues. This response was expressed in a reduced number of vesicles associated with the trans face that are capable of being induced to swell upon the addition of the monovalent ionophore, monensin. In order for monensin to induce vacuole swelling, it appeared necessary to carry out a proton/monovalent cation exchange which was effective only if the interiors of the vesicles were acidified (Mollenhauer *et al.*, 1990). Thus, the effect of LY181984 could be either on membrane flux through the Golgi apparatus or on the rate of acidification of the trans membrane compartments.

ROLE IN CELL ENLARGEMENT AND CONTROL OF MITOSIS

Physical membrane displacements are integral to the life of the cell. Processes of cell division, cell enlargement, vesicle budding, and the many pleomorphic changes associated with cell movements and changes in cell shape all involve physical membrane displacements. Enlargement of cells, especially evident in plants following cytokinesis, is a growth phase essential to sustained cell division. The rapid and expansive growth that occurs in zones just behind growing plant tips is due exclusively to cell enlargement. Tissue sections excised from these regions, when floated on water, exhibit increased rates of elongation in response to regulators of plant growth known collectively as auxins (Morré and Key, 1967). Even with mammalian cells or yeasts where growth is determined by an increase in number of cells, cell enlargement must follow or accompany cell division in order for sustained growth to occur. When unequal divisions do occur, for example, the next mitosis in the smaller of the two daughter cells is delayed until some minimum size is reached through enlargement (Baserga, 1985). Formation of a cleavage furrow during cytokinesis involves membrane displacements, as does cell plate formation in plants (Whaley, 1975).

In a few instances where it is possible to block nearly completely the NADH oxidase/thiol transferase activity of the plasma membrane, e.g., in cancer cells with drugs targeted to the oxidase, the observed phenotype appears to be a failure of cells to enlarge. When HeLa cells are treated for several days with the active antitumor drug LY181984, small cells with normal sized nuclei are found together with normal sized cells with enlarged or multiple nuclei.

MOLECULAR MODEL

Formation of membrane vesicles by budding is perhaps one of the most widely investigated examples of the vectorial displacement of membranes. Vesicle budding occurs at the transitional endoplasmic reticulum to deliver membranes to the cis Golgi apparatus and at the trans Golgi apparatus to deliver materials to the plasma membrane/cell exterior along an exocytic pathway (Farquhar, 1985). Budding occurs as well at the cell surface to internalize plasma membrane together with substances from the external milieu in an endocytic pathway (Silverstein *et al.*, 1977). Displacements evident as part of normal cellular activities and cellular movements, especially in cultured cells, include ruffling, the formation and retraction of pseudopods, and pleomorphic changes in cell shape (Hynes, 1979).

A dependency of membrane displacements on metabolic energy (e.g., ATP) is assumed. However, proof is available only for vesicle budding. Here, a requirement for ATP has been found for both permeabilized cells (Balch and Keller, 1986; Simons and Virta, 1987; Beckers *et al.*, 1987, 1990) and for cell-free systems (Morré *et al.*, 1986a; Balch *et al.*, 1987; Nowack *et al.*, 1987; Paulik *et al.*, 1988; Warren *et al.*, 1988; Wattenberg, 1991). In contrast, enlargement of plant cells generally has been assumed to occur as cell wall stretching in response to turgor (Lockhart, 1965; Taiz, 1984). Yet a strict metabolic requirement for plant cell enlargement has been indicated from certain inhibitor studies as well (Morré and Eisinger, 1968).

Very little information is available about mechanisms of physical membrane displacement

despite much investigative effort. With the budding of vesicles from transitional endoplasmic reticulum, ATP is required. Involved are low-molecular-weight G proteins as well as other cytosolic factors including coatomer (Donaldson *et al.*, 1990) and SNARE (Rothman and Warren, 1994) proteins. Thiol reagents such as *N*-ethylmaleimide (Wattenberg, 1991) and the macrolide antibiotic brefeldin A (Donaldson *et al.*, 1990; Miller *et al.*, 1990) inhibit. That the NADH oxidase activity of Golgi apparatus and plasma membranes responded to brefeldin A in the same manner as vesicle budding was one line of evidence to suggest a role of a thiol–disulfide oxidoreductase in vesicle budding (Morré *et al.*, 1994b).

A number of years ago, the author postulated a mechanism in which the physical membrane displacements leading to membrane budding could occur in an active, ATP-driven process (D. J. Morré, unpublished). Basically what was postulated was a series of membrane "extensases" or "unfoldases" arranged as an annulus as depicted in Fig. 6. Membrane proteins would be extended vectorially in the direction of the arrows as the particles slowly converged to form an annulus of decreasing diameter. The result would be a bleb or vesicle. For cell enlargement, the same membrane "extensases" or "unfoldases" could function but with extension in the plane of the membrane to increase membrane surface either uniformly or along one particular axis of the cell.

The actual mechanism postulated for how the actual extension might occur is depicted in Fig. 7. Involved was a hypothetical membrane protein, an extensase. A dynamic coiled coil region with ATP bound was postulated that was capable of extension. To prevent slippage, one end of this protein would be anchored in the membrane, perhaps to some component of the cytoskeleton. This extensase protein would "grab" a protein in the membrane by forming a disulfide linkage with the protein (Step B of Fig. 7). The ATP would be hydrolyzed by the same protein or possibly by a second protein or unfoldase and the coiled coil region of the extensase would extend to displace the bound protein within the membrane. The disulfide would then be reduced at step C (Fig. 7) to release the bound protein. The extensase could then exchange bound ADP for another ATP to reform the coiled coil in step D (Fig. 7). The system now would return to step A and would be ready to displace another membrane protein. Repeated cycles would result in membrane displacement to drive cell enlargement or, with oriented extensases (Fig. 6), in the formation of membrane vesicles or blebs.

The reason for reproducing the above model of membrane bleb formation here is that available evidence would suggest that the growth factor- and hormone-stimulated NADH oxidase (thiol–disulfide oxidoreductase or thiol–disulfide interchange protein) might very well function to drive such a cycle. The

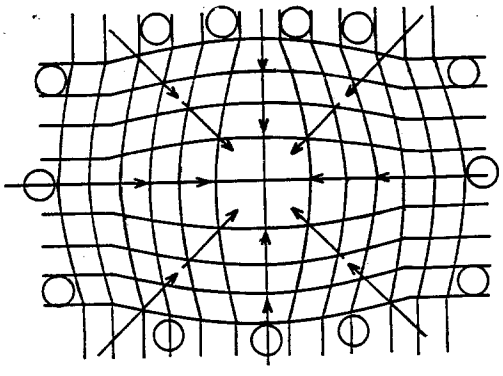

Fig. 6. Arrangement of unfoldases (circles) and directions of displacement (arrows) leading to bleb formation. For simple surface growth, requirements for specific orientations of the unfoldases or for concerted vectorial operation would be much less stringent.

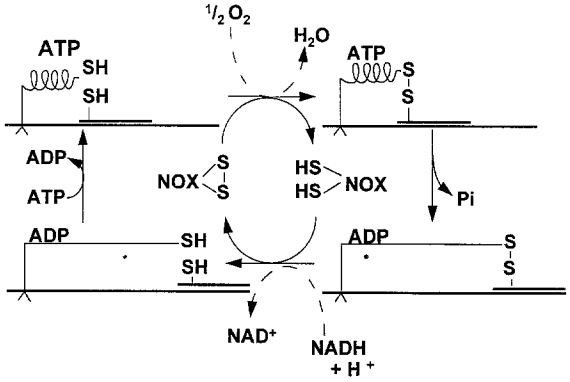

Fig. 7. A model for how the 2,4-D-stimulated thiol–disulfide interchange reaction could be important to physical membrane displacement. (A) A hypothetical membrane protein with a dynamic coiled coil region with ATP bound. (B) One end of the protein is permanently anchored (via the cytoskeleton?) and cannot slip. (C) The ATP is hydrolyzed (possibly by an unfoldase) and the coiled coil region extends, displacing the bound protein within the membrane. (D) The disulfide is reduced, releasing the bound protein, after which the coiled coil binds another ATP and is exchanged for ADP, the coiled coil re-forms, and the system is ready to displace another membrane protein. Repeated cycles would result in membrane displacement or, with oriented unfoldases (Fig. 6), in the formation of membrane vesicles or blebs.

protein (NOX) is depicted in Fig. 7 as containing one or more thiols that are reversibly oxidized and reduced in a growth factor- and hormone-responsive step with the energy coming ultimately from ATP. In the ATP bound form, the thiols of the extensase and its substrate can be used to reduce the oxidized form of the oxidase. Once reduced, the oxidase functions to restore the extensase to its free and reduced form at step C. If this is truly the manner of normal functioning, the hormone- and growth factor-stimulated NADH oxidase functions exclusively as a thiol disulfide interchange protein. However, if NADH is present, NADH can be oxidized at this same step as a competing substrate. In the normal functioning, NADH would not be required. Also, in the absence of hormone, the activity can function in the transfer of electrons and reducing equivalents to molecular oxygen as depicted at the top of the scheme either from protein thiols or from NADH, but in the normal growth factor- or hormone-directed process, this appears not to occur. Rather the NADH oxidase appears only to cycle in a series of reactions energized by ATP.

Is there evidence to support the details of the scheme of Fig. 7? Certainly, the existence of an extensase and the coiled coil transitions are hypothetical. The association of ATP with the coiled rather than the extended form is arbitrary. However, the requirement for ATP in vesicle budding is well established and Zhang and Morré (1994) have isolated, characterized, and cloned the principal ATPase of transitional endoplasmic reticulum. It is similar to a valosin-containing protein (VCP) with ATPase activity of unknown function (Koller and Brownstein, 1987) and a VCP analog, p97-ATPase from *Xenopus* oocytes (Peters *et al.*, 1992). The ATPase shares sequence homology to a yeast cell division control protein CDC48p (Fröhlich *et al.*, 1991) and to the *N*-ethylmaleimide-sensitive (NSF) protein from Chinese hamster ovary cells and Sec 18p from yeast (Wilson *et al.*, 1989). The latter two proteins are required for transport between ER and Golgi apparatus (Wattenberg *et al.*, 1991).
Erdmann *et al.* (1991) has proposed that all these proteins belong to a new family of ATPases.

Whether or not a VCP-type ATPase or a similar protein could fulfill the function of the "unfoldase" remains to be determined. However, antisera to the ATPase do block vesicle blebbing in the cell-free system (Zhang and Morré, 1994), and the antisera cross-react strongly with a plasma membrane protein currently being characterized.

An interesting aspect of the work so far is the relationship of the NADH oxidase activity to nucleotides. In addition to the response to very low concentrations of guanine nucleotides, oxidation of NADH by plasma membranes is also responsive to other nucleotides and especially to ATP. The addition of ATP, for example, shifts the K_m of the oxidase for NADH by about one order of magnitude to higher ATP concentrations. Also, the addition of 2,4-D to plant plasma membranes influences both the K_m for NADH as well as the apparent K_s for nucleotide response (Morré *et al.*, 1993b). Using pig liver plasma membranes as well as plasma membranes from rat liver and HeLa cells, we have observed that the inclusion of ATP in the assay is beneficial to maintenance of steady-state NADH oxidation rates. ATP has been added routinely in the assays for NADH oxidase with pig liver and HeLa plasma membranes. Thus, there appears to be some interaction, albeit complex, between the hormone- and growth factor-stimulated NADH oxidase (thiol–disulfide oxidoreductase) and nucleotides including ATP and ADP. Addition of ATP also reduced oxygen consumption of isolated plasma membranes in much the same manner as hormone addition.

A role for the oxidized form of the oxidase being the form favored by hormone addition comes from two lines of evidence. The first line of evidence is the apparent protection by thiols of both a 36-kD and a 72-kD protein by the addition of 2,4-D to plasma membrane preparations from soybean stems (Morré *et al.*, 1994a). Once the 2,4-D is removed, the protein apparently cycles back to the thiol form and now becomes reactive with, for example [^{14}C]*N*-ethylmaleimide. The second line of evidence comes from the activation of the NADH oxidase by the addition of small quantities of hydrogen peroxide or other mild oxidizing agents such as *N*-chlorosuccinamide (D. J. Morré, unpublished results). This occurs at least in part by changing the affinity of the active complex for NADH.

Thus, the substrate protein in the reaction scheme of Fig. 2 could be the NADH oxidase (thiol–disulfide oxidoreductase) protein itself. By undergoing progressive cycles of oxidation and reduction in a hormone- or growth factor-stimulated or constitutively activated (in cancer) manner, the protein could carry out its postulated function in growth control (Morré *et al.*, 1988c; Morré and Crane, 1990; Morré and Brightman, 1991) according to some scheme not unlike that depicted in Fig. 7. As

such, the activity provides a potentially useful target for anticancer drugs. One such target would be for the antitumor sulfonylureas that interfere in some aspects of the growth process such that the cells eventually cease dividing and undergo programmed cell death. In some more general form, it is possible that the activity may be involved in other forms of physical membrane displacement as is encountered, for example, in membrane budding events of both exocytosis and endocytosis. This interesting possibility is currently under investigation.

REFERENCES

Asard, H., Caubergs, R., Rnders, D., and DeGreef, J. A. (1987). *Plant Sci.* **53,** 109–119.

Balch E. R., and Keller, D. S. (1986). *J. Biol. Chem.* **261,** 14690–14696.

Balch, E. R., Wagner, K. R., and Keller, D. S. (1987). *J. Cell Biol.* **104,** 749–760.

Baserga, R. (1985). *The Biology of Cell Reproduction*, Harvard University Press, Cambridge.

Beckers, C. J. M., Keller, D. S., and Balch, W. E. (1987). *Cell* **50,** 523–534.

Beckers, C. J. M., Plutner, H., Davidson, H. W., and Balch, W. (1990). *J. Biol. Chem.* **265,** 18298–18310.

Brightman, A. O., Barr, R., Crane, F. L., and Morré, D. J. (1988). *Plant Physiol.* **86,** 1264–1269.

Brightman, A. O., Wang, J., Miu, R. K.-M., Sun, I. L., Barr, R., Crane, F. L., and Morré, D. J. (1992). *Biochim. Biophys. Acta* **1105,** 109–117.

Bruno, M., Brightman, A. O., Lawrence, J., Werderitsh, D., Morré, D. M., and Morré, D. J. (1992). *Biochem. J.* **284,** 625–628.

Crane, F. L., Sun, I. L., Clark, M. G., Grebing, C., and Löw, H. (1985). *Biochim. Biophys. Acta* **811,** 233–264.

Cross, A. R., and Jones, O. T. G. (1991). *Biochim. Biophys. Acta* **1057,** 218–298.

Donaldson, J. G., Lippincott-Schwartz, J., Bloom, G. S., Kreis, T. E., and Klausner, R. D. (1990). *J. Cell. Biol.* **111,** 2295–2306.

Donaldson, J. G., Finazze, D., and Klausner, R. D. (1992). *Nature (London)* **360,** 350–352.

Ellman, G. L. (1959). *Arch. Biochem. Biophys.* **82,** 70–77.

Erdman, R., Wiebel, F. F., Flessau, A., Rytka, J., Beyer, A., Fröhlich, K.-U., and Kunau, W.-H. (1991). *Cell* **64,** 499–510.

Farquhar, M. G. (1985). *Annu. Rev. Cell Biol.* **1,** 447–488.

Fröhlich, K.-U., Fries, W. W., Rüdiger, M., Erdmann, R., Botatein, D., and Meeke, D. (1991). *J. Cell Biol.* **114,** 443–453.

Gayda, D. P., Crane, F. L., Morré, D. J., and Löw, H. (1977). *Proc. Indiana Acad. Sci.* **86,** 385–390.

Ghani, F., Mandel, R., and Ryser, H. J.-P. (1993). *Mol. Biol. Cell* **4,** 320a.

Goldenberg, H., Crane, F. L., Morré, D. J., and Löw, H. (1979). *J. Biol. Chem.* **254,** 2491–2498.

Helms, J. B., and Rothman, J. E. (1992). *Nature (London)* **360,** 352–354.

Higuchi, M., Shimada, M., Yamamoto, Y., Hayashi, T., Koga, T., and Kamio, Y. (1993). *J. Gen. Microbiol.* **139,** 2343–2351.

Hynes, R. O. (1979). *Surfaces of Normal and Malignant Cells*, Wiley, New York.

Kivirikko, K. I., and Myllylä, R. (1987). *Methods Enzymol.* **144,** 96–114.

Koller, J., and Brownstein, M. J. (1987). *Nature (London)* **325,** 542–545.

Lockhart, J. A. (1965). *J. Theor. Biol.* **8,** 264–275.

Mandel, R., Ryser, H. J.-P., Ghani, F., Wu, M., and Peak, D. (1993). *Proc. Natl. Acad. Sci. USA* **90,** 4112–4116.

Miller, S. G., Carnell, L., and Moore, H.-P.H. (1990). *J. Cell. Biol.* **118,** 267–283.

Mollenhauer, H. H., Morré, D. J., and Rowe, L. D. (1990). *Biochim. Biophys. Acta* **1051,** 250–258.

Morré, D. J. (1994). *Protoplasma*, 180, 3–13.

Morré, D. J., and Brightman, A. O. (1991). *J. Bioenerg. Biomembr.* **23,** 469–489.

Morré, D. J., and Crane, F. L. (1990). In *Oxidoreduction at the Plasma Membrane: Relation to Growth and Transport. I. Animals* (Crane, F. L., Morré, D. J., and Löw, H., eds.), CRC Press, Boca Raton, Florida, pp. 67–84.

Morré, D. J., and Eisinger, W. R. (1968). In *Biochemistry and Physiology of Plant Growth Substances* (Wightman, F., and Setterfield, G., eds.), Runge Press Ltd., Ottawa, Canada, pp. 625–645.

Morré, D. J., and Key, J. L. (1967). In *Experimental Techniques in Developmental Biology* (Wilt, F., and Wessles, N., eds.), T. Y. Cromwell, New York, pp. 575–593.

Morré, D. J., and Morré, D. M. (1994). Manuscript in preparation.

Morré, D. J., Vigil, E. L., Frantz, C., Goldenberg, H., and Crane, F. L. (1978). *Eur. J. Cell. Biol. (Cytobiologie)* **18,** 213–230.

Morré, D. J., Sun, I., and Crane, F. L. (1985). In *Vitamins and Cancer: Human Cancer Prevention by Vitamins and Micronutrients* (Myeskins, F. I., and Prasad, K. N., eds.), Humana Press, Clifton, New Jersey, pp. 83–92.

Morré, D. J., Paulik, M., and Nowack, D. (1986a). *Protoplasma* **132,** 110–113.

Morré, D. J., Navas, P., Penel, C., and Castillo, F. J. (1986b). *Protoplasma* **133,** 195–197.

Morré, D. J., Crane, F. L., Sun, I. L., and Navas, P. (1987). *Ann. N.Y. Acad. Sci.* **498,** 153–171.

Morré, D. J., Crane, F. L., Barr, R., Penel, C. and Wu, L.-Y. (1988a). *Physiol. Plant.* **72,** 236–240.

Morré, D. J., Brightman, A. O., Wu, L.-Y., Barr, R., Leak, B., and Crane, F. L. (1988b). *Physiol. Plant.* **73,** 187–193.

Morré, D. J., Brightman, A., Wang, J., Barr, R., and Crane, F. L. (1988c). In *Proc. NATO Advanced Research Workshop* (Crane, F. L., Löw, H., and Morré, D. J., eds.), Alan R. Liss, New York, pp. 45–55.

Morré, D. J., Crane, F. L., Eriksson, L. C., Löw, H., and Morré, D. M. (1991). *Biochim. Biophys. Acta* **1057,** 140–156.

Morré, D. J., Morré, D. M., Paulik, M., Batova, A., Broome, A.-M., Pirisi, L., and Creek, K. E. (1992). *Biochim. Biophys. Acta* **1134,** 217–222.

Morré, D. J., Davidson, M., Geilen, C., Lawrence, J., Flesher, G., Crowe, R., and Crane, F. L. (1993a). *Biochem. J.* **292,** 647–653.

Morré, D. J., Brightman, A. O., Barr, R., Davidson, M., and Crane, F. L. (1993b). *Plant Physiol.* **102,** 595–602.

Morré, D. J., Lawrence, J., Morré, D. M., and Paulik, M. (1993c). *Mol. Biol. Cell* **4,** 211a.

Morré, D. J., Brightman, A. O., Hidalgo, A., Navas, P., Lawrence, J., Wilkinson, F. E., Penel, C., and Morré, D. M. (1994a). Manuscript in preparation.

Morré, D. J., Paulik, M., Lawrence, J. L., and Morré, D. M. (1994b). *FEBS Lett.*, **346,** 199–202.

Navas, P., Nowack, D. D., and Morré, D. J. (1989). *Cancer Res.* **49,** 2147–2156.

Njus, D., Knoth, J., Cook, C., and Kelley, P. M. (1983). *J. Biol. Chem.* **258,** 27–30.

Noiva, R., and Lennarz, W. J. (1992). *J. Biol. Chem.* **267,** 3553–3556.

Nowack, D. D., Morré, D. M., Paulik, M., Keenan, T. W., and

Morré, D. J. (1987). *Proc. Natl. Acad. Sci. USA* **84,** 6098–6102.
Paulik, M., Nowack, D. D., and Morré, D. J. (1988) *J. Biol. Chem.* **263,** 17738–17748.
Peters, J.-M., Harris, J. R., Lustig, A., Muller, S., Engel, A., Volkers, S., and Franke, W. W. (1992). *J. Mol. Biol.* **223,** 557–571.
Pupillo, P., Valenti, V., DeLuca, L., and Hertel, R. (1986). *Plant Physiol.* **80,** 384–389.
Rodriguez, M., Moreau, P., Paulik, M., Lawrence, J., Morré, D. J., and Morré, D. M. (1992). *Biochim. Biophys. Acta* **1107,** 131–138.
Rothman, J. E., and Warren, G. (1994). *Curr. Biol.* **4,** 220–233.
Schindler, T., Bergfeld, R., Hohl, M., and Schopfer, P. (1994). *Planta* **192,** 404–413.
Segal, A. W. (1989). *J. Clin. Invest.* **83,** 1785–1793.
Silverstein, S. C., Steinman, R. M., and Cohn, Z. A. (1977). *Annu. Rev. Biochem.* **46,** 669–772.
Simons, K., and Virta, H. (1987). *EMBO J.* **6,** 2241–2247.
Srivastava, M., Duong, L. T., and Fleming, P. J. (1984). *J. Biol. Chem.* **259,** 8072–8075.
Sun, I., Morré, D. J., Crane, F. L., Safranski, K., and Croze, E. M. (1984). *Biochim. Biophys. Acta* **797,** 266–275.
Sun, I. L., Navas, P., Crane, F. L., Morré, D. J., and Löw, H. (1987). *J. Biol. Chem.* **262,** 15915–15921.
Taiz, L. (1984). *Annu. Rev. Plant Physiol.* **35,** 585–657.
Van Zastrow, M., Tritton, T. R., and Castle, J. D. (1984). *J. Biol. Chem.* **259,** 11746–11750.
Vianello, A., and Macri, F. (1989). *Biochim. Biophys. Acta* **980,** 202–208.
Warren, G., Woodman, P., Pypaert, M., and Smythe, E. (1988). *Trends Biochem. Sci.* **13,** 462–465.
Wattenberg, B. (1991). *J. Electron Microsc. Tech.* **17,** 150–164.
Wells, W. W., Xu, D. P., Yang, Y., and Roque, P. A. (1990). *J. Biol. Chem.* **265,** 15361–15364.
Whaley, W. G. (1975). *Cell Biol. Monographs*, Vol. 2, Springer-Verlag, Berlin and New York.
Wilson, D. W., Wilcox, C. A., Flynn, G. C., Chen, E., Kuang, W.-J., Henzel, M., Block, R., Ullrich, A., and Rothman, J. E. (1989). *Nature* (*London*) **339,** 355–359.
Zhang, L., and Morré, D. J. (1994). Results unpublished.