Plant Physiol. (1977) 60, 54-57

Studies on the Mechanism of Action of Dinitramine

EFFECT ON SOYBEAN ROOT PLASMA MEMBRANE

Received for publication October 26, 1976 and in revised form February 7, 1977

ROBERT L. TRAVIS[1] AND WILLIAM G. WOODS
U.S. Borax Research Corporation, 412 Crescent Way, Anaheim, California 92801

ABSTRACT

The effect of dinitramine, a selective herbicide, on the plasma membrane of the soybean (*Glycine max* L.) root was studied. Used as marker systems to observe the herbicide effect were two plasma-membrane-specific enzymes, pH 6.5 ATPase and glucan synthetase.

The activity of pH 6.5 ATPase decreased significantly in membrane vesicles prepared from roots harvested 15 minutes after treatment with dinitramine. Maximum inhibition occurred in roots harvested 2 hours after treatment. Glucan synthetase activity decreased similarly within 2 hours of treatment.

Membrane permeability to ^{86}Rb was rapidly increased by dinitramine.

The activity of pH 6.5 ATPase returned to the control level within 8 hours of treatment with dinitramine.

These results show dinitramine's initial site of action to be the plasma membrane, producing an over-all reduction in membrane function through inactivation of membrane-associated proteins.

Dinitramine (N^3,N^3-diethyl 2,4-dinitro-6-trifluoromethyl-1,3-benzenediamine) is a selective preemergence herbicide for control of a broad spectrum of annual Monocotyledoneae and Dicotyledoneae weed species (16). Primary crops of interest include cotton and soybeans, as well as peanuts, dry beans, peas, and sunflower.

Morphological symptoms in susceptible broadleaf species, including soybeans, typically include root pruning and enlargement of roots in the region next to the tip. The hypocotyl or epicotyl enlarges radially in the zone of cell elongation, becoming quite turgid. The morphological response in soybean is virtually identical to that induced by the synthetic auxin, 2,4-D (5). Anatomically, the cortical cells of sensitive broadleaf species enlarge severalfold and the vascular tissue becomes disorganized. Cell enlargement typically begins 8 to 12 hr after contact with dinitramine. Little or no cell division occurs in the cortical tissue after dinitramine treatment. Dinitramine produces similar morphological symptoms in roots of sensitive grass species; coleoptile development is globular in nature as a result of reduced elongation coupled with increased lateral growth.

Little information is available on the mechanism of action of dinitramine. Velmurugu (18) suggested that a possible mechanism might include the regulation of nucleic acid synthesis. Preliminary experiments in this laboratory suggested that dinitramine did in fact stimulate cytoplasmic ribosome synthesis (to be presented elsewhere), although the response was detected only after a lag of about 12 hr. Initial or rapid herbicide-induced responses have not been studied.

Dinitramine incorporated into the soil before planting is taken up by developing seedlings. It must penetrate the plasma membrane to enter the cell. In the work reported in this communication we have attempted to characterize the early effects of dinitramine on the plasmalemma of soybean root tissue.

MATERIALS AND METHODS

Plant Tissue. Soybean seedlings (*Glycine max* L.) were germinated in darkness in flats (30 × 50 cm) of moist vermiculite at 30 C. Roots were excised below the first or second lateral root for use in studies of the plasma membrane.

Dinitramine Treatment. About 300 4-day-old seedlings/treatment were removed from the vermiculite, rinsed with distilled H_2O, and placed on moist absorbent paper. Each group of seedlings was sprayed with 25 ml of ethanol containing dinitramine. Concentrations are shown in figure legends. Control seedlings were sprayed with ethanol. All seedlings were returned to darkness before root harvest except as noted otherwise.

Isolation of Plasma Membrane Vesicles. Plasma membrane vesicles were prepared essentially as described by Hodges and co-workers (3, 6, 9, 10). Fifteen to 20 g of root tissue was chopped into small segments and homogenized for 3 to 4 min with a mortar and pestle, without added abrasives, in 4 volumes of grinding medium (25 mM tris-HEPES buffer [pH 7.2], 3 mM EDTA; 25 mM dithiothreitol, 250 mM sucrose) and filtered through Miracloth. The crude homogenate was centrifuged for 15 min at 13,000*g*. Membrane vesicles were pelleted from the resulting supernatant by centrifugation at 82,500*g* for 35 min (Spinco SW 27 rotor). Membrane vesicles were resuspended in fresh grinding medium and given a second centrifugation at 80,000*g* for 35 min (Spinco T-65 rotor) for further purification. The final pellet was resuspended in 1 ml buffer (1 mM tris-HEPES [pH 7.2], 1 mM $MgSO_4$, 20% sucrose, w/w). The resuspended membrane vesicle preparation was layered on a discontinuous sucrose gradient consisting of 8 ml of 34% sucrose (containing 0.55 mM $MgSO_4$ and 0.55 mM tris-HEPES buffer at pH 7.2), layered over 28 ml of 45% sucrose, and centrifuged for 2 hr at 82,500*g* (Spinco SW 27 rotor). Plasma membrane vesicles were recovered from the interface between the two sucrose layers. All steps were carried out at 0 to 4 C.

Potassium-activated ATPase Assay. Potassium-activated ATPase activity was assayed by the method of Leonard *et al.* (9, 10). Briefly, 1 ml of reaction mixture contained 3 mM ATP unless otherwise noted; 33 mM tris-HEPES buffer (pH 6.5); 3 mM $MgSO_4$ unless otherwise noted; 50 mM KCl, when present. The reaction was initiated by introducing 25 to 50 μg of membrane protein and incubating for 30 min at 37 C, and was terminated by adding 1 ml of cold 10% trichloroacetic acid. ATPase activity is expressed as μmol Pi released/mg protein · hr. Inorganic phosphate was determined by the Fiske-Subbarow method (4). Protein concentration was determined by the method of Lowry *et al.* (13).

[1] Present address: Dept. of Agronomy and Range Science, University of California, Davis, Calif. 95616.

Glucan Synthetase Assay. Glucan synthetase activity was determined by the method of Ordin and Hall (14). The 0.2-ml reaction mixture contained 50 mM tris-HEPES buffer (pH 8), 2 mM Na_2EDTA, 20 mM $MgCl_2$, 0.5 mM dithiothreitol, 10 mM cellobiose, 2 mM UDP-glucose, 0.3 nmol UDP-[^{14}C]glucose (240 mCi/mmol). The reaction was initiated by introducing 25 to 50 μg membrane protein and incubating 15 min at 25 C, and was terminated by placing the reaction vials in a boiling water bath. The enzyme protein was identical to that used in the ATPase assay except that membrane vesicles recovered from sucrose gradients were repelleted at 80,000g (Spinco type 65 rotor, 35 min).

The resuspension buffer contained 100 mM tris buffer (pH 8), 4 mM Na_2EDTA, and 1 mM dithiothreitol.

The assay mixture was fractionated into lipid-soluble, hot-water-soluble, and alkali-soluble extracts, and alkali-insoluble residue (17). Radioactivity was determined in a Beckman LS-100 liquid scintillation spectrophotometer. Lipid-soluble extracts were counted in 10 ml of toluene containing 0.5% PPO. Hot-water-soluble extracts were counted in toluene containing 0.5% PPO and 33% Triton X-100. Alkali-soluble extracts were counted in toluene containing 0.5% PPO, 33% Triton X-100, and 4% CAB-0-SIL (Beckman). Alkali-insoluble residues were collected on GFA glass-fiber discs, dried, and counted in toluene containing 0.5% PPO.

Membrane Permeability Studies. Membrane permeability was studied by the method of Keck and Hodges (8). Three g root tissue were cut into 0.5-cm sections and incubated in 250-ml flasks containing 100 ml aerated solution of 1 mM KCl and 1 mM $CaSO_4$. After 1 hr the solution was replaced and an aliquot of ^{86}Rb (500,000 cpm) was introduced into each flask. Root tissue was then incubated for 12 hr, removed, placed on moist filter paper, and sprayed with a 200-μg/ml solution of dinitramine in ethanol. Control tissue was sprayed with ethanol. Samples were then placed in 20-ml plastic syringes and rinsed with 5-ml portions of 1 mM KCl plus 1 mM $CaSO_4$. Radioactivity was counted in 15 ml toluene containing 0.5% PPO and 6% CAB-0-SIL.

RESULTS

This study was done to characterize the response of the plasma membrane to the herbicide dinitramine. The approach used included isolation of plasma membrane vesicles and characterization of the effect of dinitramine on K-activated ATPase activity. Supportive evidence was obtained by determining the effect of the herbicide on glucan synthetase activity, a second enzyme associated with the plasma membrane (17). Finally, the effect of dinitramine on membrane permeability was determined in ^{86}Rb efflux experiments (8).

Enrichment of K^+-ATPase Activity during Plasma Membrane Purification. The K^+-ATPase activity was enriched about 3.5-fold in the 13,000 to 80,000 postmitochondrial pellet and 13-fold in the 35 to 45% sucrose zone (Table I). These figures are in reasonable agreement with figures for oat (6) and corn (11) roots, though somewhat higher. The 34 to 45% sucrose zone has been shown by Hodges *et al.* (6) to be enriched in plasma membrane vesicles. Membrane vesicles for all subsequent experiments were recovered from this zone.

Table I. Enrichment of K^+-ATPase activity during plasma membrane purification

Membrane vesicles were purified from untreated tissue. The 13-80,000 pellet represents the total membrane fraction from the post-mitochondrial supernatant. The 34-45% gradient fraction was recovered from 34-45% sucrose interface on the discontinuous gradient.

Fraction	Experiment	ATPase Activity (μmol Pi·mg protein^{-1}·hr^{-1}) +KCl	-KCl	ΔKCl	Enrichment
Homogenate	1	10.7	8.6	2.1	---
	2	11.8	10.1	1.7	---
13-80,000 pellet	1	18.8	12.5	6.3	3.0
	2	19.8	13.4	6.4	3.8
34-45% gradient	1	46.0	18.4	27.6	12.5
	2	42.9	19.9	23.0	13.5

Characterization of K^+-activated ATPase Activity. Initial characterization of plasma-membrane-associated K^+-ATPase activity was done to determine the optima for pH, Mg^{2+}, and ATP. The aim was to determine not only the optimal assay conditions for further studies but also any similarity in activity to enzymes as reported in other plant systems (1, 10-12).

The pH optimum for K^+-activated ATPase activity (obtained by measuring the activity in the presence and absence of KCl) was 6.5 (Fig. 1). An increase or decrease in pH of 0.5 reduced enzyme activity greatly. Leonard and Hodges (10) and Leonard and VanDerWoude (11) reported optimum pH values of 6 to 7 for oat root and 6.5 for corn root. In this study, all subsequent assays were done at pH 6.5.

ATPase activity was further characterized as to ATP and Mg^{2+} requirements (Fig. 2, A, B, C). Activity was maximum at 3 mM Mg whether or not K was present in the reaction mixture (Fig. 2, A and B). Magnesium concentrations greater than 3 mM gave no further increase in ATPase activity (data not included). The optimum ATP concentration ranged from 1.5 to 3 mM. Figure 2 C represents K-stimulated ATPase activity. The data plotted were obtained by subtracting ATPase activity in the absence of K^+ (Fig. 2B) from activity in its presence (Fig. 2A). Activity was measured in the presence and absence of K in all subsequent experiments. Figures 3 to 5 represent the K^+-activated component of the activity. All assays were carried out at 3 mM Mg^{2+} and 3 mM ATP in experiments involving dinitramine. These results indicate that the K^+-activated ATPase of the plasma membrane was similar in soybean root to that in roots of other higher plants (1, 10-12).

Inhibition of K^+-ATPase by Dinitramine. The activity of plasma membrane ATPase from tissue treated with 200-μg/ml dinitramine is shown in Figure 3. Enzyme activity of membrane vesicles prepared from control tissue was essentially linear throughout the 30-min assay period. For the first 5 min of assay the ATPase activity of vesicles from tissue treated for 2 hr was similar to that of the control but then declined rapidly, ultimately to about one-third that of the control.

Time course experiments were performed by returning treated seedlings to darkness for various periods before harvest. About 15 min was required for treatment, harvest, and homogenizing root tissue, so K^+-ATPase activity could not be assessed sooner than 15 min after treatment. Since membrane vesicle purification required nearly 4 hr, actual assay was about 4 hr after harvest.

The K^+-ATPase enzyme activity of membrane vesicles from dinitramine-treated tissue (Fig. 4) was reduced by approximately 15% within 15 min. The dotted portion of the curve represents an extrapolation from 15 min back to zero time. Inhibition was maximal (60%) 2 hr after treatment. The K^+-ATPase activity of

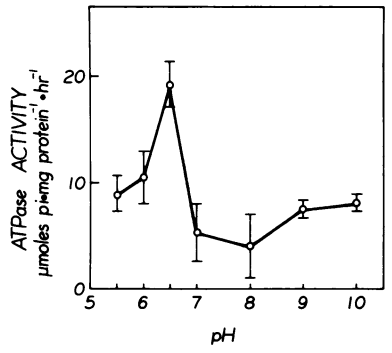

FIG. 1. Effect of pH of reaction mixture on K^+-activated ATPase activity. Plasma membrane vesicles were isolated from untreated soybean roots. Results are expressed as the difference in activity in the presence and absence of KCl. Error bars indicate standard deviation.

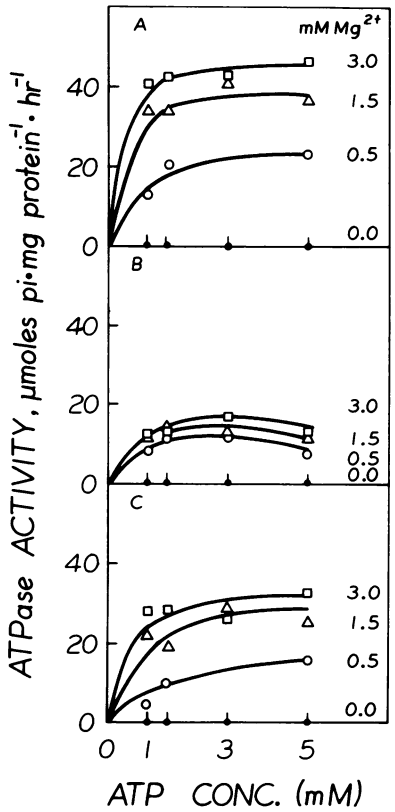

FIG. 2. Effect of ATP and Mg^{2+} concentrations and K^+ on soybean root plasma membrane ATPase activity. A: K included in reaction mixture. B: K omitted from reaction mixture. C: data calculated by subtracting activity in absence of KCl from activity in presence of KCl.

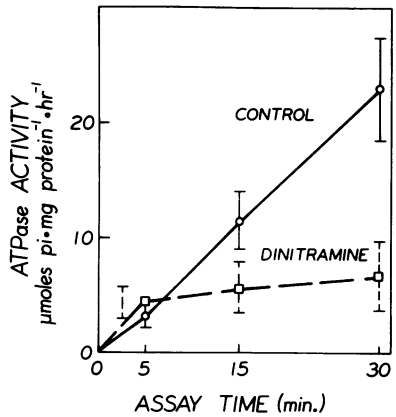

FIG. 3. Effect of dinitramine on kinetics of K^+-activated ATPase activity of plasma membrane vesicles of soybean root. Roots were sprayed with a 200-μg/ml solution of dinitramine and returned to darkness for 2 hr before membrane preparation. Control roots were sprayed with ethanol. Error bars indicate standard deviation.

vesicles from treated tissue began to recover between 2 and 4 hr after treatment, returning to the initial level by 8 hr.

Effect of Dinitramine Concentrations on K^+-ATPase Activity. Figure 5 shows the effect of various dinitramine concentrations on K^+-ATPase activity. Maximum inhibition was at dinitramine concentrations of 50 μg/ml or greater.

It would be of interest to determine the effect of dinitramine on plasma membrane vesicles in an *in vitro* system. Unfortunately, its solubility in water is less than 1 μg/ml. Attempts to solubilize dinitramine in DMSO and/or alcohol at solvent levels low enough to prevent enzyme inactivation were unsuccessful.

Effect of Dinitramine on Glucan Synthetase Activity and Permeability to ^{86}Rb. The effect of dinitramine on plasma membrane K^+-ATPase activity suggests an interaction between the herbicide and the membrane system. It thus seemed of interest to assess the effect of dinitramine on other membrane activities. Selected as test systems were glucan synthetase, a second enzyme activity associated with the plasma membrane (14), and permeability to ^{86}Rb (8).

The inhibition of glucan synthetase by dinitramine correlated with the inhibition of K^+-ATPase activity. Water-soluble, lipid-soluble, alkali-soluble, and alkali-insoluble products of the glucan synthetase system were respectively inhibited by 69, 47, 58, and 54% (Table II). The data presented in Table II were obtained from three separate experiments. The data clearly show a dinitramine inhibition although the variability in control tissue was greater than expected. For this reason the per cent inhibition is noted for each experiment.

The effect of dinitramine on the permeability of the plasma membrane was determined with an ^{86}Rb efflux procedure (8). Figure 6 shows the time course of ^{86}Rb loss from control and treated root segments, plotted as percentage of counts remaining in the tissue at the end of the efflux period. Rate of loss was initially quite rapid from both tissues, presumably reflecting the

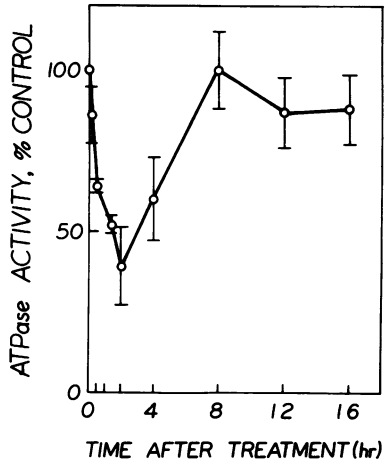

FIG. 4. Time course of dinitramine effect on K^+-activated ATPase activity of plasma membrane vesicles of soybean root. Roots were sprayed with a 200-μg/ml solution of dinitramine in ethanol and returned to darkness for various periods before membrane preparation. Control roots were sprayed with ethanol. Error bars indicate standard deviation.

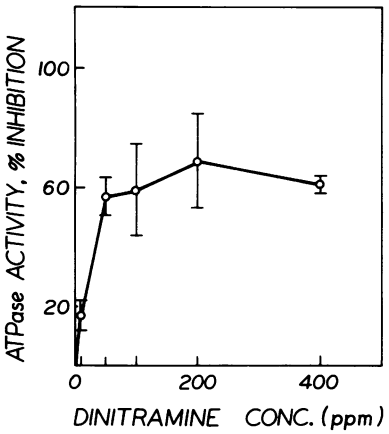

FIG. 5. Effect of concentration of dinitramine on K^+-activated ATPase activity of plasma membrane vesicles of soybean root. Roots were sprayed with various concentrations of dinitramine in ethanol and returned to darkness for 2 hr before membrane preparation. Control roots were sprayed with ethanol. Error bars indicate standard deviation.

Table II. Effect of dinitramine on soybean root plasma membrane glucan synthetase activity
Tissue was treated with ethanol plus or minus 200 ug/ml dinitramine. The plasma membrane vesicles were prepared 2 hr after treatment.

Fraction	Exp. 1	Exp. 2	Exp. 3	Average
	Glucan synthetase activity nmol glucose incorporated/mg protein·hr			
Water-soluble				
Control	290	165	204	
Treated	123 (58)[1]	58 (65)	17 (85)	(69)
Lipid-soluble				
Control	25	80	91	
Treated	13 (51)	--	52 (43)	(47)
Alkali-soluble				
Control	281	720	346	
Treated	127 (55)	165 (77)	205 (41)	(58)
Alkali-insoluble				
Control	640	876	1080	
Treated	426 (34)	227 (74)	---	(54)

[1] percent inhibition

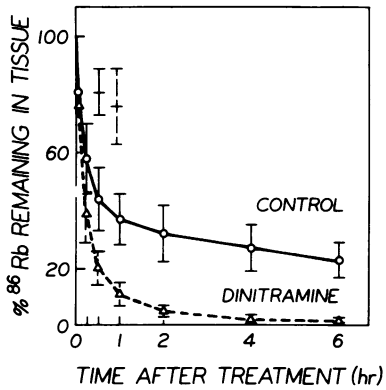

FIG. 6. Effect of dinitramine on ^{86}Rb efflux from soybean root segments. Root segments were incubated in ^{86}Rb for 12 hr, sprayed with a 200-μg/ml solution of dinitramine in ethanol and rinsed with 5-ml portions of 1 mM KCl plus 1 mM $CaSO_4$-$7H_2O$. Control root segments were sprayed with ethanol. Error bars indicate standard deviation.

loss of Rb loosely adsorbed to the tissue surface plus that occupying the free space. Within 15 min, however, ^{86}Rb loss from treated tissue began to exceed that of the control tissue, becoming nearly 50% greater within 30 min. At 6 hr, control tissue retained about 25% of initial ^{86}Rb, *versus* only 2% in treated tissue.

DISCUSSION

The results suggest that the herbicide had its initial effect at the level of the plasma membrane. Hodges and co-workers have established that a K^+-stimulated ATPase, localized in the plasma membrane of oat roots, mediates energy transfer to the ion transport system (3). Thus, an initial effect of dinitramine may involve a reduction in ion uptake or at least an inhibition of energy generation necessary for ion uptake. The inhibition of glucan synthetase activity indicates that dinitramine does not specifically affect ion uptake. The synthesis of glucans (glucan synthetase activity) is a membrane-associated activity involved in cell wall biogenesis (17). Thus, the initial effect of dinitramine is likely to be an over-all reduction in membrane function. This conclusion is supported by the dinitramine-enhanced permeability to ^{86}Rb. Preliminary studies in this laboratory (to be presented elsewhere) have shown that an early effect of dinitramine (within 15 min of treatment) is the release of membrane-bound ribosomes. Ribosome attachment to membranes in mammalian systems reportedly occurs through an interaction of ribosomal subunits and protein components of the ER (7, 15). In higher plants the interaction reportedly involves the nascent or growing peptide of the ribosome and the ER (2). Dinitramine-induced release of membrane-bound ribosomes suggests an effect on the ribosome-membrane interaction. Thus, we can document several "early" responses to dinitramine, all involving protein components of the membrane system. The plasma membrane is suggested as the initial site of action because the compound must penetrate the membrane before it can induce ribosome release from the ER.

The final result of dinitramine treatment of soybean roots is the radial enlargement of cortical cells coupled with an increase in cytoplasmic ribosomes in the region of cell elongation. The results presented suggest that the final outcome may be mediated by an early membrane response. A critical aspect of this argument is that membrane-associated ATPase activity recovered from the initial dinitramine treatment. The precise nature of recovery cannot be determined from these results. Preliminary experiments in this laboratory with [^{14}C]dinitramine suggest that the herbicide rapidly binds to the plasma membrane. Whether or not dinitramine remains bound to the membrane is not known. Thus, several possible explanations for K^+-ATPase recovery exist. It appears that the initial activity of the herbicide is the inactivation of membrane-associated proteins. The reappearance of enzyme activity may represent reactivation of preexisting proteins or the synthesis of new proteins. In either case recovery may depend upon release of dinitramine from the membrane and/or its detoxification.

Future studies will consider: (*a*) the nature of herbicide-membrane interaction; (*b*) the nature of membrane recovery, *i.e.* whether proteins inactivated by dinitramine are resynthesized or simply reactivated; (*c*) the nature of the dinitramine-induced increase in cytoplasmic ribosomes; and (*d*) a potential link between an initial membrane response and an ultimate nuclear response as manifested by cytoplasmic ribosome synthesis.

LITERATURE CITED

1. CHRISTIANSEN JL, S LINDBERG 1976 Kinetic studies of (Mg^{2+}-ATPase) in oat roots. Physiol Plant 36: 110-112
2. DOBBERSTEIN B, D VOLKMANN, D KLAMBT 1974 The attachment of polyribosomes to membranes of the hypocotyl of *Phaseolus vulgaris*. Biochim Biophys Acta 374: 187-196
3. FISHER JD, D HANSEN, TK HODGES 1970 Correlation between ion fluxes and ion-stimulated adenosine triphosphatase activity of plant roots. Plant Physiol 46: 812-814
4. FISKE CH, Y SUBBAROW 1925 The colorimetric determination of phosphorus. J Biol Chem 66: 375-400
5. GUILFOYLE TJ, CY LIN, YM CHEN, RT NAGAO, JL KEY 1975 Enhancement of soybean RNA polymerase I by auxin. Proc Nat Acad Sci USA 72: 69-72
6. HODGES TK, RT LEONARD, CE BRACKER, TW KEENAN 1972 Purification of an ion-stimulated adenosine triphosphatase from plant roots: association with plasma membranes. Proc Nat Acad Sci USA 69: 3307-3311
7. JOTHY S, J-L BILODEAU, H SIMPKINS 1975 The role of membrane proteins and phospholipids in the interaction of ribosomes with endoplasmic reticulum membranes. Can J Biochem 531: 1039-1045
8. KECK RW, TK HODGES 1973 Membrane permeability in plants: changes induced by host-specific pathotoxins. Phytopathology 63: 226-230
9. LEONARD RT, D HANSEN, TK HODGES 1973 Membrane-bound adenosine triphosphatase activities of oat roots. Plant Physiol 51: 749-754
10. LEONARD RT, TK HODGES 1973 Characterization of plasma membrane-associated adenosine triphosphatase activity of oat roots. Plant Physiol 52: 6-12
11. LEONARD RT, WJ VANDERWOUDE 1976 Isolation of plasma membranes from corn roots by sucrose density gradient centrifugation. Plant Physiol 57: 105-114
12. LINDBERG S 1976 Kinetic studies of a (Na^+ + K^+ + Mg^{2+}) ATPase in sugar beet roots. Physiol Plant 36: 139-144
13. LOWRY OH, NJ ROSEBROUGH, AL FARR, RJ RANDALL 1951 Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265-275
14. ORDIN L, MA HALL 1968 Cellulose synthesis in higher plants from UDP glucose. Plant Physiol 43: 473-476
15. ROLLESTON FS 1972 The binding of ribosomal subunits to endoplasmic reticulum membranes. Biochem J 129: 721-731
16. SMITH RA, WS BELLES, KW SHEN, WG WOODS 1973 The degradation of dinitramine (N^3,N^3-Diethyl 2,4-dinitro-6-trifluoromethyl-*m*-phenylenediamine) in soil. Pest Biochem Physiol 3: 278-288
17. VANDERWOUDE WJ, CA LEMBI, DJ MORRÉ, JI KINDINGER, L ORDIN 1974 β-Glucan synthetases of plasma membrane and golgi apparatus from onion stem. Plant Physiol 54: 333-340
18. VELMURUGU V 1974 Studies on the mode of action and selectivity of dinitramine. MS thesis. Univ Wales, Bangor, UK