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Effect of Diethylstilbestrol on Ion Fluxes in Oat Roots[1]

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NELSON E. BALKE[2] AND THOMAS K. HODGES
Department of Botany and Plant Pathology, Purdue University, West Lafayette, Indiana 47907

ABSTRACT

Effects of diethylstilbestrol (DES) on ion fluxes in oat roots (*Avena sativa* L.) were investigated by measuring K^+ and Cl^- absorption and K^+ efflux. DES rapidly decreased the absorption of K^+ (^{86}Rb) and $^{36}Cl^-$ by excised roots; 10^{-4} molar DES inhibited Cl^- absorption in 1 minute and K^+ absorption in 1 to 2 minutes. With a 10-minute incubation period, K^+ and Cl^- absorption were inhibited 50% by 1.1×10^{-5} molar and 8.4×10^{-6} molar DES, respectively. Treatment for 3 minutes with 10^{-4} molar DES caused irreversible inhibition of K^+ absorption. Increasing concentrations of KCl in the absorption media decreased the DES inhibition. Experiments with the DES analogs, DES dipropionate, dienestrol and hexestrol, showed that the steric configuration and the hydroxyl groups of the DES molecule are important in determining the inhibitory capacity of the compound.

DES increased the efflux of ^{86}Rb from excised roots only after a 10-minute lag period. In 10^{-4} molar DES, roots lost 82% of their radionuclide content in 1 hour. Comparison of efflux curves for roots loaded for 20 hours and those loaded for 15 minutes suggested that DES increased the permeability of the plasma membrane after about 10 minutes and the permeability of the tonoplast after 10 to 20 minutes. Oligomycin and dinitrophenol also increased the loss of ^{86}Rb, but the lag period was about 4 hours.

The rapid effect of DES on ion absorption and the slower effect on ion efflux suggest that DES initially inhibits ion uptake by affecting the transport mechanism at the plasma membrane in some manner other than alteration of membrane permeability.

The synthetic estrogen diethylstilbestrol inhibited ion absorption by organelles of animal cells (6, 22), and recently we found that it inhibited ion absorption by oat roots (2, 3). This compound increased the permeability of bacterial (32) and artificial phospholipid membranes (30, 31) and also depolarized the electrical potential and increased the resistance across cellular membranes of *Chara* (18). Thus it appears that DES[3] may be a useful probe for studying ion absorption in plants as well as animals.

In this paper we report an investigation into the effects of DES on ion transport in excised oat roots. DES inhibited the absorption of ions and increased the loss of ions from roots, but the times required for the two effects were different.

MATERIALS AND METHODS

Plant Material. Seeds of *Avena sativa* L. cv. Goodfield were germinated and grown for 5 to 6 days in the dark at 24 C over 3 liters of aerated 1 mM $CaSO_4$ in a 4-liter beaker (15). Each seed was individually positioned into a layer of cheesecloth stretched across the top of the beaker. This procedure permitted individual plants to be removed and rinsed in ice-cold deionized H_2O prior to excision for the experiments.

Ion Absorption. The apical 5 cm of nine roots (primary root and two seminal roots from three plants) were cut into 1-cm segments and stored on moistened filter paper on ice for no longer than 30 min before being used. The standard absorption solution contained 0.25 mM $CaSO_4$, 0.5 mM KCl, ^{86}Rb or ^{36}Cl (about 2×10^6 cpm/μmol KCl) and 5.0 mM $Tris_L$-MES_L (pH 6.5); inhibitors were added as indicated. ^{86}Rb was the tracer for K^+, and ^{36}Cl for Cl^-. Changes in concentrations of KCl or Tris-MES are indicated in table and figure legends.

Root segments (about 65 mg fresh weight) were submerged in 10 ml aerated absorption solution for 10 min at 24 C. The segments were then collected by suction filtration on a Büchner funnel and transferred to ice-cold absorption solution without the radionuclide or inhibitor. After exchanging for 1 min, which was sufficient to remove K^+ and Cl^- from the "free-space," the segments were collected by suction filtration and weighed in tared scintillation vials. The radioactivity of the segments was measured by liquid scintillation spectroscopy in a dioxane-methyl Cellosolve cocktail (7). Cab-O-Sil (600 ml/liter scintillation fluid) was added to produce a thixotropic gel; this gel kept the root segments suspended. Most experiments were done at least twice with each treatment in triplicate; exceptions are noted in the figure legends. Standard errors of the mean were less than 10% of the mean for each experiment.

Ion Efflux. For efflux experiments the apical 5 cm of 36 roots (12 plants) were cut into 2.5-cm segments. The roots (about 225 mg fresh weight) absorbed ions from an aerated solution of 0.25 mM $CaSO_4$, 0.5 mM KCl, ^{86}Rb (about 105,000 cpm/μmol KCl), 5 mM $Tris_L$-MES_L (pH 6.5) at 24 C for either 0.25 or 20 hr. The segments were removed, blotted with tissue paper to remove the adhering absorption solution, and placed in the barrel of a 25-ml plastic syringe fitted with a piece of surgical tubing and a pinch clamp. Ten ml of nonradioactive absorption solution plus inhibitor was added to the syringe, drained, and replaced at specific times. The solutions containing root segments were aerated during the entire efflux period. Root segments were weighed at the end of the period. Radioactivity of the root segments and 1-ml aliquots of the drained solutions was measured as described above except Cab-O-Sil was added only to the vials containing root segments.

Dissolution of Compounds. All inhibitors were dissolved in absolute ethanol because of low solubility of the compounds in water. The concentration of ETOH in the absorption and washout solutions was 1%. This concentration of ETOH caused less than 5% inhibition of K^+ and Cl^- absorption and did not affect ion efflux (see Fig. 10). The concentrations of the oligomycin solutions were calculated based on the purchased compound being 15% oligomycin A and 85% oligomycin B. All of the inhibitors were purchased from Sigma Chemical Co., St. Louis, Mo.

At concentrations above 10^{-4} M, DES and DES analogs precipitated due to the low solubility of these compounds in water (21). However, a partition of DES between the ETOH solution and the

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[2] Present address: Department of Agronomy, University of Wisconsin, Madison, Wisconsin 53706.

[3] Abbreviations: DES: diethylstilbestrol; DNP: 2,4-dinitrophenol; ETOH: ethanol; $Tris_L$-MES_L: buffer made by mixing solutions of equal concentrations of Tris and MES to give the desired pH.

membrane lipid of the oat roots should have been established regardless of precipitation. Because these compounds are very lipid-soluble (19) and readily dissolve in membrane lipid (1), it is possible that some precipitated DES could have adsorbed to and been absorbed by plasma membranes.

RESULTS

Experimental Conditions. The pH of an unbuffered absorption solution decreases as ions are absorbed (16, 25). To prevent a 0.1-pH unit decrease in our absorption solution, we buffered the solutions with Tris-MES (pH 6.5). K^+ was absorbed more rapidly from the buffered than unbuffered solutions (Table I). This may have been due to stabilization of the pH or stimulation of uptake by Tris or MES. This experiment did not distinguish between these two possibilities. Tris-MES buffer was shown previously to have little effect on K^+ absorption by oat roots in 1 mM Ca^{2+} (28). However, Van Steveninck (29) found that the Ca^{2+} concentration affected the Tris stimulation of ion uptake in beet storage tissue. Thus, the different Ca^{2+} concentrations may have caused the difference between previous (28) and present (Table II) results with Tris-MES buffers. In the subsequent absorption and efflux experiments, $Tris_L$-MES_L (5.0 mM, pH 6.5) containing 0.25 mM $CaSO_4$ was used to buffer all solutions.

In order to study effects of DES on ion absorption at the plasma membrane, we used initially a 10-min absorption period and a 5-min wash period (9). Inasmuch as DES might affect permeability of plasma membranes (32), we reduced the wash period as much as possible to prevent loss of ^{86}Rb or ^{36}Cl from the cytoplasm. A 1-min wash was sufficient to remove K^+ or Cl^- from the "free space" of the tissue (Fig. 1). In subsequent experiments the root

Table I. K^+ absorption by excised roots from solutions containing various concentrations of $tris_L$-MES_L, pH 6.5, buffer.

Absorption was for 10 min in standard absorption medium with various buffer concentrations. The solution without buffer was brought to pH 6.5 with KOH. The wash period following absorption was 1 min.

$tris_L$-MES_L (mM)	$\left(\frac{\text{nmoles } K^+}{\text{g fresh wt}}\right)$	%
0.0	349	100
1.0	475	136
5.0	428	123
10.0	475	136

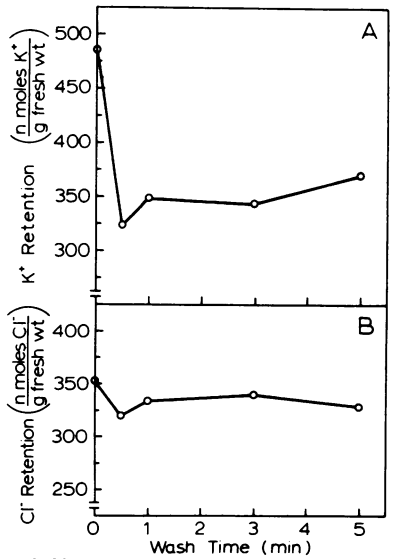

FIG. 1. K^+ and Cl^- retention by excised roots exchanged in ice-cold absorption solution. The wash followed a 10-min absorption period. A: K^+ retention, B: Cl^- retention. Experiment done once in triplicate.

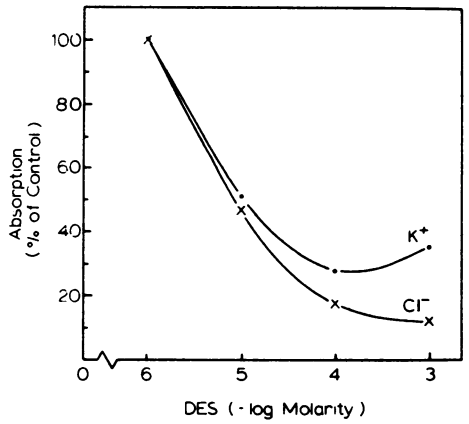

FIG. 2. K^+ and Cl^- absorption by excised roots in the presence of various concentrations of DES. Controls absorbed 359 nmol K^+/g fresh weight and 218 nmol Cl^-/g fresh weight in 10 min.

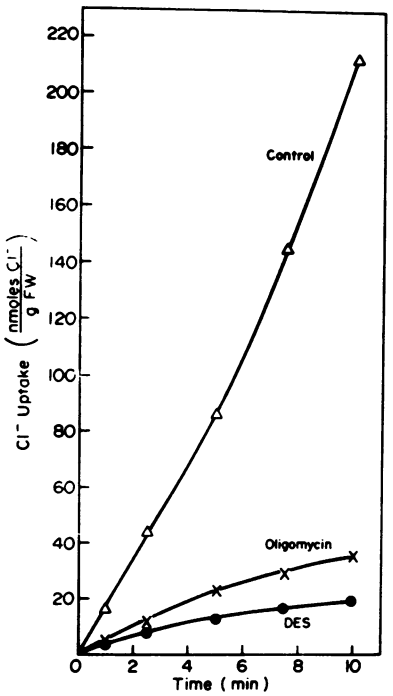

FIG. 3. Time course of Cl^- absorption by excised roots in the presence of 10^{-4} M DES, 1.2×10^{-5} M (5 μg/ml) oligomycin, or 1% ETOH (control). Original data intersected the ordinate at 3 nmol Cl^-/g fresh weight. This value was subtracted from all data points to give Figure 3.

segments were rinsed in ice-cold absorption solution without radionuclides for 1 min prior to being weighed and counted for radioactivity.

Absorption Experiments. Increasing concentrations of DES drastically inhibited both K^+ and Cl^- absorption (Fig. 2). K^+ absorption was inhibited 50% at 1.1×10^{-5} M DES, and Cl^- absorption was inhibited 50% at 8.4×10^{-6} M. Above 10^{-5} M, DES inhibited Cl^- absorption more than K^+ absorption. K^+ absorption was inhibited less at 10^{-3} M DES than at 10^{-4} M DES, but Cl^- did not show this peculiarity. This unexpected result for K^+ absorption may have resulted from high concentrations of DES preferentially increasing the permeability of the plasma membrane to K^+ in 10 min. Also, DES inhibited ion absorption from unbuffered absorption solutions (not shown). In the unbuffered solutions, ion absorption at DES concentrations above 10^{-6} M was 10 to 15% greater than in the buffered solutions.

DES, as well as oligomycin, inhibited anion and cation absorption very rapidly (Figs. 3 and 4). Both compounds inhibited Cl^-

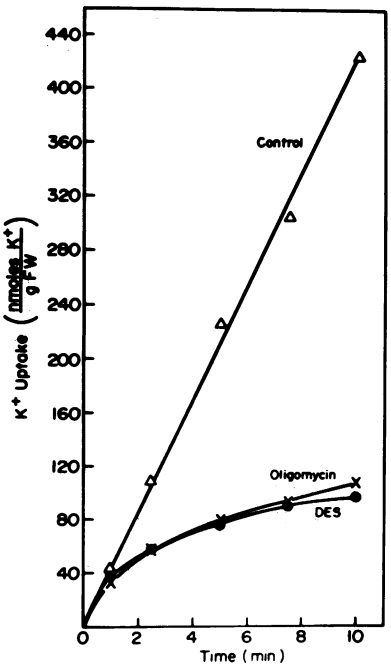

FIG. 4. Time course of K^+ absorption by excised roots in the presence of 10^{-4} M DES, 1.2×10^{-5} M (5 μg/ml) oligomycin, or 1% ETOH (control). Original data intersected the ordinate at 12 nmol K^+/g fresh weight. This value was subtracted from all data points to give Figure 4.

absorption in 1 min (Fig. 3) and K^+ absorption in 1 to 2 min (Fig. 4). The two compounds inhibited K^+ absorption to the same extent, but DES inhibited Cl^- absorption more than oligomycin did.

Roots treated with DES for 3 min, rinsed, and transferred to 1% ETOH were unable to recover their capacity to absorb K^+ (Fig. 5). The inhibition by DES was not reversible in 1 hr. Roots treated for the entire 1 hr with DES showed a slight loss of K^+ (^{86}Rb) after 30 min. This result suggested that extended exposure to DES increased the permeability of the tissue and allowed K^+ to wash out of the tissue although a 10-min exposure to DES did not appear to increase permeability.

Other experiments verified the lack of a DES effect on permeability in 10 min. Absorption of K^+ from ice-cold absorption solution was inhibited only 7% by DES in 5 min (data not shown). Hence, passive absorption was not altered significantly by DES. Due to the large concentration gradient ($K^+_{external}/K^+_{cytoplasm}$) at 200 mM KCl, if DES had increased the permeability, the absorption of K^+ should have been much greater in the presence of DES than in the presence of 1% ETOH. K^+ absorption in the presence of DES was never greater than the ETOH control (Table II). In contrast, gramicidin D, which is known to increase ion absorption (14) and produce channels in membranes (11), did increase the absorption of K^+ by the root segments (Table II). DES inhibition of K^+ absorption during the 10-min exposure does not appear to be due to increased permeability of the plasma membrane.

A Lineweaver-Burk plot of the data from Table II revealed a nonlinear relationship between absorption and KCl concentration in the presence or absence of DES (Fig. 6). The nonlinearity of these data was even more evident when the data points for 0.5 mM KCl were plotted, but this is not presented because of the restricted scale of the abscissa. Other Lineweaver-Burk plots have also shown nonlinearity of ion absorption at high KCl concentrations (20, 23). DES was not as inhibitory at the higher concentrations of KCl as shown by the convergence of the two lines toward the origin.

In an attempt to determine the structural components of the DES molecule that are necessary for inhibition, we determined the effects of three DES analogs (Fig. 7) on K^+ and Cl^- absorption (Table III). Hexestrol inhibited both K^+ and Cl^- absorption as effectively as DES, but dienestrol was less inhibitory, and DES dipropionate was the least inhibitory. DES, dienestrol, and hexestrol inhibited Cl^- absorption more than K^+ absorption.

Efflux Experiments. Because DES might affect the permeability of the plasma membrane when the treatment time exceeds 10 min (Fig. 5), we investigated the effect of DES on loss of ^{86}Rb from root segments loaded with ^{86}Rb for 20 hr. Roots exchanged in 1% ETOH (control) for 12 hr lost only 13% of the ^{86}Rb originally absorbed by the tissue (Fig. 8). However, DES caused a dramatic loss of ^{86}Rb from the root segments. In 1 hr, 82% of the absorbed ^{86}Rb was lost from the roots, and in 12 hr, 99% was lost.

DNP and oligomycin also increased the loss of ^{86}Rb, but there was a lag of about 4 hr before the effect was evident (Fig. 8). After the 4-hr lag, DNP rapidly increased the loss of ions, and the total loss after 12 hr was as great with DNP as with DES. On the other hand, oligomycin did not increase the rate of ion loss as much as either DES or DNP, and the lag time was longer. After 12 hr of exchange, 66% of the ^{86}Rb was removed from the root segments in the presence of oligomycin.

The first 40-min efflux period for the same experiment as shown in Figure 8 is shown in Figure 9. Oligomycin and DNP did not increase ^{86}Rb loss in this time period. However, DES began to increase the loss of ions after about 10 min, and by 20 min an effect by DES was evident.

It is impossible to perform a compartmental analysis (8, 24) of the efflux data because the tissue did not remain in steady-state

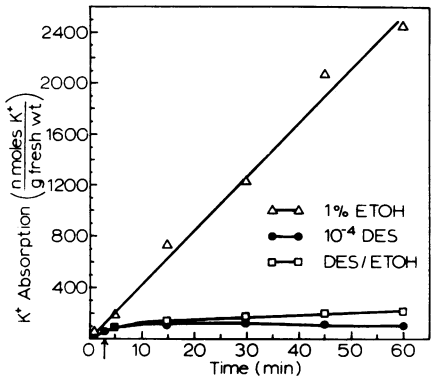

FIG. 5. Time course of K^+ absorption by excised roots in the presence of 10^{-4} M DES, 1% ETOH (control), or after being transferred from 10^{-4} M DES to 1% ETOH. DES/ETOH refers to roots that absorbed K^+ for 3 min from absorption medium plus 10^{-4} M DES; were rinsed 2 min in 30 ml 0.25 mM $CaSO_4$, 5 mM $Tris_L$-MES_L (pH 6.5), 1% ETOH at 24 C (at the arrow); and were transferred to absorption medium plus 1% ETOH.

Table II. K^+ absorption by excised roots from solutions of various concentrations of KCl in the presence of DES or gramicidin D or ETOH.

This experiment was done once in triplicate.

KCl Concentration in Absorption Medium (mM)	Treatment	K^+ Absorbed ($\frac{\text{nmoles } K^+}{\text{g fresh wt}}$)	K^+ Absorbed % of Control
0.5	1% ETOH	371	100
0.5	10^{-4} M DES		27
0.5	5 μg/ml gramicidin D		188
5.0	1% ETOH	1345	100
5.0	10^{-4} M DES		48
50.0	1% ETOH	4615	100
50.0	10^{-4} M DES		77
100.0	1% ETOH	5857	100
100.0	10^{-4} M DES		102
100.0	5 μg/ml gramicidin D		127
200.0	1% ETOH	11859	100
200.0	10^{-4} M DES		96

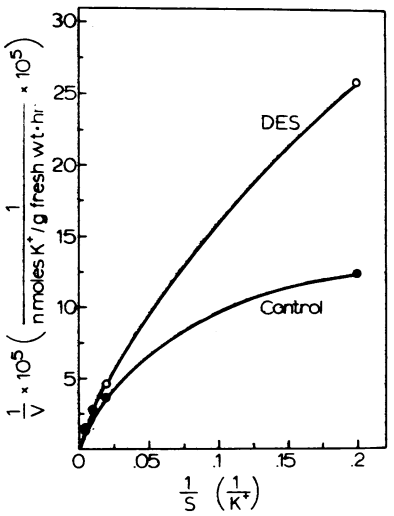

FIG. 6. Lineweaver-Burk plot of K^+ absorption rate *versus* K^+ concentration in the presence of 10^{-4} M DES or 1% ETOH (control). K^+ concentration in the absorption media ranged from 5 to 200 mM. These data are from Table III.

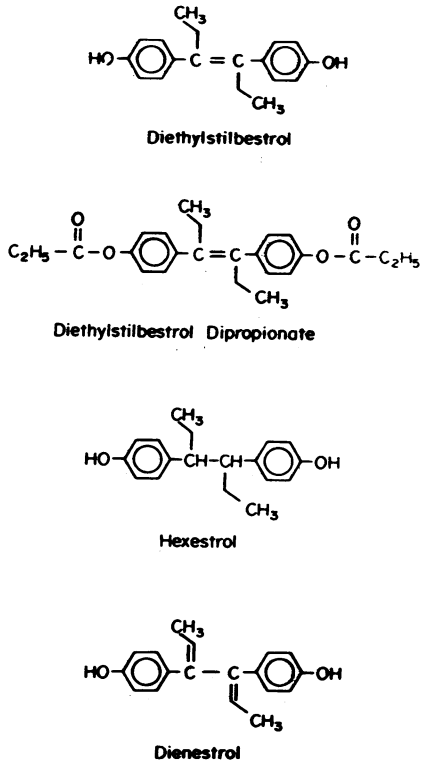

FIG. 7. Structures of DES analogs.

with respect to ion content. Once treated with DES, the tissue lost ions so fast that net absorption probably did not equal net loss of ions. The rapid loss of ^{86}Rb indicated that the permeability of both the plasma membrane and the tonoplast were increased at about the same time (Fig. 9).

To separate the effect of DES on the permeabilities of the plasma membrane and the tonoplast, the loss of ^{86}Rb was measured with roots which were loaded for 15 min instead of 20 hr. The ^{86}Rb concentration should have been much greater in the cytoplasm than in the vacuole with the short loading period. In contrast, ^{86}Rb concentration in the cytoplasm and vacuole should have been about equal with the long loading period (24). The ion efflux from the roots loaded for 15 min again showed that 10 min was required for DES to increase the loss of ^{86}Rb (Fig. 10). Also, 1% ETOH did not increase the loss of label as compared to the water control.

Comparison of the efflux of ^{86}Rb from roots loaded for 15 min and those loaded for 20 hr showed that the initial rapid loss of label in 1 min (*i.e.* loss from the free space) was more extensive in the tissue loaded for 15 min than loaded for 20 hr (Fig. 11A). This probably resulted from the percentage of label in the free space (free space total) being higher in roots loaded for 15 min. The roots loaded for 20 hr should have had much more label in the cytoplasm and vacuole than free space (24), and thus the percentage loss from the free space was lower in that tissue. Roots loaded for 15 min and those loaded for 20 hr were affected by DES at about 10 min, although the roots loaded for 20 hr were not affected as much (Figs. 10 and 11B). Between 10 and 20 min the rate of ^{86}Rb loss was much greater for the roots loaded for 15 min than the roots loaded for 20 hr. Between 20 and 30 min the rates were almost identical, but after 30 min the tissue loaded 20 hr lost ^{86}Rb more rapidly than the tissue loaded 15 min.

DISCUSSION

The inhibition of ion absorption by DES showed several important characteristics. First, DES inhibited both K^+ and Cl^- absorption (Fig. 2), and the inhibition was rapid (Figs. 3 and 4). Second, the inhibition of K^+ absorption was irreversible (Fig. 5). Third, increased KCl prevented the DES inhibition of K^+ absorption (Table II and Fig. 6).

Table III. K^+ and Cl^- absorption by excised roots in the presence of DES analogs.

Controls absorbed 400 nmoles K^+/g fresh weight and 247 nmoles Cl^-/g fresh weight in 10 min.

Compound (10^{-4} M)	K^+ Absorption (% of Control)	Cl^- Absorption (% of Control)
Control (1% ETOH)	100	100
DES	25	16
Hexestrol	30	16
Dienestrol	80	72
DES dipropionate	88	96

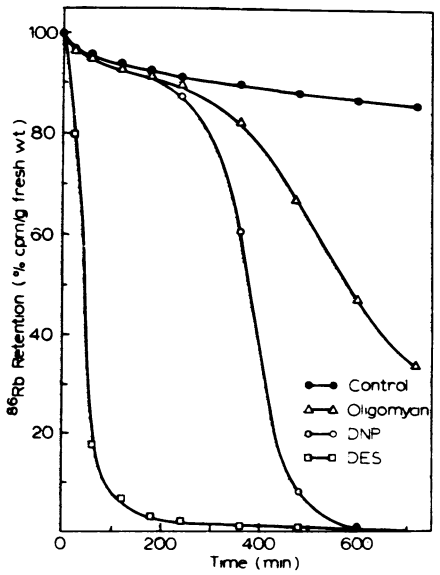

FIG. 8. Twelve-hr efflux of ^{86}Rb from roots loaded for 20 hr. ^{86}Rb content at the beginning of wash-out was 3.25×10^6 cpm/g fresh weight. Inhibitors in the wash-out medium were 10^{-4} M DES, 10^{-4} M DNP, 1.2×10^{-5} M (5 μg/ml) oligomycin, or 1% ETOH (control). Data are presented as ^{86}Rb retained by the root tissue after a given time of efflux. Data are from one experiment.

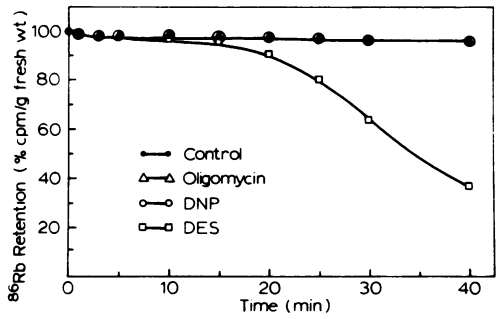

FIG. 9. Forty-min efflux of ^{86}Rb from roots loaded for 20 hr. Inhibitors in the wash-out medium were 10^{-4} M DES, 10^{-4} M DNP, 1.2×10^{-5} M (5 μg/ml) oligomycin, or 1% ETOH (control). Data are presented as ^{86}Rb retained by the root tissue after a given time of efflux and were obtained from the same experiment as Figure 8.

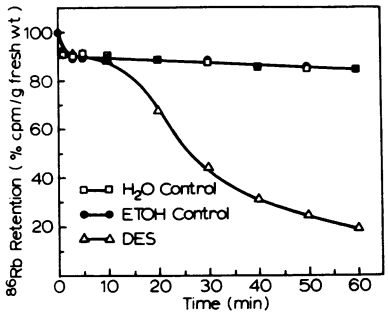

FIG. 10. Sixty-min efflux of ^{86}Rb from roots loaded for 15 min. Inhibitors in the exchange medium were 10^{-4} M DES, 1% ETOH (ETOH control) or none (H_2O control). Data are presented as ^{86}Rb retained by root tissue after a given time of efflux. Data are from one experiment.

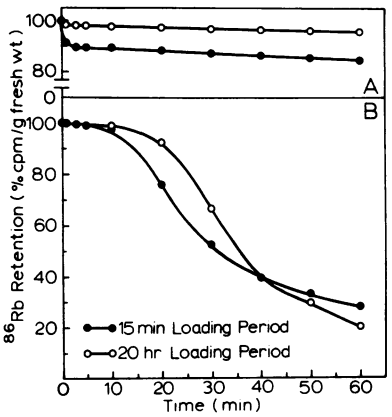

FIG. 11. Sixty-min efflux of ^{86}Rb from roots loaded for 15 min and roots loaded for 20 hr. A: efflux in the presence of 1% ETOH (control). B: efflux in the presence of 10^{-4} M DES. Loss from free space has been subtracted from the curves in B.

Comparison of the inhibition by DES to the inhibition by other compounds showed that oligomycin and DES analogs were less inhibitory than DES (Figs. 3 and 4, and Table III). The results with the analogs showed that the terminal hydroxyl groups were essential for inhibition, the C=C was not essential, and steric interference by rigid side chains reduced the ability of DES to inhibit absorption (Table III).

DES did not seem to alter membrane permeability of oat roots during the first 10 min of exposure to the compound. Loss of ^{86}Rb from roots loaded 20 hr (Fig. 9) or 15 min (Fig. 10) was not evident until 10 min of treatment (Fig. 11). Also, DES inhibited ion absorption from ice-cold absorption media only 7% in 5 min. If DES had altered the passive permeability of the plasma membrane, this effect should have been greater. DES did not produce the same effect as gramicidin D, which increased absorption above the control (Table II). Thus, DES did not act like gramicidin D, which produced channels in artificial membranes (11) and thus increased the permeability.

The effects of the DES analogs on absorption also support our contention that membrane permeability was not altered in 10 min. The more lipid-soluble compounds should have a greater effect on permeability because those compounds more readily disperse the lipid matrix of membranes. DES and DES dipropionate have octanol-water partition coefficients of 1.2×10^5 (19) and 1.2×10^7 (calculated from Leo, *et al.*, [19] and Fujita, *et al.* [10]), respectively. In contrast to DES, DES dipropionate inhibited K^+ and Cl^- absorption very little in 10 min. The inhibition did not correlate with the lipid solubility of the compounds.

In addition to DES, neither DNP nor oligomycin affected ^{86}Rb loss from roots in 10 min (Fig. 8). As with DES, these compounds did not rapidly alter membrane permeability. The inhibition of absorption by oligomycin (Figs. 3 and 4) was probably caused by some mechanism other than a change in permeability (17).

DES did affect membrane permeability when root segments were exposed to the compound for more than 10 min. The ^{86}Rb content of roots absorbing ions in the presence of 10^{-4} M DES decreased slightly after 30 min (Fig. 5). This result suggested that DES increased the permeability of the tissue and allowed ^{86}Rb to leak out of the tissue. Our hypothesis that DES increased the membrane permeability after 10 min is supported by the efflux experiments. After about 10 min, DES increased the loss of ^{86}Rb from roots loaded for either 20 hr (Fig. 9) or 15 min (Fig. 10). The loss of radionuclide was rapid and massive after the 10-min lag period.

Comparison of ^{86}Rb loss from tissue loaded for 20 hr and tissue loaded for 15 min (Fig. 11) aided in separating the effects of DES on the permeabilities of the tonoplast and the plasma membrane. The rate of loss of ^{86}Rb between 10 and 20 min was greater for the roots loaded for 15 min than those loaded for 20 hr. Inasmuch as most of the ^{86}Rb should have been in the cytoplasm of the roots loaded for 15 min, the greater rate of loss in that tissue reflected increased permeability of the plasma membrane. The increased rate of loss between 20 and 30 min was probably caused by DES increasing the permeability of the tonoplast. This conclusion is supported by the increased rate of loss from tissue loaded for 20 hr which should have had the majority of the label in vacuoles (24). After 30 min the rate of loss was greater for tissue loaded for 20 hr than tissue loaded for 15 min because the former tissue should have contained more label to be exchanged out of vacuoles. These data suggest that the permeability of the plasma membrane was increased in about 10 min and the permeability of the tonoplast was increased in about 20 min.

Taken collectively, the absorption and wash-out experiments suggest the following mechanism of action for DES. DES inhibits ion absorption very rapidly by diffusing into the plasma membrane and disrupting the transport systems for both K^+ and Cl^-. This rapid inhibition does not appear to be due to altered membrane permeability. With time the DES concentration in the plasma membrane increases to the point that the membrane loses its structural integrity. The plasma membrane becomes permeable allowing ions to move out of and DES to move into the cytoplasm. Shortly after the permeability of the plasma membrane increases, the permeability of the tonoplast also increases, resulting in a massive loss of ions from the tissue. The increase in permeability resulting from a direct effect of DES on membranes may be similar to the increased permeability of artificial liposomes in the presence of DES (30, 31).

Prior to increasing the membrane permeability, DES may inhibit transport systems at the plasma membrane as it did the ($Na^+ + K^+$)-ATPase of liver plasma membranes (12) and brain microsomes (26) of rats. Also, DES may decrease the energy supply for

absorption by inhibiting mitochondrial electron transport or oxidative phosphorylation as it did with mitochondria of rat liver (27). Because the inhibition of absorption by DES is very rapid, indicating effects at the plasma membrane, DES inhibition of the plasma membrane ATPase that is associated with ion absorption across the plasma membrane of oat roots (13) has been studied in the following paper (5). The effects of DES on mitochondrial functions have been addressed in a subsequent paper (4).

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