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Infrared Spectroscopic Evidence for a Conformational Alteration of Plant Plasma Membranes Upon Exposure to the Growth Hormone Analog, 2,4-Dichlorophenoxyacetic Acid

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Summary: Infrared spectroscopy of highly purified fractions of plasma membrane vesicles from hypocotyls of etiolated soybean (Glycine max L.) seedlings revealed changes in bands assigned to proteins and phospholipids upon exposure to the growth hormone analog, 2,4-dichlorophenoxyacetic acid (2,4-D). The changes included a concentration dependent broadening of amide I absorbance and a change in the absorbance ratios of amide I and amide II indicative of a change in protein conformation. Band broadening of amide I was observed at 2,4-D concentrations as low as 10^{-8} M, and the optimal 2,4-D concentration to evoke the change was 1 μM whereas the amide peak ratios (amide II/amide I) declined steadily over the range of concentrations (10^{-8} to 10^{-3} M) tested. An alteration in hydrocarbon chains (CH_2 scissoring) was seen only at 1 mM (10^{-3} M) 2,4-D. In contrast, the vibrational frequency of the choline stretch declined proportionally over the range 10^{-6} to 10^{-3}. The findings provide evidence for a conformational change in the plasma membrane in response to the hormone demonstrable in a cell-free system.

Effector (hormone)-responsive systems usually are considered to include at least three essential elements: (1) A receptor site to recognize and bind the extracellular signal (hormone); (2) Some form of transduction mechanism that recognizes a change in configuration or conformation of the receptor; and (3) An amplifier that usually translates the received message into an increase (or decrease) in the intracellular concentration of a chemical species capable of exerting control over intracellular processes (1). There is now general agreement that membrane fragments, including plasma membranes, bind the plant growth hormones of the auxin type in a manner that is both reversible and auxin specific (2). However, direct evidence for a conformational change in the receptor leading to the initiation of a transducing response has been more difficult to obtain.

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Ultrastructural evidence for an alteration of plant plasma membranes induced by auxins was observed both in isolated and in situ plasma membranes of etiolated hypocotyls of soybean (3). In these studies, fixed and embedded preparations were stained by a phosphotungstate-chromate procedure (4) to identify and accentuate plasma membranes. Isolated plasma membrane vesicles treated with physiological concentrations of an auxin (e.g. 1 μM) for 2 to 10 min were 10 to 15% thinner than the untreated vesicles. The response was auxin-specific, temperature- and time-dependent, and reversible. The dose-dependency of the response paralleled that of the growth response to auxin with an optimum at 1 μM. Subsequently, it was shown for isolated fractions of the plasma membrane vesicles that 1 μM auxin caused an increase of 25% in the fluorescence polarization of the membrane-bound probe N-phenyl-naphthylamine (5), with no measurable change in fluorescence lifetime. The amplitude of the polarization increase was maximal in the temperature range 12 to 22°C and confirmed the cell-free response indicated from membrane measurements.

More recent studies on the nature of the transducing mechanism using the isolated membrane vesicles responsive to auxin has indicated various enzymatic changes involving phospholipid degradation (6) and activation of plasma membrane redox activities (7). These findings prompted a reinvestigation of the response of the isolated plasma membrane vesicles to 2,4-D to provide additional information on its interaction with the membrane and to determine what membrane constituents might be involved. Infrared spectroscopy provides a powerful, noninvasive technique for this type of study where membrane phospholipids and proteins and their interactions with an extrinsic effector are to be investigated (8-12).

Materials and Methods: Soybean (Glycine max L., Merr. cv. Williams) seedlings were grown in darkness in Vermiculite for 4 to 5 days. Two cm long segments, cut 5 mm below the cotyledons, were hand homogenized (35 g) using a chilled mortar and pestle in an equal volume (w/v) of medium containing 50 mM HEPES, pH 7.5, 400 mM sucrose, 100 mM KCl and 1 mM $MgCl_2$. The homogenates were centrifuged at 6,000 rpm (4500 x g) and the pellet containing debris, nuclei, starch, plastids and mitochondria was discarded. The supernatant was then centrifuged at 18,000 g for 45 min, and this pellet was used for isolation of plasma membranes by aqueous two-phase partition based on Kjellbom and Larsson's (12) adaptation of the general method (14). The crude microsomal pellets were

mixed with 6.4% (w/w) Dextran T500 (Pharmacia), 6.4% (w/w) Polyethylene Glycol 3350 (Fisher), 0.25 M sucrose and 5 mM potassium phosphate, pH 6.8. After mixing of the contents of the two-phase system by 40 inversions of the tubes, the two phases were separated by centrifugation at 1,500 g for 5 min. The upper phase, enriched in plasma membrane, was rewashed against a fresh lower phase with centrifugation to separate the phases after the second set of 40 inversions. Likewise, the first lower phase was repartitioned once against fresh upper phase. Finally the two upper phases, with purified plasma membrane, were diluted with water and centrifuged at 18,000 g for 45 min to collect the membranes.

Infrared spectra were taken with a Perkin-Elmer Fourier Transform Infrared Spectrometer (Model 1750) and data were acquired with a Perkin-Elmer 7500 data station and the Perkin-Elmer CDS-3 software. Spectra were the result of 1 to 10 co-added spectra and each treatment condition was repeated at least three times. BaF windows with a teflon 0.05 mm spacer were used in a sealed demountable cell for all spectra. The data are aqueous spectra from which a water spectrum was subtracted automatically, using the interactive difference routine in the CDS-3 software. The data were collected over the IR region 4000 cm^{-1} to 600 cm^{-1}, with trapezoidal apodization. Particular regions of interest were then copied out from this region, converted to absorbance and processed through the abscissa expansion (ABEX) in the CDS-3 software. This routine expands the spectra so that relative heights of the bands are maintained while the most intense band is expanded to a predetermined absorbance. Unless indicated otherwise, other data manipulations such as spectral enhancement or smoothing were avoided. 2,4-D was added in aqueous solution without aid of cosolvent.

Results: The infrared spectrum of soybean plasma membranes appears qualitatively similar to published spectra for rabbit (15) or lobster (9) sarcoplasmic reticulum and other biological membranes (Fig. 1). Bands relevant to intrinsic protein structure include the amide I (1653 cm^{-1}) and amide II (1549 cm^{-1}) regions. A band relevant to alterations of phospholipid head groups is the choline stretching at about 1000 cm^{-1}.

Spectra in the amide I and amide II regions showed a consistent pattern of change to the presence of 2,4-D (Fig. 2). The changes were similar if the 2,4-D was added directly to the membrane contained in the sample cell or if the membrane was resuspended in 2,4-D and repelleted from the 2,4-D-containing solution. In the untreated membranes, the integrated intensity of the amide I band was about twice that of the amide II (Fig. 2). Upon addition of 1 μM 2,4-D, the amide I band was appreciably broadened and the relative ratios of amide I and amide II were greatly reduced (Fig. 2). The broadening was proportional to auxin concentration over the range 10^{-8} to 10^{-6} M as evidenced by direct measurement and by an apparent frequency shift in the carbonyl stretch frequency at 1735 cm^{-1} (see Fig. 2). Maximum broadening was achieved at auxin concentra-

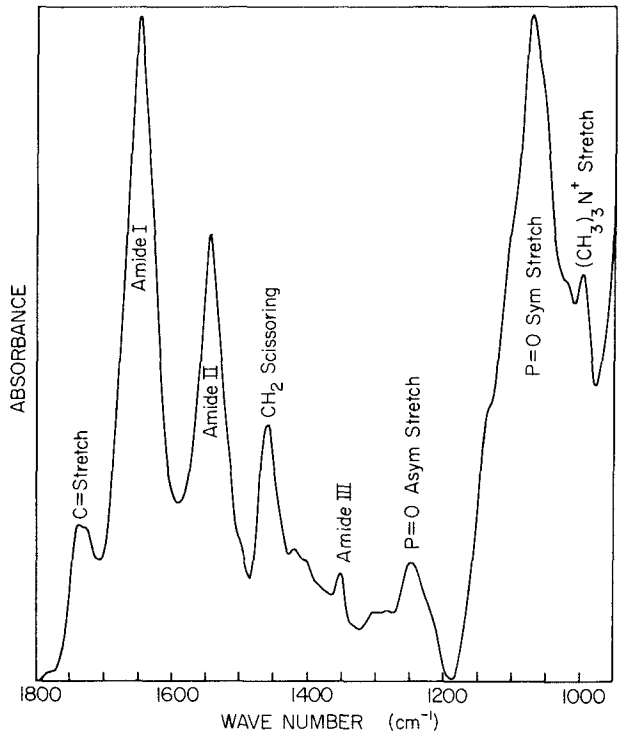


Figure 1. Infrared difference spectrum of fully hydrated plasma membrane vesicles (1 mg protein, pelleted at 18,000 g for 45 min but not resuspended) of soybean. Band assignments are given in the text.

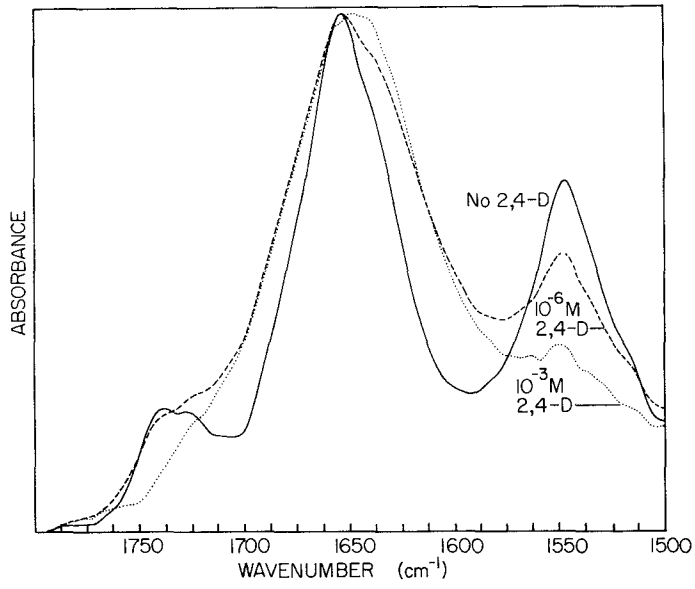
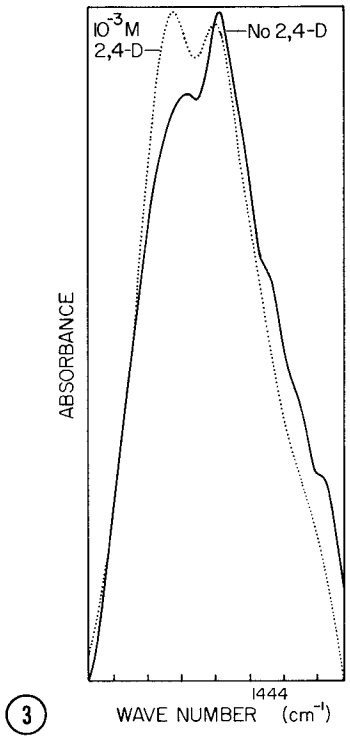


Figure 2. The amide band region of the infrared difference spectrum for soybean plasma membrane vesicles with and without exposure to 1 μM or 1 mM 2,4-D. Both band broadening and a reduced peak I to peak II ratio were seen. Broadening was maximal at 1 μM but the altered peak ratios were proportional to the negative logarithm of the auxin concentration.



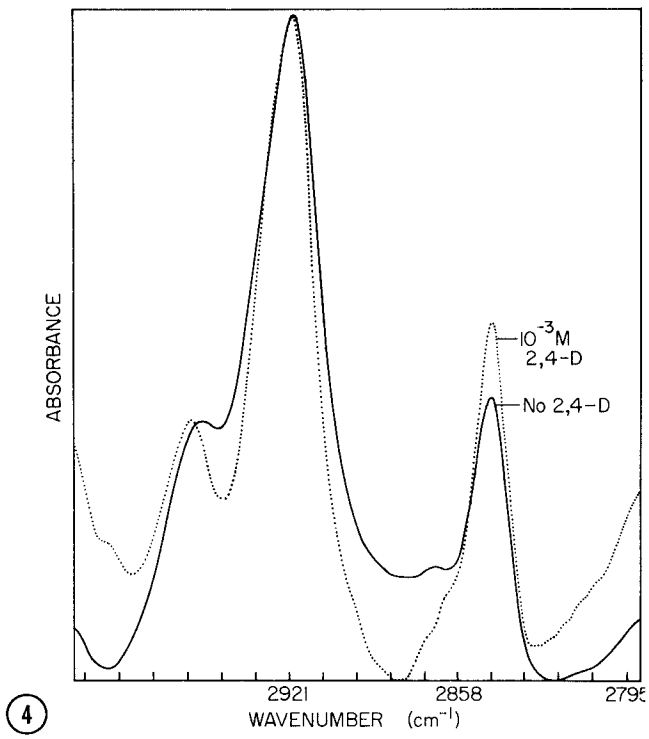


Figure 3. Difference spectra in the CH_2 scissoring region of soybean plasma membrane vesicles with and without exposure to 1 mM 2,4-D. With this concentration there was a reversal of the anisotropy of splitting. At lower 2,4-D concentrations differences were not obvious.

Figure 4. The hydrocarbon chain region of soybean plasma membrane vesicles with and without exposure to 1 mM 2,4-D. The CH_2 symmetric stretch was at about 2853 cm^{-1} suggesting that the membranes were in a liquid crystalline phase. There were no obvious frequency shifts among the CH_2 and CH_3 vibrations, only small changes in the peak height ratios. The only frequency shift observed for a lipid domain of the membrane involved the major choline coupled vibration at 1000 cm^{-1}.

tions of about 10^{-6} whereas the relative ratios of amide I and amide II declined proportionally to the negative logarithm of 2,4-D concentration over the entire range of 10^{-8} to 10^{-3} M.

In the hydrocarbon chain region, there was little effect between 10^{-6} and 10^{-4} M 2,4-D but at 10^{-3} M there was a clear reversal in the anisotropy of splitting in CH_2 scissoring region (Fig. 3). From the frequency of the CH_2 symmetric stretch of about 2853 cm^{-1}, it was apparent that the membrane lipids were in the liquid crystalline phase (Fig. 4). However, no frequency shift was apparent due to 2,4-D in the hydrocarbon region. The only alteration was the change in the peak height ratios at high auxin concentrations (Fig. 4). In

contrast, the vibrational frequency of the choline stretch at 1000 cm^{-1} (Fig. 1) declined in parallel to the relative ratios of amide I and amide II.

Discussion: We have examined the possibility that the plant growth hormone analog 2,4-dichlorophenoxyacetic acid (2,4-D), when added to isolated vesicles of plant plasma membrane can induce conformational changes in the lipid or protein regions of the membrane. The results show that 1 μM 2,4-D added to the membrane resulted in spectral broadening of the amide I region of the infrared spectrum and a decrease in the ratio of amide II to amide I integrated intensities. This interaction may be indicative of protein conformational alterations. Those contributing to the spectral broadening exhibit a maximum at the growth optimal 2,4-D concentration of 10^{-6} M. Those contributing to the declining amide II to amide I peak ratios increase in magnitude over the entire range of hormone concentrations from 10^{-8} to 10^{-5} M in proportion to the negative logarithm of the auxin concentration.

In the hydrocarbon chain region, small changes in the peak height ratios of asymmetric to symmetric stretching were found together with a reversal of anisotropy of the splitting in the CH_2 scissoring region but only at the highest 2,4-D concentration (1 mM) tested. Such changes may be indicative of formation of an interdigitating phase although this would not be expected to occur as readily for membranes with lipids in the liquid crystalline phase (CH_2 symmetric stretch at 2853 cm^{-1}) as if the membranes were in the gel phase (CH_2 symmetric stretch at 2848 cm^{-1}). No large frequency shifts were seen in this region as might have been expected were the increased fluorescence polarization measurements with the N-phenyl-napthylamine probe due to changes in the lipid region accompanying 2,4-D treatment of isolated membrane vesicles (Helgerson et al., 1976).

Jones et al. (16,17) and Jones and Paleg (18,19) have used proton nuclear magnetic resonance chemical shifts to investigate the interaction between the plant hormone indole-3-acetic acid (IAA) and artificial vesicles containing soybean phosphatidylcholine (PC). The auxin addition to sonicated aqueous dispersions of the phospholipid vesicles caused upfield changes in chemical

shifts of the protons of the choline head group. These were proportional to molar ratio of IAA/PC in the range 0.1 to 1 and greatest below pH 5. 2,4-D, which is equal in effectiveness to indole-3-acetic acid in inducing growth responses in tissue segments and enzymatic responses in isolated vesicles (6,7) induces only a small change in the chemical shift of the N^+ $(CH_3)_3$ protons of phosphatidylcholine (18) and was therefore chosen for use in the present study to avoid complications. Nevertheless, at the higher 2,4-D concentrations a shift of the vibrational frequency of the choline stretch to lower frequencies also was observed in our findings. This does not necessarily indicate a direct 2,4-D-choline interaction but may reflect a much broader conformational change extending beyond the interacting proteins to include possibly phospholipid head groups at the lipid/protein phase boundary.

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