

Evidence for an Increase in Microviscosity of Plasma Membranes from Soybean Hypocotyls Induced by the Plant Hormone, Indole-3-Acetic Acid¹

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ABSTRACT

The plant hormone indole-3-acetic acid (IAA or auxin) added at a concentration for half-maximal promotion of cell elongation (1 μ M) caused an increase of 25% in the fluorescence polarization of the membrane-bound probe *N*-phenyl-1-naphthylamine, when added to fractions enriched in plasma membranes from soybean hypocotyls (*Glycine max* L. var. Wayne), with no measurable change in fluorescence lifetime. The amplitude of the polarization increase was maximal in the temperature range 12 to 22 C. The findings provide evidence for a cell-free response of isolated plasma membranes to the hormone and imply that the response involves an increase in the microviscosity of hydrocarbon regions of the membrane.

Changes caused by adding auxins such as IAA to isolated plasma membranes can be demonstrated by direct measurements from electron micrographs, as shown in the preceding paper (7). Independent confirmation of a plasma membrane response to auxin was sought through an application of fluorescence probe techniques. In this study, polarization of the lipophilic probe, *N*-phenyl-1-naphthylamine, bound to isolated preparations of plasma membranes was studied. The findings show an increase in polarization of bound probe molecules which correlates with the apparent thinning of the membrane (7). Taken together, the findings indicate a change in membrane conformation resulting from treatment with the hormone.

MATERIALS AND METHODS

Plant Material. Soybean seeds (*Glycine max* L. var Wayne) were soaked in water overnight, planted in moist vermiculite, and germinated in the dark at 29 C in a constant temperature, high humidity chamber (3). Four days after the beginning of germination, hypocotyls were excised and used for membrane isolations.

Membrane Isolations and Morphometry. Approximately 40 g of tissue were finely chopped with razor blades and suspended in homogenizing medium (1 ml/g tissue). The medium consisted of freshly prepared 0.1 M K_2HPO_4 and 20 mM Na_2 EDTA in Millipore-filtered coconut milk, pH 7.4, containing 0.5 M sucrose. Gradient solutions were prepared by appropriate dilution with the buffered coconut milk of a 2 M solution of sucrose

prepared in buffered coconut milk (5). Homogenization was for 2 min at approximately 5,000 rpm with a Polytron 20 ST (Kinematica, Lucerne, Switzerland).

After homogenization, the brei was filtered through Miracloth (Chicopee Mills, New York) and the filtrate was centrifuged at 7,000g (6,750 rpm, Sorvall HB-4 rotor) for 15 min. The resulting supernatant was applied to a discontinuous gradient of sucrose in buffered coconut water (5) and centrifuged at 90,000g (26,000 rpm, Spinco SW 27.1 rotor) for 1 hr. Fractions were removed with a Pasteur pipette and pelleted at 90,000g for 45 min. Pellets from the 1.0/1.2 and 1.2/1.4 M sucrose interfaces of the gradients consisted of two distinct layers. The top layers rich in plasma membranes (Fig. 1) were removed by careful resuspension with a Pasteur pipette in 2 ml of a medium containing 0.25 M sucrose, 80 mM tris-acetate, 25 mM MES, and 14 mM mercaptoethanol, pH 6, with a final protein concentration of about 2.5 mg/ml. The bottom layers, rich in mitochondria and endoplasmic reticulum, were discarded. The above procedure results in a purer preparation than that used in the preceding paper. This was thought to be necessary for the fluorescence experiments which involved a membrane suspension.

For morphometric analyses of fraction composition, electron micrographs were printed at a final enlargement of 35,000 \times and examined under a transparent overlay bearing lines 1 cm apart (5). Intersections with different membrane types were counted. The isolated fractions contained 40 to 55% plasma membrane vesicles (Fig. 1) with mitochondria, endoplasmic reticulum, and unidentified membranes comprising the rest of the fraction. Measurements of membrane thickness were as described in the preceding report (7).

Fluorescence. Fluorescence polarization measurements were made with an instrument described previously (2, 4). The excitation light was defined by a Bausch and Lomb high intensity monochromator with UV grating and deuterium source, a Corning 7-39 filter, and a 363-nm interference filter. Emission was defined by a 2-mm-thick 2 M $NaNO_2$ liquid filter and a Corning 3-74 filter. The polarization parameter,

$$p = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}}$$

is defined relative to vertically polarized exciting light. The cuvette containing the membrane suspension was constantly stirred during the measurements using a magnetic stirring bar. *N*-Phenyl-1-naphthylamine (Eastman) was used without further purification.

The temperature of the sample cuvette was controlled by a Lauda K2-R circulator with proportioning control. A constant rate of 0.5 C/min change was maintained when polarization was measured as a function of increasing and decreasing temperature. Using a copper-constantan thermocouple immersed in the

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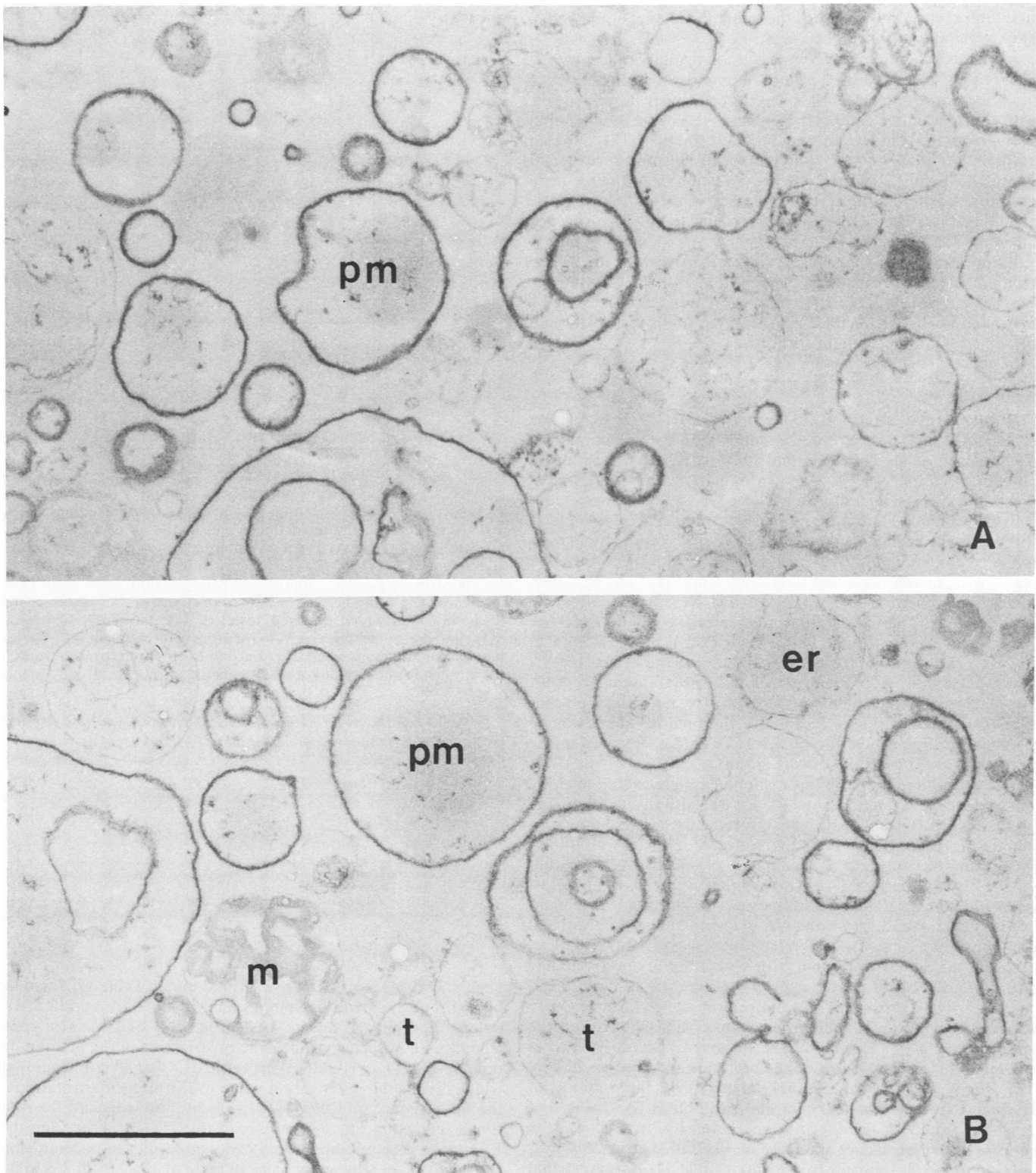


FIG. 1: Electron micrograph of isolated plasma membrane fraction from the 1.0/1.2 M (A) and 1.2/1.4 M (B) gradient interfaces stained by the procedure of Roland *et al.* (9). Plasma membrane vesicles (pm) stain intensely by this procedure. Mitochondria (m), endoplasmic reticulum (er) with attached ribosomes, and thick, unstained membranes resembling tonoplast (t) contaminate the fraction from the 1.2/1.4 M interface. The identity of the nonstaining vesicles in the fraction from the 1.0/1.2 M interface is unknown. Scale bar = 1 μm .

cuvette and Keithley 153-microvoltammeter, the sample temperature was measured continuously to an accuracy of 0.25 C.

N-phenyl-1-naphthylamine was added to a final concentration of 2 μM and the fluorescence polarization was measured as the temperature was varied reversibly between 25 to 10 C. IAA was

then added to a concentration of 1 μM and approximately 10 min allowed to elapse before the second temperature run was begun. This procedure was repeated three times on different membrane preparations with consistent results. Also, two control experiments were done in which no IAA was added after the

first temperature run. Instead, the membranes were subjected to another temperature run as a control.

Fluorescence lifetimes were measured in collaboration with J. Harris and F. Lytle of the Purdue Chemistry Department with a modified TRW nsec spectral source as previously described (6), with the same filter combinations employed in the polarization measurements. The measurements were made at 25 C to an accuracy of ± 0.5 nsec. The fluorescence intensities recorded at 25 and 17 C in the fluorescence polarization experiments were used to estimate the fluorescence lifetime in the sample at 17 C, assuming that the lifetime is proportional to the fluorescence intensity.

Fluorescence emission spectra were recorded with the instrument described previously (4), except that an EMI 9524A photomultiplier tube was used. The emission monochromator had a half-bandwidth of 4.8 nm, and the wavelength scale was calibrated with a mercury light source.

RESULTS

Within 5 min after the addition of $1 \mu\text{M}$ IAA to plasma membranes at 25 C, there was an increase in the polarization of fluorescence of membrane-bound PhNap.² The increase in polarization caused by IAA is shown by polarization values measured in the absence and presence of IAA (triangles) systematically as a function of temperature. Two similar experiments of this nature are shown in Figure 2, a and b. Control and hormone-treated membranes are each measured during consecutive scans with increasing (closed symbols) and decreasing (open symbols) temperature. The IAA scans were made after the control scans. The polarization increase after addition of IAA was largest between 12 and 22 C. In the ranges 8 to 12 C and 22 to 26 C, the results were more variable and the effect of IAA less pronounced. A control experiment in the absence of IAA was performed, in which four consecutive temperature scans similar to those of Figure 2, a and b were made. There was no significant difference in the polarization values obtained in the first and second pairs of scans (Fig. 2c).

The membrane preparations exhibited a high level of intrinsic fluorescence and the addition of $2 \mu\text{M}$ PhNap caused a doubling of the fluorescence signal. The fluorescence polarization of a membrane preparation in the absence of PhNap was 0.045, 0.047, and 0.049, at 25, 17, and 12 C, respectively, independent of the presence of IAA (experiment not shown). Fluorescence emission spectra and lifetimes were measured in the membranes with no addition, with IAA present, and with PhNap and IAA present (Table I). The membranes with IAA showed an emission maximum at 444 nm with a shoulder at approximately 520 nm; addition of PhNap caused the intensity to double and the major peak to shift to 430 nm. Fluorescence lifetimes were measured at 25 C in membranes alone, and then after the addition of PhNap and IAA. The lifetime appeared to have a similar value, 8 ± 0.5 nsec, in the presence and absence of dye. The lifetime was not measurably changed by the addition of IAA, as expected from the fact that addition of IAA caused at most a small ($\sim 2\%$) change in signal intensity. In addition, the ratio of the fluorescence intensity at 17 C to that at 25 C was approximately 1.1 for all treatments (Table I). Assuming that fluorescence lifetimes were proportional to fluorescence yields, the lifetime at 17 C was 8.8 nsec in the presence or absence of dye.

The presence of a high background level of fluorescence intensity means that the polarization value measured for the membrane bound dye is calculated from a difference of the weighted polarization functions $(1/\text{Pi}-1)^{-1}$, obtained in the pres-

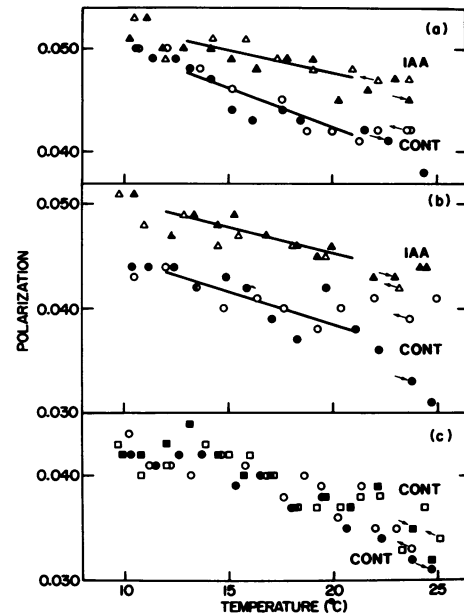


FIG. 2. Polarization of membrane-bound PhNap ($2 \mu\text{M}$) as a function of temperature. The polarization was measured with increasing (\bullet , \blacktriangle) and decreasing (\circ , \triangle) temperature and in the absence (\bullet , \circ) and presence (\blacktriangle , \triangle) of IAA. The temperature scan was started 10 min after addition of IAA. (a) and (b) are from two different preparations showing the approximate range of variation comparing auxin-treated ($1 \mu\text{M}$ IAA) and untreated membranes. (c) is a control preparation in which the temperature is lowered, elevated, lowered, and elevated in the manner of (a) and (b), but without IAA, to show the reversibility of the polarization increase due to temperature. Each of the four temperature scans in (a) through (c) took about one-half hour.

Table I. Fluorescence Characteristics of Soybean Hypocotyl Plasma Membranes with and without PhNap and IAA

Additions	Emission Maximum (a)	$I_{17^\circ}/I_{25^\circ}$ (b)	τ_{25°	τ_{17° (c)
none	—	1.1	8.0	8.8
$1 \mu\text{M}$ IAA	444 (520)	1.1	8.0	8.8
$2 \mu\text{M}$ PhNap	—	1.1	8.0	8.8
$2 \mu\text{M}$ PhNap + $1 \mu\text{M}$ IAA	430 (520)	1.1	8.0	8.8

^aNumber in parentheses indicates approximate position of a shoulder in the emission spectrum.

^b I_T is the fluorescence intensity at $T^\circ\text{C}$; addition of PhNap to the membranes caused a two-fold increase in the fluorescence intensity

^cAssuming $\frac{I_{17^\circ}}{I_{25^\circ}} = \frac{\tau_{17^\circ}}{\tau_{25^\circ}}$

ence and absence of dye as described under Table II. Since the background polarization is not changed by IAA addition, the polarization change due to IAA (Fig. 2, a and b) must come from the fluorescence probe. The polarization of the membrane-bound dye, (P_D) is shown in Table II. The Perrin equation, which relates the polarization value to the molecular rotation of the fluorescence molecules (10), was used to calculate the rotational relaxation time (ρ) in nsec (Table II). It can be seen that IAA causes the motion of the PhNap molecules to become more restricted as seen by an increase in the ρ at 17 C from 2.8 to 3.6 nsec (data from Fig. 2a and Table II) and from 2.3 to 3.3 nsec (data from Fig. 2b and Table II). These calculated ρ values should be considered as a qualitative evidence for a significant change in the environment of the probe molecules, although the absolute values for the ρ are probably not correct because of the approximate nature of the calculations.

When stained by the PACP procedure (9), the isolated plasma membranes in these studies were thinner when treated for 20 min with $1 \mu\text{M}$ IAA than comparable control preparations (Table III). The control membranes showed a distribution of mem-

² Abbreviations: ρ : rotational relaxation time; PhNap: *N*-phenyl-1-naphthylamine. Other abbreviations are explained with the tables.

Table II. Corrected Polarization Values (P_D) for Membrane-bound PhNap Dye and Calculated Rotational Relaxation Times (ρ) of Bound Dye

Conditions	P_{MD}	P_M	α	P_D	ρ (nsec)
Fig. 2a: 17°; - IAA	0.044	0.047	0.5	0.041	2.8
Fig. 2a: 17°; +IAA (1 μ M)	0.049	0.047	0.5	0.051	3.6
Fig. 2b: 17°; -IAA	0.041	0.047	0.5	0.035	2.3
Fig. 2b: 17°; +IAA (1 μ M)	0.047	0.047	0.5	0.047	3.3

$$a \left(\frac{1}{P_{MD}} - \frac{1}{3} \right) = \frac{1}{\alpha \left(\frac{1}{P_M} - \frac{1}{3} \right)^{-1} + (1-\alpha) \left(\frac{1}{P_D} - \frac{1}{3} \right)^{-1}}$$

where P_{MD} = measured polarization

obtained from the intrinsic emission of membranes plus that of the dye; P_M = measured polarization from the intrinsic emission of membranes alone; P_D = calculated polarization of bound dye alone; α = ratio of the total fluorescence intensity of the membranes alone in the absence of the dye to that measured in the presence of dye (10).

^bFrom the Perrin equation (10), substituting values of P_D , limiting polarization of $P_0=0.38(4)$, and lifetime at 17°=8.8nsec.

brane thickness with a mean of about 100 Å. The IAA-treated membranes were significantly thinner with a mean of about 90 Å. These findings show that the plasma membrane fragments in the preparations responded to the hormone in terms of changes in membrane thickness in a manner similar to that shown in the preceding report (7).

DISCUSSION

The increase in fluorescence polarization of PhNap resulting from IAA-treatment of plasma membranes of soybean hypocotyl is interpreted in terms of an increase in membrane microviscosity since: (a) the probe is located in the hydrocarbon region of the membranes (1, 8); and (b) fluorescence polarization is a measure of the rotational mode of the probe if the fluorescence lifetime and ρ are similar. The calculated ρ value of the PhNap molecules increased due to IAA-treatment (Table II), indicating a more restricted environment of higher microviscosity in the membranes. These ρ values are approximate for the following reasons: the emission spectrum from membranes without PhNap shows the presence of significant quantities of 444-nm and 520-nm emitting species, which may be NADH and possibly flavin. Since flavins have a strong absorption peak at 450 nm, they could accept energy from PhNap through a resonance transfer mechanism. In general, energy transfer and subsequent reemission tend to depolarize and alter the lifetime of the polarized emission under consideration. It should also be pointed out that the Perrin equation is valid only for spherical molecules, so that with the more plate-like PhNap molecules, the calculated ρ values are approximate.

As pointed out in a previous application of these fluorescence probe techniques (4), the increase in apparent microviscosity could result either from: (a) a real increase in microviscosity of the isolated membranes; or (b) from redistribution of dye molecules within the membrane. The second interpretation would

Table III. Effect of a 20-Min Incubation with Water or 1 μ M IAA in Water on Thickness of Plasma Membranes of Soybean Hypocotyl *In Vitro*

Plasma membrane fractions were isolated and prepared in a manner identical to those for the fluorescence polarization measurements. Results are from measurements from 10 different membrane preparations. The membranes were stained and identified by the PACP procedure of Roland *et al.* (9).

Treatment	Membrane thickness $\bar{X} \pm$ standard deviation
None	106 \pm 3
Control	102 \pm 3
Auxin	91 \pm 5

also account for our findings. Either interpretation would be consistent with the conformational change in the membrane measured by electron microscopy. Thinning of the membrane might: (a) result in closer packing of lipid molecules in the membrane interior—a more ordered and space-saving arrangement of lipid chains with a corresponding increase in microviscosity; or (b) result in a reorientation of the dye molecules from regions of lower microviscosity to regions of higher microviscosity. Regarding possible mechanisms of hormone action, these experiments provide evidence independent of electron microscopy for a cell-free response of isolated plasma membranes to the hormone IAA.

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