Induction of “Pore” Formation in Plant Cell Membranes by Toluene

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HENRI R. LERNER, DAVID BEN-BASSAT, LEONORA REINHOLD, AND ALEXANDRA POLIJKOFF-MAYBER
Department of Botany, The Hebrew University, Jerusalem, Israel

ABSTRACT

Treatment with aqueous toluene-ethanol has been shown to induce “pore” formation in plant cell membranes. The evidence is as follows:

1. Toluene treatment induced preloaded vital dyes to leak from cells as observed under the microscope. It also brought about inward diffusion of 1-anilinonaphthalene-8-sulfonate.

2. Compartmental analysis indicated that the space within the tissue accessible to the free diffusion of a molecule of the size of thiourea was increased about 3-fold by the toluene treatment.

3. Gel filtration on Sephadex G-25 showed that a substantial fraction of the low molecular weight cellular solutes leaked out as a result of toluene treatment, while no leakage of macromolecules (proteins) could be detected.

4. The results with gel filtration, as well as in situ measurements of enzymic reactions, indicated that the pores induced by toluene in the cytoplasmic membranes were smaller than the Stokes’ radius nicotinamide adenine dinucleotide.

While the principal experimental material was roots of Atriplex nummularia Lindl., the fact that similar results were also observed with leaves of Pisum sativum L. and with the alga Chlorella pyrenoidosa ChlK. suggests that the phenomenon is general.

Although the phenomenon of pore induction is qualitatively similar to that in microorganisms, the pores induced appear to be smaller. It is proposed that induced leakage could be the basis for the development of simple and rapid methods for plant biochemical studies.

MATERIALS AND METHODS

The plant material used in the experiments was mainly excised roots of Atriplex nummularia Lindl. When other plant material was used it is stated in the text. Atriplex and Phaseolus vulgaris L. were grown in aerated half-strength Hoagland solution.

The procedure for leak induction by toluene-ethanol was essentially similar to that described by Serrano et al. (16). The toluene stock solution was made of 1 volume of toluene to 4 volumes of ethanol. One volume of the stock solution was added with stirring to 20 volumes of distilled H2O at room temperature. Roots were incubated for 5 min in this medium.

Preloading of the cytoplasm with fluorescein was achieved by incubating the roots for 10 min at room temperature in a 10 mm solution of KH2PO4 containing 1 mg of fluorescein/10 ml.

Loading with ANS2 was achieved by incubating the roots for 10 min at room temperature in a 50 μm solution of ANS brought to pH 6 by addition of dilute NaOH containing 1% toluene and 4% ethanol.

Cytoplasmic fluorescence induced by either fluorescein or ANS was observed with a Leitz Ortholux fluorescence microscope using transmission filters BG38 (4 mm) plus BG12 (3 mm) and suppression filter K530.

Preloading of the vacuoles with neutral red was achieved by incubating the roots in an aqueous solution of the dye (1 mg/10 ml). Potassium phosphate buffer (pH 7.5) was then added to a final concentration of 10 mm and incubation continued for 1 hr. Although neutral red precipitates at this pH, the non-ionized species which is formed is lipid-soluble and penetrates the cellular membranes. After incubation the roots were rinsed several times in distilled H2O.

The procedure for compartment analysis was essentially similar to that described by Glinka (3). Course of influx of 14C-thiourea into Atriplex root at room temperature was found to be considerably faster than into carrot root. After 1 hr the labeled thiourea had reached equilibrium with the tissue. Atriplex roots were therefore loaded by incubation in 50 mm 14C-thiourea for 1 hr at room temperature. Efflux was measured by transferring the roots, in a holder, sequentially through a series of vials containing 6 ml of 50 mm nonradioactive thiourea. The vials were shaken constantly and were maintained at 4°C to slow down the process. During the first 2 min the roots were transferred every 15 sec to wash off the radioactivity adhering to the holder and to the surface of the roots. Subsequent efflux periods were progressively longer and continued for 3 hr. Aliquots from each vial, as well as the root tissue itself at the conclusion of the experiment, were added to Instagel and counted.  

Biochemical studies of cells provided with hard outer walls are hindered by the technical difficulties involved in homogenization. In the case of microorganisms a way of bypassing these difficulties has been developed. The formation of small holes is induced in the cell membrane by suitable techniques, the simplest being treatment with toluene (2, 5–7, 10, 12, 14–16, 19, 20). This treatment is particularly mild: low mol wt cellular solutes are induced to leak out without the general structural organization of the cell being disturbed.

Similar techniques hitherto have not been applied in studies of plant cells. The method could, however, be of considerable potential usefulness since, apart from allowing in situ measurements of intracellular enzyme activities, it could permit the rapid analysis of intracellular solutes.

In this report we describe the induction of leakiness in the cells of various plant tissues by toluene treatment and present information concerning the size of the pore induced.

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2 Abbreviations: ANS: 1-anilinonaphthalene-8-sulfonate; TNS: 2-p-toluidinynaphthalene-6-sulfonate; PMS: phenazine methosulfate; MTT: 3-(4,5′-dimethylthiazol-2-yl)-2,4-diphenyltetrazolium bromide; O.D.: change in optical density per minute.
counted in a Packard liquid scintillation spectrometer. The curve relating log radioactivity remaining in the root to time may be resolved into a series of straight lines (see, e.g. ref. 3). The slope of a line indicates the rate constant for efflux from a cellular compartment while the relative volume of the compartment may be calculated from the intercept of the line extrapolated to zero time. In order to obtain from the intercept the approximate per cent of the total volume of the tissue occupied by each compartment

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\log \left( \frac{\text{dpm per mg of root}}{\text{dpm per mm}^3 \text{ of incubation solution}} \right) \times 100
\]

was plotted on the vertical axis.

Separation of solutes according to size was achieved on a Sephadex G-25 column, 2.5 cm in diameter and about 35 cm in height. A peristaltic pump controlled the rate of flow which was between 1 and 2 ml/min. The eluant was passed through a 10-mm path flow cell (Gilford model 203 or Sturmy type 44) which was placed in a Cary 15 spectrophotometer, recording O.D. at 198 nm against time. When required a fraction collector was placed so as to receive 2-3 min fractions of elution liquid flowing out of the cell.

Certain molecules, for example α-chymotrypsin, remain absorbed on Sephadex G-25 when filtered in the absence of buffer. Addition of buffer prevents absorption but considerably increased the O.D. of the background during the recording of the elution profile since mineral as well as organic molecules absorb at 198 nm. The supernatant of crude Atriplex root homogenate did not contain discernible concentrations of molecules requiring the addition of buffer, and elution of columns has been carried out using distilled H2O.

Root homogenate was prepared by cutting the roots into small pieces in distilled H2O followed by hand grinding with a glass homogenizer. The homogenate was centrifuged 15 min at 15,000g and the supernatant collected.

Protein was measured according to the method of Lowry et al. (8). TLC of the leaked solutes was performed on silica gel using the solvent: 96% ethanol-water-25% ammonium hydroxide (78 + 9.5 + 12.5). Organic acids were detected with bromocresol green (reagent 25 in ref. 18); sugars with anisaldehyde-sulfuric acid (reagent 11 in ref. 18); and amino acids with ninhydrin (reagent F78 in ref. 18).

The in situ enzymatic activities of phosphatase, alcohol dehydrogenase as well as the oxidation of NADH were measured by adapting to plant tissues methods similar to those described by Bergmeyer (1). Root samples were immersed in the reaction medium contained in a reaction vessel which was maintained at 28°C. The reaction medium was continuously circulated (in a closed cycle) through a flow cell placed in a Gilford spectrophotometer, which recorded O.D. against time at the appropriate wavelength.

All compounds used were of the purest grade commercially available. PMS, MTT, NAD, NADH and p-nitrophenyl phosphate were obtained from Sigma; Instagel from Packard; Sephadex G-25 from Pharmacia; ANS from Kodak; purified Triton X-100 from Koch-Light; the vital dyes fluorescein and neutral red from National Aniline.

RESULTS AND DISCUSSION

Microscopic Observation of Toluene-induced Membrane Leakage. Incubation of Atriplex root for 5 min in toluene-ethanol caused the rapid loss of preloaded neutral red from the vacuoles of all the cells, while preloaded fluorescein was slowly lost by most of these cells, a few root cells remaining fluorescent.

Plasmalemma leakage may also be made apparent under the fluorescent microscope by following the entry into the cell of a compound such as ANS or TNS which does not permeate through undamaged membranes and which fluoresces when dissolved in the hydrophobic regions of certain proteins (9, 13). Incubation of an Atriplex root for 10 min in toluene-ethanol containing 50 μM ANS caused the cells to become fluorescent, while control roots in 50 μM ANS did not show any fluorescence.

Compartment Analysis of Toluene-treated Cells. That the volume of “free space” of the tissue, i.e. the space freely accessible to small molecules by diffusion, is greatly enlarged by toluene treatment has been demonstrated by the technique of compartment analysis.

For this purpose we have used the nonmetabolized solute, thiourea, which reaches diffusion equilibrium with plant tissues (3). After preloading Atriplex roots with 3H-thiourea the curve for efflux under the standard conditions of compartment analysis was clearly resolvable into at least three linear components. Table 1 sets out the half-times for efflux from the various compartments as well as their relative volumes as estimated from intercepts on the vertical axis (see under “Materials and Methods”). It will be seen that the half-times were not greatly altered by the toluene treatment: the most marked effect of the latter was on the relative volumes. The “slowest compartment,” which is likely to have been the vacuole, was reduced by about two-thirds by the 5-min toluene treatment. The somewhat larger effect of the continuous toluene incubation is probably due to the nonuniform population of root cells—a certain number of “resistant” cells apparently responded to more prolonged treatment. On the other hand the free space, estimated as the difference between 100% volume and the sum of the percentage volumes of the slow and intermediate compartments, was enlarged nearly 3-fold. Toluene treatment had thus substantially reduced the “osmotic volume” of the tissue for a molecule the size of thiourea; this molecule could now readily diffuse through most of the tissue.

Gel Filtration of Leaked Solutes. The cellular solutes leaked by the toluene-ethanol treatment from Atriplex roots were visualized by means of their UV spectra, after lyophilization of the incubation medium in order to eliminate the toluene and the ethanol. The leaked solutes showed an increase in O.D. as wavelength was decreased from 230 to 200 nm (Fig. 1). The spectrum of the supernatant of crude Atriplex root homogenate in distilled H2O (Fig. 1) is similar to that of the leaked solutes except for an additional shoulder of low O.D. in the 290 to 250 nm region.
We have exploited the fact that all cellular solutes absorb at 198 nm to monitor the separation of the high and low mol wt solutes during gel filtration.

The Sephadex G-25 column was calibrated by percolating, in distilled H₂O, a mixture containing BSA, glycerol, and tryptophan (Fig. 2). The BSA peak indicates the void volume, while the glycerol peak indicates the total volume. The position of the tryptophan peak demonstrates how solutes may be retarded on the column owing to interactions with the gel. It is therefore not possible to determine the Stokes radii of solutes unless it is certain that there are no gel-solute interactions.

The profile of the G-25 filtration of the supernatant of crude root homogenate, Figure 3A, shows two maxima, one around the void volume, the other about 5-fold greater around the total volume. A shoulder between these two peaks is also present. Figure 4 shows the UV spectra of these two peaks. The shoulder between 290 and 250 nm in Figure 1 is seen in Figure 4 to be due to the macromolecules, while the high absorption below 240 nm is mainly attributable to the low mol wt solutes.

The solutes retained in Atriplex roots after toluene-ethanol treatment were examined by gel filtration of the supernatant of root homogenates. The Sephadex G-25 profile showed two peaks (Fig. 3B), but the peak for the low mol wt solutes was diminished, now being of approximately twice the height of the peak for the macromolecules.

The gel filtration profile of solutes leaked from Atriplex root by toluene-ethanol treatment shows, after lyophilization, only the low mol wt solute peak (Fig. 5).

Colorimetric assays which allow more sensitive detection of protein were performed on the macromolecular fractions coming off the column. These assays confirmed that no protein leaked out of the cells as a result of the treatment. TLC indicated that sugars, amino acids, and short chain organic acids were among the leaked solutes.

**Fig. 1.** UV spectra of Atriplex root solutes. Δ--Δ: lyophilized solutes leaked by toluene-ethanol treatment; O--O: supernatant from roots homogenized in distilled water. 1.25 mg root/ml in both cases.

**Fig. 2.** Sephadex G-25 elution profile at 198 nm of 100 μg of BSA, 40 mg of glycerol, and 100 μg of tryptophan. For details see under "Materials and Methods."

**Fig. 3.** Sephadex G-25 profile at 198 nm of supernatant from Atriplex root homogenates passing through a 5-mm path flow cell. A: Supernatant after centrifugation of homogenate of 500 mg of root tissue at 15,000g; B: supernatant as above from homogenate of 500 mg of roots which had been previously incubated 5 min in 1% toluene-4% ethanol at room temperature and thoroughly rinsed in distilled water. O.D. in B have been multiplied by 0.85 to normalize the peak of the macromolecules to the same value as in A.

**Fig. 4.** UV spectra of fractions from gel filtration on Sephadex G-25 of supernatant from Atriplex root homogenate (see Fig. 3A) in 10-mm path cuvette. O--O: spectrum of the peak of the macromolecular solutes; Δ--Δ: spectrum of the peak of the low mol wt solutes.

**Fig. 5.** Sephadex G-25 elution profile at 198 nm of lyophilized solutes leaked from 2.8 g of Atriplex roots by toluene-ethanol treatment. A relatively large amount of root tissue was used in this experiment in order to detect any possible leakage of macromolecular solutes.

Some experiments were also carried out on the leaves of *Pisum sativum* L. and the alga *Chlorella pyrenoidosa* Chik. Gel filtration of the lyophilized medium after incubation in toluene-ethanol showed in both of these cases that only low mol wt solutes had leaked out, indicating that a wide range of plant cells may be affected in a similar manner by this treatment.

**In Situ Enzyme Measurements.** In the case of microorganisms, reactions catalyzed by enzymes located in the cytoplasm have been studied, after toluene treatment, by the diffusion of substrates into the plant cells and concomitant spectrophoto-
metric measurement of the rate of formation of the products diffusing out. We have examined whether substrates may similarly diffuse through the pores produced in the cellular membranes of plant roots by toluene treatment.

For this purpose it was useful to have an estimate of the total cellular activity of the enzymes being measured. Triton X-100 is known to dissolve membrane components while usually not inhibiting enzymes (4, 11, 17); and indeed intracellular enzymes in Atriplex roots are exposed by Triton X-100. Figure 6 shows the progressive exposure of intracellular phosphatases in Atriplex roots with increasing concentration of Triton X-100. Further increase in Triton X-100 concentration, above 0.1%, did not increase activity appreciably. Triton X-100 at 0.1% was therefore added routinely to the reaction vessel after toluene-ethanol in order to determine the total cellular activity of the enzymes being measured.

It should be pointed out that the enzymic activities measured in the presence of 0.1% Triton X-100 are not in situ and are not comparable with the enzymic measurements made in microorganisms after treatment with toluene. While toluene induces the formation of small pores through membranes, Triton X-100 dismantles the membranes by forming mixed micelles with hydrophobic membranal components thereby liberating in the incubation medium low mol wt as well as macromolecular cellular solutes. However, the method using 0.1% Triton X-100, described in this paper, provides a simple and rapid way of determining enzymic activities in plant cells.

Table II shows phosphatase activity measured in situ in Atriplex and bean roots using p-nitrophenyl phosphate as substrate. Toluene treatment increased the hydrolysis 2- to 3-fold. In the case of Atriplex root it appeared that all of the phosphatases in the cells had been made accessible, as indicated by the lack of further effect of Triton X-100. In bean, on the other hand, approximately 60% of the intracellular phosphatases had apparently been made accessible by toluene treatment.

Table III shows the oxidation of NADH measured in situ and Atriplex roots. While it is evident that NADH is oxidized by the addition of untreated root to the reaction vessel, toluene-ethanol doubles the oxidation rate. Addition of 0.1% Triton X-100 further increases the rate of oxidation.

In the case of alcohol dehydrogenase (Table IV) apparently only the addition of 0.1% Triton X-100 made the enzyme accessible to its substrate NAD.

With regard to the phosphatase two interpretations of the results would be possible: either the increase in rate produced by the addition of toluene-ethanol is due to increased accessibility of an intracellular enzyme to its substrate, or it is due to the activation of the surface enzyme. However, the second explanation is not applicable in the case of alcohol dehydrogenase which is known to be a soluble cytoplasmic enzyme. The lack of activity displayed by the toluene-treated roots indicates that the pores made were not large enough to admit NAD to the site of the enzyme. The fact that enhanced NADH oxidation was observed after toluene treatment suggests that alcohol dehydrogenase may be compartmented differently from the NADH-oxidizing system exposed by the toluene treatment.

**CONCLUSIONS**

From the observations reported above it is evident that toluene induced pore formation in membranes of higher plant cells in a manner qualitatively similar to that reported for microorganisms. Not much information is available concerning the size of the pores induced in the membranes of microorganisms. The present work suggests that the pores produced in the membranes of Atriplex roots were probably in the range of 0.5 to 0.7 nm in radius since sugars, amino acids, and short chain organic acids could diffuse out relatively freely, whereas NAD was not able to reach the site of alcohol dehydrogenase. The pores induced in the membranes of microorganisms would appear to be larger since NAD passes freely (16, 19, 20). The slow outward diffusion of fluorescein from the cytoplasm observed under the fluorescence microscope is in accordance with the conclusion that the pores induced are not much larger than the Stokes radii of this molecule. There are two possible interpretations for the rapid leakage of neutral red from the vacuoles following the toluene treatment as compared with the

![Image](image_url)
slow leakage of fluorescein from the cytoplasmic compartment. Either larger pores are formed in the tonoplast than in the multimembranes separating the subcompartments of the cytoplasm; or leakage is the result of deprotonation of neutral red following the pH increase in the vacuole which results from leakage of hydrated protons. It will be remembered that it is the pH profile of the cell (neutral cytoplasmic compartment and acidic vacuole) which is responsible for the accumulation of fluorescein and neutral red in specific cellular compartments.

In spite of the quantitative difference in the size of the pores induced, the qualitative similarity in the response of the two types of cells suggests that induced leakage could be the basis for the development of simple and rapid methods for plant biochemical studies as it has in microbiology. An additional advantage might accrue in the case of higher plant cells: if differential leakage of the various cellular membranes could be achieved, the method might yield valuable information with regard to the location within the various cellular compartments of particular solute pools, enzymes, and enzyme substrates.

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LITERATURE CITED

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