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Kinetics of Toluene-Induced Leakage of Low Molecular Weight Solutes from Excised Sorghum Tissues[1]

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ABSTRACT

The relationship between toluene concentration and the rate of leakage of solutes from toluene-treated roots and leaves of *Sorghum bicolor*, L. Moench, was studied to determine the effect of toluene on plant cell membranes. A threshold concentration of 0.2% toluene was needed to induce leakage. Maximal leakage rates were obtained with 0.5% toluene. Low molecular weight solutes, such as amino acids, sugars, and inorganic ions, leaked from treated tissue, while macromolecules, such as protein were retained. The rates at which the low molecular weight solutes diffused from treated cells decreased with increasing molecular weight. At 25°C, treatment of roots and leaves with 0.5% toluene resulted in the quasi-quantitative leakage of solutes within 180 minutes. At 1°C, roots and leaves differed in their response to toluene. The rates of leakage from roots at 1°C were much lower and the total amounts much smaller than at 25°C, while in leaves the difference between the two temperatures was very small.

The procedure of treating tissues with 0.5% toluene for 180 minutes at 25°C proved to be a rapid and simple technique for quantitative extraction of water-soluble, low molecular weight solutes from plant cells into the extracting medium while macromolecular constituents are retained inside the cells.

Treatment of living cells with aqueous toluene to induce pore formation and leakage has been used successfully with microorganisms for over two decades (1–3, 6, 8, 13–17, 19, 22, 26). This method was also successfully applied to liver cells (5) and to mitochondria (10). In all cases, the leakage of water-soluble low mol wt solutes was achieved, while macromolecules were apparently retained within the cell membrane. Although toluene treatment destroyed selective permeability of membranes, it did not seem to cause a general structural breakdown. The holes or pores formed in the membranes by the treatment were of limited size so that membranes acted as sieves. Not only did low mol wt solutes diffuse out of the cells, but other compounds could be introduced into the cells to serve as substrates for *in situ* enzymic reactions. Lerner *et al.* (7) have applied the toluene treatment method to plant roots. From their experiments they concluded that the size of the pores formed by toluene in *Atriplex* root cell membranes are smaller than those formed in membranes of microbial or mammalian cells. The mechanism for this difference in the degree of response of plant membranes from those of micro-organisms or liver cells is unknown, but it may be a function of the chemical composition of the membrane (20).

To determine whether there was any difference in the response of various plant tissues to toluene extraction, the leakage of various solutes from roots and leaves of *Sorghum bicolor* was followed as a function of time of extraction. Such a difference could be expected if the composition of the cellular membranes of roots and leaves is not identical. Also, an attempt was made to correlate the rate of extraction of several solutes with their molecular size or charge.

MATERIALS AND METHODS

Plant Material. Seeds of *S. bicolor*, Hazera 610, were spread on moist cheesecloth supported by a rigid plastic screen and incubated at room temperature over water in a closed chamber so that the RH of the air approached 100%. In 5 days, when the roots were about 5 cm long, the seedlings with the cheesecloth and plastic screen were placed over 8 L aerated 1:2 dilution of Hoagland solution with about 2.5 cm or more of the roots immersed in the liquid. The plants were allowed to grow in a greenhouse. Any liquid lost from the growth medium was replaced with water to keep the volume relatively constant at 8 L. When the plants were 14 to 18 days old, they were removed from the growth medium and the tissues used for the experiments.

Roots were cut off about 1 cm below the cheesecloth, washed, and carefully blotted dry. The youngest leaf blades were cut from the plants in lengths of 10 cm. Care was taken not to damage the root or leaf tissues anywhere but at the cut edges.

Time Course Leakage Experiments. In the experiments in which the effect of concentration of toluene on solute leakage was measured, 4 g tissue was placed in plastic vessels containing 100 ml H_2O plus increasing amounts of toluene from 0 to 2 ml in increments of 0.2 ml, and also in amounts of 3 and 10 ml. In the following, for convenience, we will refer in the text to toluene content as percentage (v/v), an expression used by microbiologists. In the kinetic experiments, the conditions were the same except that only 1 ml toluene was added to all reaction mixtures. The vessels were large enough to hold the tissue without mutilation by creasing or folding. The reaction mixture was equilibrated to the temperature desired (either 1°C or 25°C) for a particular experiment before adding tissue. The vessels were placed on a reciprocal shaker and shaken gently enough to avoid splashing, but rapidly enough to ensure aeration and to keep leaked solutes well mixed. Volumes of 5 ml were removed from reaction mixtures at intervals between 1 and 180 min from the beginning of the experiment. These aliquots were used for the assays for solutes (to be described later). In calculating the amounts of solutes leaked, corrections were made to compensate for the reduction in volume of the reaction mixture as the experiment proceeded while the fresh weight of tissue remained constant. After 180 min, the tissues were removed from the reaction mixtures, washed in cold water, and then placed in 25 ml boiling water for 10 min. The liquid extract

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was decanted and the boiling water extraction of tissue repeated. The two boiling water extracts were pooled, concentrated under vacuum to 10 ml, and assayed for solutes.

In early experiments, concentrations of ethanol ranging from 0 to 10% in water were included in reaction mixtures with and without 1% toluene. However, ethanol was omitted in subsequent experiments when it became evident that it had no effect on leakage.

Quantitative Leakage Experiments. Two g tissue was placed in 50 ml water plus 0.5 ml toluene at 25°C. The reaction mixture was shaken for 60 min. The liquid was decanted and saved. This procedure was repeated two more times. The liquid reaction mixtures were pooled, filtered through ashless filter paper (Whatman 42) supported by a plastic funnel, diluted to 150 ml with water, and used for solute assays.

As controls, the same amount of fresh tissue was extracted with either 25 ml boiling water or 25 ml boiling 80% ethanol for 10 min. The liquid was decanted and the extraction procedure repeated two more times. The three hot water extracts of each tissue were pooled, filtered, and diluted to 75 ml. The three hot ethanol extracts of each tissue were pooled and dried under vacuum. The residue was dissolved or suspended in 75 ml H_2O and filtered.

Extraction of Macromolecules. Fresh and toluene-treated tissues were extracted in a cold Duell conical tissue homogenizer as described previously (25).

Chemical and Enzymic Assays. Potassium ion concentrations were determined by flame photometry; phosphate, by reaction with molybdate (23); sugars, with anthrone (4), and amino acids, by their reactions with ninhydrin (21). Protein was measured, after precipitation from reaction mixtures with $HClO_4$ and after the precipitate was washed with alcohol (24), by the Lowry method (9). Glucose-6-P dehydrogenase and glutamate dehydrogenase activities were measured optically by following reduction of NADP and oxidation of NADH at 340 nm, respectively (24). Alkaline phosphatase was assayed by measuring the rate at which Na pyrophosphate was hydrolyzed to inorganic phosphate at pH 8.5 (25).

RESULTS

Effect of Ethanol on Toluene-Induced Leakage. The claim that ethanol had an enhancing effect on toluene-induced leakage of solutes from yeasts (19) and *Atriplex* roots (7) was examined. It was found that the rates of leakage and the quantities of solutes leaked from sorghum tissues in the presence of 1% toluene were the same whether or not ethanol, ranging in concentration from 0 to 10%, was included in the reaction mixtures. Treatment of tissue with ethanol at the same concentrations in the absence of toluene did not induce any leakage from tissue. Therefore, ethanol was not used in further experiments.

Effect of Toluene Concentration on Leakage of Endogenous Solutes. To determine the minimal and optimal concentrations of toluene needed for effective extraction of solutes, roots and leaves of sorghum were treated with various concentrations of toluene. The results of a representative experiment showing the leakage of K^+, Pi, amino acids, and sugars are shown in Figure 1. The data for two other experiments (but with different intervals between the several toluene concentrations used) are presented in Table I. No leakage of solutes occurred before toluene concentration reached a threshold level of 0.2 to 0.3%. Above this amount of toluene, rates of leakage increased markedly with increasing toluene up to 0.5 to 1%. Above 1%, the effect of toluene on the roots and leaves differed somewhat. In leaves, the rates of leakage decreased with increasing toluene concentrations above 1%. At 10% toluene, the decrease in rate of leakage from leaves was so marked that there was actually a lag of approximately 30 min before leakage began (data not included). In roots, on the other hand, the initial rates of leakage were relatively constant or

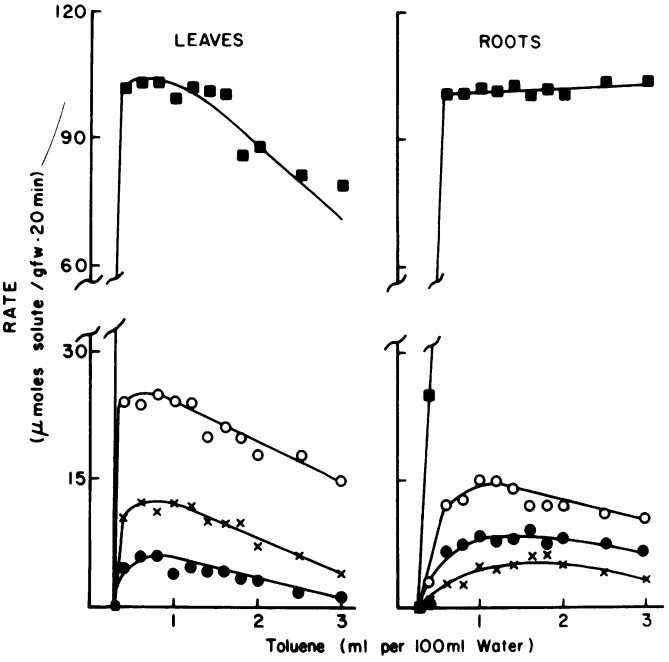

FIG. 1. Leakage of potassium, phosphate, amino acids, and sugars from sorghum roots and leaves at 25°C as a function of toluene concentration. Sorghum roots and leaves were incubated for 20 min at various toluene concentrations. Leaked solutes were measured in the supernatant (■), potassium; (×), phosphate; (●), amino acids; (○), sugars. Results are expressed as average rates for 20 min.

decreased slightly from 0.5 to 3% toluene. At 10% toluene, rates of solute leakage were depressed by only 5 to 10% of the rates at 3% (data not shown).

Kinetics of Leakage Induced by Toluene. Figures 2 and 3 show the amounts of the four solutes leaked as a function of time from tissues exposed to 1% toluene at 25°C and 1°C. At both temperatures, maximal rates of leakage were reached in less than 1 min incubation. The only exception was the leakage of Pi from leaves at 1°C where there appeared to be a slight, but measurable, lag. At 25°C, about 90% total amount of extractable solutes was leaked within the first 60 min. The rest leaked out slowly in the subsequent 120 min incubation. Tissues heated in boiling water after 180 min toluene treatment released little or no additional solutes. The $t_{1/2}$ values (the time required for one-half of the total amount of a particular solute in the tissue to leak out) were 7.5, 19, 20, and 26 min for K^+, Pi, amino acids, and sugars, respectively, for leaves, and 10, 11, 13, and 13 min, respectively, for roots.

Incubation at 1°C reduced the rates of leakage and reduced the total amounts leaked from roots much more than from leaves on a percentage basis. In leaves, the rates were only slightly less than the rates at 25°C with $t_{1/2}$ values of 10, 23, 22, and 27 min for K^+, Pi, amino acids, and sugars, respectively, and total amounts leaked in 180 min were less than 10% lower. In roots, however, the rates of leakage at 1°C were less by values ranging from 35% for K^+ to 63% for sugars. The total amounts that leaked at 1°C were considerably less than those at 25°C. The amounts of solutes leaked from roots during 180 min treatment at 1°C as compared with treatment at 25°C were: K^+, 76%; Pi, 47%; amino acids, 47%; and sugars, 44%. Continued incubation of roots in reaction mixtures beyond 180 min did not significantly change the total amounts leaked. Also, increasing the concentration of toluene to 3% did not change the pattern of solute leakage from roots from that shown in Figure 2 except that approximately 5% more total solute leaked from the tissue. Higher toluene concentrations, therefore, were not tested.

Table I. *Replicate Experiments for Determining Rate of Leakage of Solutes from Sorghum Roots and Leaves at 25°C as a Function of Toluene Concentrations*

Conditions same as Figure 1.

	Amount Toluene	Potassium		Phosphate		Amino Acids		Sugars	
		Exp. I	Exp. II	Exp. I	Exp. II	Exp. I	Exp. II	Exp. I	Exp. II
	ml	*μmol/g fresh wt·20 min*							
Roots	0		0		0		0		0
	0.2	0		0		0		0	
	0.3		30.8		1.0		1.1		1.8
	0.4	25		0.5		1.5		3.0	
	0.5		76		5.0		7.4		3.4
	0.6	84		3.3		6.8		12.0	
	1.0	91	81	8.2	8.8	8.4	10.3	15.1	16.7
	2.0	84		8.7		10.5		11.8	
	3.0		80		8.5		9.3		15.3
Leaves	0		0		0		0		0
	0.2	0		0		0		0	
	0.3		69.5		9.7		2.9		20.5
	0.4	108		10.3		9.5		24.5	
	0.5		87		14.4		11.6		22.6
	0.6	108		12.2		9.9		23.8	
	1.0	97	86.5	18.5	16.6	10.0	11.6	24.0	22.6
	2.0	80		8.4		5.3		16.0	
	3.0		58.3		6.3		3.3		11.0

Table II. *Comparison of Amounts of Solutes Extracted from Roots and Leaves of Sorghum Seedlings by Various Methods*

For detail see "Materials and Methods."

Extraction Procedure	Potassium	Phosphate	Amino Acids[a]	Sugars[a]
	μmol fresh wt/g			
Roots				
1% Toluene	75.1 ± 13.9[b]	17.4 ± 0.4	13.4 ± 1.3	15.5 ± 1.5
Hot water	73.9 ± 15.9	17.4 ± 0	13.6 ± 2.5	18.7 ± 0.7
Hot 80% ethanol	66.8 ± 18.8	17.7 ± 0.3	12.5 ± 1.7	22.6 ± 0.2
Leaves				
1% toluene	111 ± 26.1	41.7 ± 4.0	36.5 ± 9.8	61.8 ± 7.6
Hot water	111 ± 32.4	42.3 ± 8.7	32.4 ± 7.6	57.0 ± 6.3
Hot 80% ethanol	115 ± 28.8	43.5 ± 10.3	31.4 ± 8.8	68.8 ± 6.8

[a] The standards were leucine for the amino acids and glucose for the sugars.

[b] Data are averages of three replicate experiments with plants grown at different times of the year ± SE.

Table III. *Protein and Enzyme Activity Extracted from Control and Toluene-Treated Roots and Leaves*

Treatment	Protein	Glc-6-P Dehydrogenase	Alkaline Phosphatase	Glutamate Dehydrogenase
	mg/g fresh wt	*μmol/min·g fresh wt*		
Roots				
Untreated	0.7	0.4 (0.6)[a]	0	0.4 (0.6)
Toluene-treated[b]	0.5	0.3 (0.6)	0	0.3 (0.6)
Leaves				
Untreated	17	1.1 (0.06)	19.1 (1.1)	0.2 (0.01)
Toluene-treated[b]	14	0.9 (0.06)	16.8 (1.1)	0.2 (0.01)

[a] Values in parentheses are specific activities (μmol/min·mg protein).

[b] Tissues extracted three times for 60 min each time with 1% toluene before being ground to extract proteins and enzymes.

Evaluation of Selective Leakage of Low and High Molecular Weight Solutes by Toluene. The percentage of low mol wt solutes leaked from tissues after treatment with 1% toluene at 25°C were compared with the amounts extracted from fresh tissues with boiling water or hot 80% ethanol (Table II) using the accepted times and procedures of extraction. It is evident that the three methods of extraction were equally efficient for extracting K^+, Pi, and amino acids. Only the anthrone reaction for sugars was higher in the hot alcohol extract which could be extracting also other anthrone-reacting compounds. Similar results were obtained several times with different lots of plants and at different seasons. In all cases, practically no further solutes could be extracted from toluene-treated tissues by boiling water at the end of the treatment period.

To determine whether any macromolecules leaked from toluene-treated tissue, the amount of protein and several enzymes were extracted from treated tissue and were measured and compared to amounts extractable from untreated tissue (Table III). Between 70 and 80% of the amount of soluble protein in untreated cells was extracted from toluene-treated cells. Thus, a major portion of the tissue-soluble protein was still associated with treated tissue. It could not be determined whether the lower recovery of protein in treated tissue was due to protein leakage or *in situ* denaturation. However, three enzymes, glucose-6-P dehydrogenase, alkaline phosphatase (in leaves only), and glutamate dehydrogenase were all extracted from treated tissue with specific activities unchanged from those of enzymes extracted from untreated tissue. Chl was not measured quantitatively, but visual observation showed that most, if not all, of this high mol wt cell constituent remained associated with toluene-treated leaves.

DISCUSSION

Lerner *et al.* (7), showed that toluene treatment induced pore formation in *Atriplex* roots and allowed the leakage of low mol wt substances. Only compounds of mol wt less than that of NAD were able to leak out of roots. However, when roots were treated with Triton X-100, there was a much larger degree of cell membrane damage because molecules of larger size than NAD could now leak out. Our work indicated that the action of toluene on

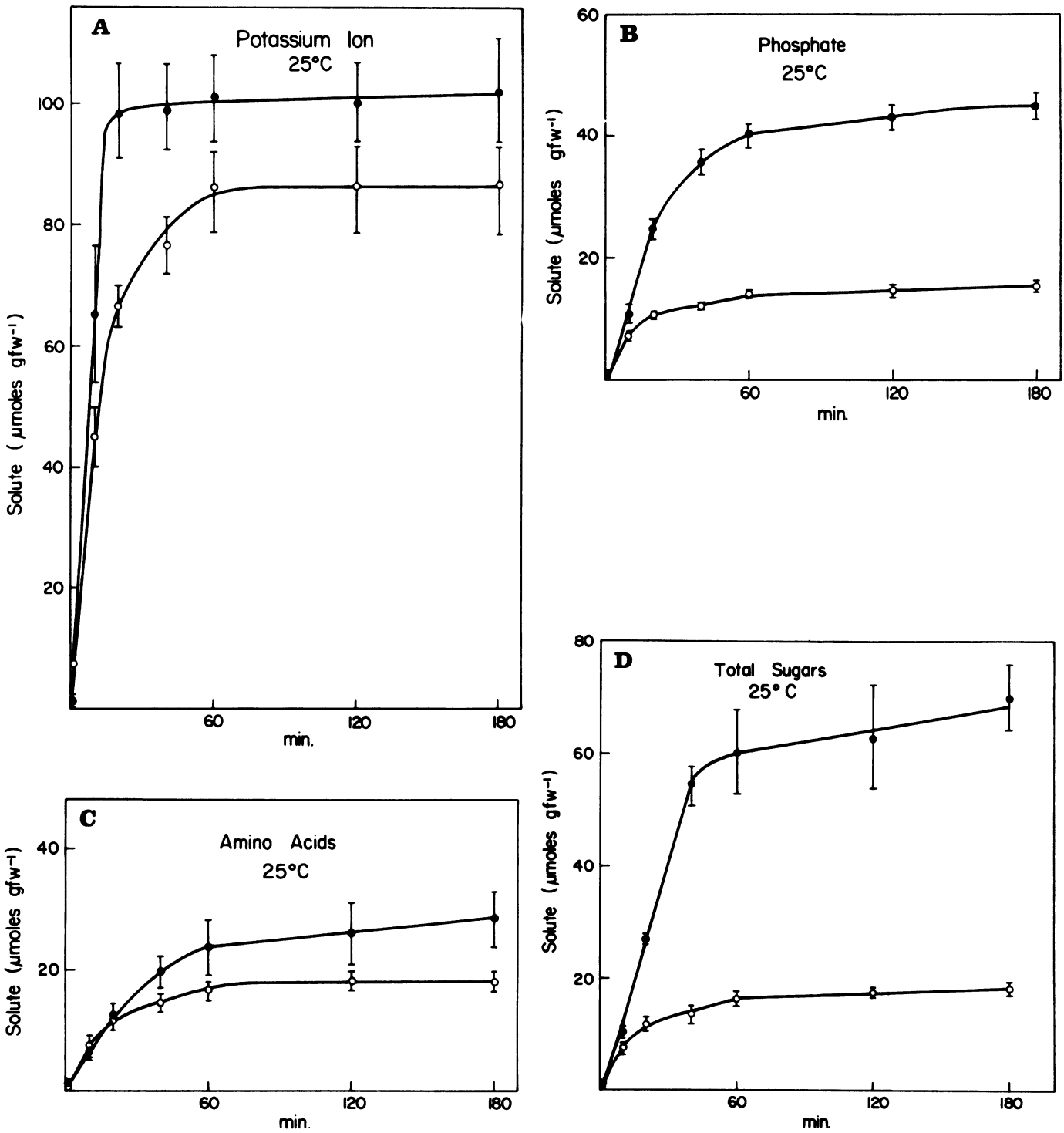

FIG. 2. Kinetics of leakage of potassium (A), phosphate (B), amino acids (C), and sugars (D) from sorghum roots and leaves at 25°C. Roots and leaves were incubated in 1% aqueous toluene at 25°C. At various time intervals, aliquots were taken for assay; (○), roots; (●), leaves.

sorghum roots and leaves was similar to that reported for *Atriplex* roots. Sugars, amino acids, Pi, and K^+ leaked out of treated tissues at rates that seemed to be approximately related to their molecular sizes. The action of toluene was very rapid; in 1 to 2 min, maximal leakage rates were reached. The rate of leakage of each compound measured at 25°C, therefore, can be considered as the rate of diffusion of the specific compound through the pores into the external environment rather than the rate of action of toluene on the membranes. Also, since there was essentially a 100% extraction of the solutes by toluene treatment (Table II), we concluded that toluene was able to diffuse rapidly into the cells to reach all inner membranes and make them leaky as well.

At lower temperatures, both the rates of leakage and the total amounts of solutes leaked were lower than at 25°C. For leaves, the amount of a solute leaked at 1°C was only about 10% lower than that leaked at 25°C. In roots, however, the difference in the amounts at the two temperatures were greater in that it was 25% lower for K^+, and 50% lower for Pi, amino acids, and sugars. The data do not provide an explanation for this lower yield from roots. It may be that lipids of root membranes at 1°C are in a different

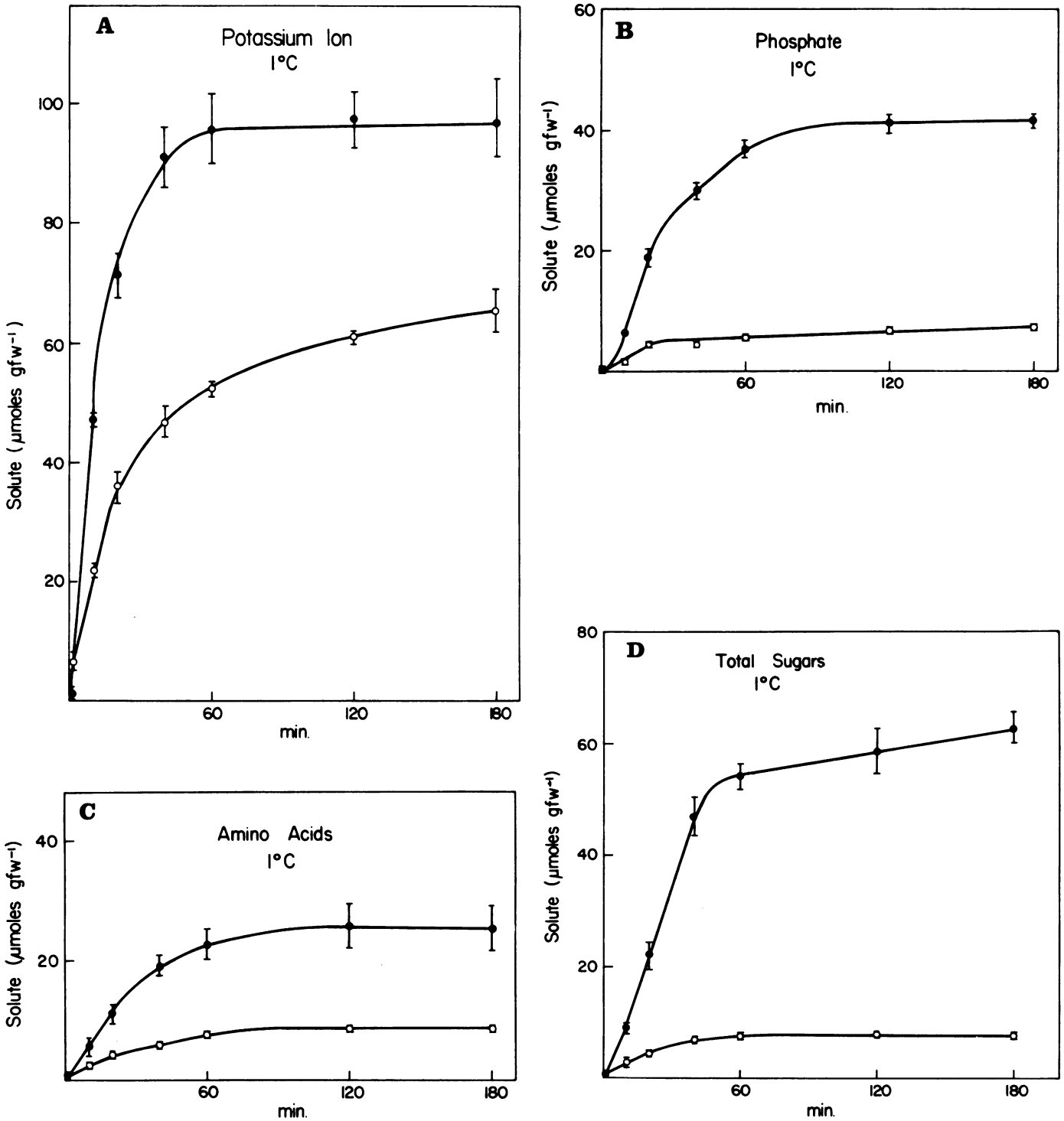

FIG. 3. Kinetics of leakage of potassium (A), phosphate (B), amino acids (C), and sugars (D) from sorghum roots and leaves at 1°C. Roots and leaves were incubated in 1% aqueous toluene at 1°C. At various time intervals, aliquots were taken for assay; (○), roots; (●), leaves.

state than at 25°C and therefore respond differently.

Extraction of low mol wt solutes required a threshold concentration of toluene below which no extraction occurred. Above this concentration, the rate of extraction increased dramatically until some optimal concentration of 0.5 to 1% toluene. Higher concentrations were either equally or less effective than the optimal one. There is no good explanation for this decrease in effectiveness of leakage. But these results are in agreement with the electron microscopic findings showing less membranal damage by 4% toluene than by 1% (unpublished results). In addition these effects of toluene show some similarity to the biphasic effect of general anaesthetics on membranes of erythrocytes and vesicles (11, 18). From what is known concerning the interaction of general anaesthetics with lipid bilayers (11, 12, 20), it is possible to speculate that, below the threshold for pore formation, toluene partitions reversibly into the lipophilic regions of the membranes causing swelling; when a critical amount of toluene has accumulated into the lipid bilayer it triggers an irreversible transformation of the lipid arrangement. This transformation is probably from a lipid bilayer to an inverted micelle arrangement thereby creating

aqueous pores which are large enough to allow the leakage of low mol wt solutes from cells while small enough to retain cellular polymers.

The treatment of roots and leaves with toluene appears to be as useful a technique as hot water or hot 80% ethanol for quantitative extraction of water-soluble solutes from the tissues. Toluene extraction may be an improvement over the other two methods because toluene extraction takes place at room temperature, thus avoiding chemical changes of solutes that might take place at high temperatures. Also, many pigments and other high mol wt compounds that hot water or ethanol extract do not leak out of cells during toluene treatment. Thus, it is easier to separate the individual low mol wt compounds in the leachate for qualitative and quantitative identification. The major disadvantage of the toluene technique is that, since the extraction at room temperature requires several hours, enzymes in the cells remain active and may attack substrates, particularly those that cannot leak through the pores as the semipermeable properties of compartment membranes are destroyed. On the other hand, since polymers such as proteins are retained within the tissue structure (7, Table III) and the substrates that are small enough to diffuse through the pores are released into the very large volume of the aqueous environment, such substrates are physically separated from the enzymes that may act on them. Once a substrate has leaked out, it is probably relatively stable. It would, indeed, be better if the extraction were done at lower temperatures to reduce enzyme transformation of substrates, but maximal leakage of solutes from roots occurred only at room temperature.

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