Dimethy sulfoxide as a Potential Tool for Analysis of Compartmentation in Living Plant Cells

DEBORAH P. DELMER

MSU-DOE Plant Research Laboratory, Michigan State University, East Lansing, Michigan 48824

ABSTRACT

Data are presented which indicate that dimethy sulfoxide (DMSO) acts selectively on the plasma membrane of cultured tobacco cells, rendering it more permeable to small molecules, while having a far smaller effect on the permeability of the vacuolar membrane. The results which support this conclusion are: (a) DMSO (5 to 10%, by volume) causes complete release of $^{14}$C-tryptophan newly synthesized from $^{14}$Clindole while causing efflux of only about 20% of the total intracellular tryptophan pool; (b) similar concentrations of DMSO do not cause substantial release from these cells of phenolic compounds or preloaded neutral red, nor of $\beta$-cyanin from fresh beet discs; (c) kinetic studies of release of tryptophan and neutral sugars and of efflux of $^{86}$Rb show that DMSO selectively promotes rapid release of a portion of the total pool, followed by a substantially slower release of the remaining pool; (d) when tobacco cell protoplasts are incubated in the presence of 7.5% (by volume) DMSO, rapid lysis is observed concomitant with the release of intact vacuoles. These data indicate that a procedure involving a brief treatment of intact plant cells or tissues with DMSO may be used to assess the distribution of metabolites between cytoplasmic and vacuolar compartments.

It has been accepted for many years that metabolites are not uniformly distributed within cells. Such compartmentation of metabolites may be particularly pronounced in higher plant cells containing a large central vacuole, and this fact adds an additional level of complexity to the study of metabolic regulation in plants. Recent technical advances have made possible the isolation of vacuoles from some plant tissues (3, 13, 21), and this technique has been used recently to assess the compartmentation of enzymes within this organelle (3, 4). However, uncertainty about the extent of loss of low mol wt metabolites during isolation makes this technique less desirable for study of compartmentation of these compounds. Other techniques of analysis using intact tissues have been utilized which include the efflux analyses used primarily for ions (17) and radioisotope studies which have indicated the presence of "storage" pools not in easy equilibrium with "metabolic" or turnover pools of amino acids (19), organic acids (16), or sugars (20). In other studies, Ferrari et al. presented data which indicated the existence of two distinct pools of nitrate in cultured tobacco cells (9; later work has supported this concept for other plant tissues such as leaves (1)). The two nitrate pools have been called the metabolic and storage pools; the metabolic pool is defined as that containing nitrate which can be converted anaerobically to nitrite by endogenous nitrate reductase, with the storage pool defined as the remainder of unconverted nitrate in the cells. The metabolic pool and storage pools have been suggested to represent the cytoplasmic and vacuolar pools, respectively (9), although direct confirmation of these localizations is lacking.

Some years ago, I described a phenomenon whereby plant cells grown in tissue culture could be rendered permeable to small molecules by treatment with DMSO, and a technique was developed to use intact, but leaky, DMSO-treated cells for the assay of enzymes (5, 8). A preliminary experiment was performed which indicated that, although the plasma membrane of these cells is made freely permeable to certain small molecules by DMSO, the tonoplast was relatively unaffected (5). In this paper, results are presented which indicate that DMSO has a differential effect on these membranes and therefore may hold some promise for future use as a tool for analysis of compartmentation in plant cells.

MATERIALS AND METHODS

Growth of Cells and Preparation of Protoplasts. Unless otherwise indicated, all experiments were performed with cells on Nicotiana tabacum L., line XD, grown in liquid suspension culture on M-I-D medium. The cells were obtained from P. Filner; a history of the derivation of this cell line, the conditions for growth, and composition of the M-I-D medium is given by Filner (10). All weights of cells given are fresh weight. Protoplasts were derived from a culture of N. tabacum L. cv. Wisconsin 38; procedures for growth of this cell line and preparation of protoplasts have recently been described in detail (3).

Enzyme Assays and Chemical Determinations. Tryptophan synthetase was assayed as described by Delmer and Mills (8) except that the temperature of incubation was lowered to 30 C and the specific radioactivity of $[^{14}]Clindole$ (purchased from International Chemical and Nuclear Corp.) was 5 $\mu$Ci/ mumol. L-Tryptophan was quantified as described previously (7), and it was determined that DMSO in concentrations up to 15% (v/v) did not affect the results of the assay. Prior to assay, cells were washed twice by gentle shaking for 5 min in an aqueous solution containing 0.79 mM KCl and 0.85 mM CaCl$_2$ (CaK solution) initially devised by Ferrari et al. (9). DMSO was obtained from J. T. Baker Chemical Corporation. Reducing sugars were determined by a modified Nelson-Somogyi assay (11). Sucrose was determined by measuring the increase in total reducing sugars following treatment of the sample for 30 min at 40 C with 100 $\mu$g/ml invertase (Sigma, Grade X) in 0.05 M Na-acetate buffer (pH 4.5). Nitrate, nitrite, nitrate reductase, and metabolic pools of nitrate were determined as described previously (9, 22). Amino acids were quantified by GLC of the heptfluorobutyyl derivatives (15) with piperidyl acid as an internal standard. The derivatives were separated on 3% SP-2100 with a temperature program from 85 to 215 C at 4 C/min. Prior to derivatization, the amino acid fraction was partially purified by binding and elution of samples on a Dowex 50-X2-200 column as described previously (23). Such samples consisted either of that

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$^2$ Abbreviations: DMSO: dimethy sulfoxide; CaK: aqueous solution containing 0.79 mM KCl and 0.85 mM CaCl$_2$. 

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material released from cells in CaK containing 7.5% (v/v) DMSO or that material not released which was extracted as described by Widholm (23). Previously described procedures were used for the quantification of malate (12) and ATP (6) following their partial purification by binding to a DEAE-Sephadex A-25 column equilibrated in water and eluted with 0.5 M ammonium bicarbonate. The ammonium bicarbonate was removed by repeated evaporation of the eluate.

Efflux Kinetics. For studying the kinetics of release of L-tryptophan and reducing sugars, cells were subcultured for 9 days on M-I-D medium supplemented with 0.2 mM L-tryptophan, harvested by filtration, and weighed. Each aliquot (5 g) was suspended in 10 ml CaK solution containing 7.5% DMSO (or water in the control) in a sintered glass funnel attached to a suction flask, and aeration was begun by bubbling air from the outlet port of a vacuum pump. At the times indicated, the vacuum line was switched to the air intake port on the vacuum pump and the solution filtered from the cells into a collection tube. The cells were immediately resuspended in 10 ml fresh solution and aeration and sampling repeated at each of the times indicated. At the end of the experiment, the cells were placed in 20 ml of CaK solution and heated at 100°C for 10 min to release the remaining L-tryptophan and reducing sugars. For studying the kinetics of efflux of Rb⁺, cells which had been subcultured for 4 days on M-I-D medium were harvested by filtration and preloaded with Rb⁺ by incubation for 16 h in M-I-D medium containing 1 μCi/ml RbCl (obtained from New England Nuclear; specific radioactivity, approximately 1 Ci/mmol). The concentration of K⁺ in M-I-D medium was 0.79 mM. These cells were then harvested by filtration and efflux analyses were carried out in M-I-D medium by the same technique described above. Rb⁺ was quantified by liquid scintillation counting.

RESULTS

Effect of DMSO on Leakage of Tryptophan. The original observation which led to these studies came from an experiment which was designed to test whether prolonged growth of tobacco cells on L-tryptophan resulted in a repression of tryptophan synthetase activity as assayed by an in vivo assay utilizing DMSO as described by Delmer and Mills (8). Although enzyme repression was not observable, the experiment indicated that essentially all of the newly synthesized tryptophan derived from [14C]indole was released in the presence of 10% (v/v) DMSO and was recovered simply by filtration or low speed centrifugation of the cells; however, the bulk of the unlabeled tryptophan that had accumulated in the cells during prolonged growth on L-tryptophan was not released unless the cells were broken or placed briefly in boiling water prior to filtration or centrifugation. However, the enzyme, tryptophan synthetase, was not released by the DMSO treatment. Those studies (5) were done with callus tissue of N. tabacum var. Wisconsin 38 which had been grown on agar medium. The results of Table I confirm this initial observation using cells of N. tabacum XD grown in a liquid suspension culture. The results show, first, that the presence of DMSO enhances the total assayable activity by making the substrates more accessible (5, 8). Second, essentially all of the radioactive tryptophan, newly synthesized presumably in the cytoplasm, is released without boiling in the presence of 5 to 10% (v/v) DMSO, yet only about 20% of the total tryptophan is released by DMSO.

Kinetcs of Release of Tryptophan and Reducing Sugars. Figure 1 shows the kinetics of release from cells of L-tryptophan and reducing sugars in the presence and absence of 7.5% DMSO. In the absence of DMSO, 13% of the total L-tryptophan and 56% of the total reducing sugars are rapidly released, and no substantial further release is observed after about 10 min of incubation. Since the cells were not washed before the start of incubation, this release is interpreted to represent removal from the free space (cell walls and surface film) compartment. In the presence of DMSO, an additional 25% of the total L-tryptophan and 16% of the total reducing sugars were rapidly released, followed by a very slow continued release of these components. If one subtracts the free space content released in the absence of DMSO from the total amount released in the presence of DMSO, one can calculate that of the total intracellular pools, 29% of the L-tryptophan and 30% of the reducing sugars were present in the DMSO-accessible compartment.

Table I. Effect of DMSO on Leakage of Tryptophan from Tobacco Cell Suspensions

<table>
<thead>
<tr>
<th>DMSO in Assay</th>
<th>Newly Synthesized [14C]Tryptophan in Filtrate</th>
<th>Total Tryptophan in Filtrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>% (v/v)</td>
<td>cm³/g cells</td>
<td>μmol/g cells</td>
</tr>
<tr>
<td>0</td>
<td>28,850</td>
<td>59,930</td>
</tr>
<tr>
<td>2.5</td>
<td>82,160</td>
<td>94,950</td>
</tr>
<tr>
<td>5.0</td>
<td>105,840</td>
<td>101,480</td>
</tr>
<tr>
<td>7.5</td>
<td>108,530</td>
<td>96,450</td>
</tr>
<tr>
<td>10.0</td>
<td>105,180</td>
<td>95,980</td>
</tr>
</tbody>
</table>

FIG. 1. Kinetics of release of L-tryptophan and reducing sugars from cultured tobacco cells. Analyses were performed with cells subcultured for 9 days on M-I-D medium supplemented with 0.2 mM L-tryptophan: A: release of tryptophan; B: release of reducing sugars.

Microscopic Examination of DMSO-treated Cells; Effect on Release of Neutral Red from Vacuoles. Cells (7 days after subculture) were allowed to absorb neutral red for 30 min, were washed, and then incubated with shaking in Ca solutions containing various concentrations of DMSO. The cells were periodically observed and photographed under a phase contrast micro-
DMSO, MEMBRANES, AND COMPARTMENTATION

Fig. 2. Visual observation of tobacco cells preloaded with neutral red and incubated in the presence of varying concentrations of DMSO. Cells were observed and photographed using a Zeiss phase microscope. Cells in parts 1, 2, 3, 4, 5, and 6 were incubated in 0, 5, 10, 15, 20, and 30% DMSO, respectively.

Fig. 3. Quantitation of neutral red release from tobacco cells incubated in CaK solution containing various concentrations of DMSO. Cells were preloaded with dye by incubation with shaking for 30 min in 0.1% neutral red in CaK solution. They were then washed on a sintered glass filter and incubated with shaking in CaK solution containing various concentrations of DMSO. Neutral red remaining in cells after 120 min was released by heating the cell suspension in CaK solution at 100°C for 10 min.

scope (Fig. 2); neutral red released from cells was measured by the A at 530 nm of the incubation solution freed of cells (Fig. 3). Concentrations of DMSO up to 10% (v/v) do not impair the capacity of the vacuolar membrane to retain neutral red (Figs. 2 and 3). This stability of the tonoplast is apparent for up to 2 h at room temperature (Fig. 3); the fact that less neutral red is released at the later time seems to be caused by absorption of some neutral red which had been released prior to the 30-min sampling. At higher concentrations of DMSO, a shrinkage of the vacuoles resembling plasmolysis occurs (Fig. 2, parts 4–6), suggesting that the DMSO itself is not readily permeating the vacuolar membrane and is imposing an osmotic stress on this compartment. Above 15% DMSO, all cell membranes look visibly damaged and neutral red is no longer retained by the cells. One consistent observation is that there appears to be some heterogeneity in the cell popula-
immediately
were then
presence M-1-D medium, were
bation or% absenc of 5%

stabilized the vacuoles for
occurred, medium

30
the vacuoles

FIG. 5. Kinetics of efflux of "Rb" from cultured tobacco cells in the presence or absence of 5% DMSO. Cells, subcultured for 4 days on M-1-D medium, were harvested by filtration and preloaded with "Rb" by incubation for 16 h in M-1-D medium containing 1 μCi/ml 86RbCl. These cells
were then harvested by filtration and efflux analyses were carried out in M-1-D medium as described under "Materials and Methods."

Effects of DMSO on Isolated Protoplasts. The most compelling argument for a differential effect of DMSO on the plasma and tonoplast membrane comes from observations on the effect of the DMSO on isolated tobacco cell protoplasts. Such protoplasts, osmotically stabilized in 0.6 m mannitol, were diluted into the same medium containing DMSO (final concentration was 7.5% [v/v]) and observed by phase contrast microscopy (Fig. 4). Within minutes, the plasma membranes of the majority of the protoplasts began to expand, presumably due to the increased permeability to mannitol; the tonoplast membranes showed little swelling. Within 30 min, the plasma membranes expanded to the extent that lysed occurred, and the preparation then appeared to consist almost entirely of vacuoles and isolated debris. However, once liberated from the cells, the vacuoles appeared to be relatively fragile, and eventually they too began to swell and lyse. A similar result was obtained when protoplasts derived from cultured soybean cells
were tested, but in this case the effect was less pronounced with only about 25% of the protoplasts releasing vacuoles in the 30 min of observation, although virtually all of the protoplasts showed swelling.

Effects of DMSO on Efflux of Rb+ from Cells. The effect of DMSO on the kinetics of efflux of 86Rb+ was examined in order to see if DMSO would convert the so-called "cytoplasmic" compartment to a "free space" compartment while having little effect on the "vacuolar" compartment of 86Rb+. The results (Fig. 5) indicate that the half-time for efflux of 86Rb+ from the "slow" or "vacuolar" compartment is decreased, although not drastically, in the presence of 5% (v/v) DMSO. Unexpectedly, the content of this compartment, as indicated by the intercept on the ordinate (17), appears to be decreased in the presence of DMSO by about 30%. The inset of Figure 5 shows the kinetics of efflux from the "fast" (free space) and "intermediate" (cytoplasmic) compartments, and shows that in the presence of DMSO, the two compartments appeared to be converted into a single compartment with a considerably shorter half-time for 86Rb+ loss than that of the "intermediate" compartment in the absence of DMSO. A similar result was obtained in the presence of 10% DMSO, except that after 90 min of efflux in 10% DMSO, the vacuolar compartment appeared to begin to disintegrate since the rate of efflux sharply increased at that time (data not shown).

Effects of DMSO on the Total and Metabolic Pools of Nitrate. If the interpretation of Ferrari et al. (9) is correct, it might be expected that preincubation of cells in DMSO would lead to a selective leaching of the metabolic pool of nitrate from cells. The data of Table II show, however, that this is not the case. The size of the metabolic pool (as assayed by anaerobic nitrite production) remained unchanged after preincubation in DMSO, even though such pretreatment caused leakage of about 80% of the total nitrate from the cells. The measurement of the metabolic pool depends upon the activity of endogenous nitrate reductase; it was determined that DMSO had a relatively small effect on the activity, causing a 30% inhibition at 7.5% DMSO (data not shown); since production of nitrite was allowed to proceed to completion anaerobically, this effect does not influence the validity of the results. When DMSO was present during the metabolic pool determination, it caused an elevation of the nitrate in this pool but not so much as did treatment with butanol which had previously been reported to increase the metabolic pool, presumably by alteration of the permeability barrier between the metabolic and storage pools (9).

Size of Molecules Released by DMSO Treatment. The fact that no substantial release of either nitrate reductase or tryptophan synthetase from cells occurred when cells were preincubated for 30 min in DMSO indicates that these proteins are not leached by DMSO. The results shown in Figure 6 further support the concept that little or no protein of mol wt >5,000 is released from cells by DMSO (7.5%). This figure shows the elution profile on a Sephadex
Table II. Effects of DMSO on Leakage of Total Nitrate and Nitrate from the Metabolic Pool of Suspension-cultured Tobacco Cells

Cells were used 4 days after subculture on M-1-D medium. Results presented are the average of duplicate samples. Prior to preincubation, cells were briefly washed three times with CaK, filtered, and divided into 200-mg aliquots for preincubation with gentle shaking in 5.0 ml CaK containing 7.5% (v/v) DMSO. Total and metabolic pools were measured as described in references 10 and 22.

<table>
<thead>
<tr>
<th>Time of Preincubation in DMSO</th>
<th>Metabolic Nitrate Pool after Preincubation in DMSO</th>
<th>Total Nitrate Remaining in Cells after Preincubation in DMSO</th>
</tr>
</thead>
<tbody>
<tr>
<td>min</td>
<td>mmol/g fresh weight</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>155* (271; 837*)</td>
<td>4520</td>
</tr>
<tr>
<td>15</td>
<td>157*</td>
<td>940</td>
</tr>
<tr>
<td>30</td>
<td>188*</td>
<td>980</td>
</tr>
<tr>
<td>60</td>
<td>137*</td>
<td>740</td>
</tr>
</tbody>
</table>

* Measured in absence of DMSO.
* Measured in presence of 7.5% of DMSO.
* Measured in presence of 0.1% butanol.

G-25 column of \(A_{260}\)-absorbing material released or remaining in cells after a 30-min treatment in DMSO. Essentially all of the \(A_{260}\)-absorbing material of mol wt >5000 (void volume) was not released by DMSO.

Effects of DMSO on Leakage of Other Metabolites. Preliminary experiments on the release of sucrose, malate, ATP, and total pool amino acids indicate that only a portion of the total pools of these compounds is released from cells as a result of DMSO treatment. (Values given below do not include the free space compartment.) Using cells harvested 8 days after subculture, it was found that of the 6.78 mmol of sucrose present per g fresh weight of cells, only 1.32 mmol (20%) was released by treatment for 30 min with 7.5% DMSO in CaK solution; of the 1.53 mmol of L-malate present per g fresh weight of cells, 0.27 mmol (18%) was released; and of the 0.86 mmol of ATP present, only 0.50 mmol (58%) was released.

Three experiments were performed in which the free amino acids released by a 30-min treatment with 7.5% DMSO were quantified by GLC of the heptfluorobutyl derivatives. No pool of amino acid was completely released by DMSO treatment, but some amino acids were present in higher proportions in the DMSO-accessible pool than others. Proline, alanine, and \(\gamma\)-amino butyric acid were found to be more highly concentrated in the DMSO-accessible pool (40-75% of total released), whereas phenylalanine, arginine, lysine, and leucine were found in lowest proportions in the DMSO-accessible pool (8-30% released). Phenolic compounds which show a brown color at high pH were not extracted at all by DMSO. Two other general observations which have not yet been explored in detail are that the proportion of metabolites found in the DMSO-inaccessible pool increases with culture age, and that young cells (1 to 2 days after subculture) are in general rather leaky; in some experiments, controls of young cells lacking DMSO showed almost as much leakage of amino acids as the samples containing DMSO.

DISCUSSION

The results presented here clearly show that incubation of plant cells in DMSO (5-10%, v/v) causes a rapid release of low mol wt constituents from the cells. The components released may be...
neutral (sucrose, reducing sugars), anionic (nitrate, ATP, malate), cationic (Rb⁺/K⁺), or zwitterionic (amino acids). However, for most small molecules examined, DMSO causes a rapid release of only a fraction of the total intracellular pool. The only component studied here which was completely released by such DMSO treatment was a pool of [14C]-tryptophan newly synthesized from [14C]-indole. Since tryptophan synthetase in cultured tobacco cells is a soluble enzyme (7), and therefore presumably cytoplasmic, localization of newly synthesized tryptophan would most likely also be cytoplasmic. This localization is further supported by other results of mine (5) which indicated that tryptophan synthesized endogenously from supplied [14C]-indole is used preferentially for protein synthesis when compared with exogenously supplied tryptophan. Other studies have indicated that the total pool of tryptophan (2, 23) as well as of other amino acids (19) is compartmented into at least two distinct pools in plant cells. Thus, the DMSO treatment appears to cause a preferential release of only one of these pools, presumably the cytoplasmic pool, from these plant cells. The remaining, DMSO-inaccessible pool, would appear to be localized in the vacuole based on the results discussed below.

Other data presented herein support the conclusion that DMSO renders the plasma membrane permeable to small molecules while having a far less pronounced effect on the vacuolar membrane. Thus, components known to be concentrated in vacuoles, such as phenolic compounds or the β-cyanin of beet discs, are not released to any significant extent by the DMSO treatment; the same holds true for neutral red, a vital stain which preferentially accumulates in vacuoles. Furthermore, visual observation of protoplasts shows that DMSO (7.5%, v/v) causes a rapid lysis of the plasma membrane while vacuoles remain intact for substantially longer periods. It seems that a DMSO treatment of plant cells (5–10%, 30 min) could effectively be employed as a means of assessing the compartmentation of a variety of low mol wt metabolites in plant cells.

Some words of caution are in order, however. First, I do not yet have data on the effect of DMSO on other, smaller intracellular compartments such as nuclei, mitochondria, or chloroplasts. Since a vacuolar localization for ATP seems rather unlikely, it may be that the portion of ATP not extracted from the tobacco cells by DMSO may be localized in one or more of these compartments, the membranes of which may be more resistant to the effects of DMSO. It is also clear that the so-called “metabolic pool” of nitrate exists in a compartment not accessible to DMSO; this result is of some importance and suggests that the current interpretation—namely that the “metabolic” pool is equivalent to a cytoplasmic pool—should be reexamined.

As another note of caution, it should be stressed that the differential effect of DMSO on plasma and vacuolar membranes is not absolute. Thus, vacuoles do eventually lyse in DMSO, and the kinetics of release of tryptophan and reducing sugars indicates a slow, but measurable, release of constituents from the DMSO-inaccessible compartment(s). The t₁/₂ of 86Rb⁺ efflux from the vacuolar compartment is also decreased in the presence of DMSO. It should be noted that this experiment measures a passive efflux in the presence of external K⁺ where the efflux or exchange rate for K⁺ is governed by other factors as well, such as membrane potential and external activity of the ion in question; factors which are likely to change upon addition of DMSO. In this sense, the experiment is not precisely equivalent to the studies of Figure 2 showing release of tryptophan and reducing sugars where the external solution did not initially contain the compounds in question; under those conditions the effect of DMSO on release of components from the inaccessible compartment was much less than in the 86Rb⁺ efflux experiment.

As a final note of caution, there appears to be some heterogeneity in the tobacco cell population with respect to sensitivity to DMSO. Visual observation shows that a small number of cells are capable of retaining neutral red at concentrations of DMSO > 20% (v/v). It also seems possible that certain cells may be more sensitive to DMSO; one interpretation of the decreased content of the vacuolar 86Rb⁺ compartment measured in the presence of DMSO is that a portion of the cells contain vacuoles possessing membranes more sensitive to DMSO and which release their contents at a rate comparable to that from the cytoplasm. However, since visual observation indicates that DMSO can actually cause a shrinking of vacuoles in intact cells, this factor as well may contribute to this result. Cells in tissue culture are known to be rather heterogeneous with respect to a variety of parameters, and heterogeneity with respect to the effect of DMSO may be less pronounced in other tissues.

In spite of these limitations, the use of a DMSO treatment of cells does appear to hold some promise for future use in analyses of compartmentation provided that conditions are carefully controlled. Preliminary studies indicate that DMSO does affect the permeability of leaves and roots, suggesting that the technique may be applicable to such tissues. The obvious differential effect of DMSO on different cellular membranes may also prove useful in studying the physical and chemical properties of these membranes. The effects of DMSO seem to be different from toluene which appears to alter the permeability of both plasma and vacuolar membranes (14). Preliminary studies indicate that the composition of vacuolar membranes is different from other membranes (18). Determining how that compositional difference contributes to stability to DMSO may offer insights into the role of various lipid classes in determining membrane characteristics.

Note Added in Proof. With regard to the intracellular localization of tryptophan synthetase, a reference has recently come to my attention which presents data indicating a localization of this enzyme in plastids of peas (W. Grosse, 1976 Z. Pflanzenphysiologie 80: 463-468). If this were true also for the tobacco cells used in my studies, it would indicate that the plastids may also be included in the DMSO-accessible compartment.

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LITERATURE CITED

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