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Permeabilization of plant cells for release of intracellularly stored products: viability studies

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Summary. The effects of various chemical substances on the permeability of plasma membranes and tonoplasts of three suspension cultures (*Catharanthus roseus, Thalictrum rugosum* and *Chenopodium rubrum*) have been studied. The permeability of the plasma membrane is monitored by measuring the activity of the cytosolic enzyme isocitrate dehydrogenase and the permeability of the tonoplast is measured by determining the release of substances stored in the vacuoles (inorganic phosphate, berberine and betanin for the three cell lines, respectively). The minimum concentration required for quantitative release of vacuolar products have been established for five different permeabilization agents. Cell viability is lost upon permeabilization except for treatment of *Catharanthus roseus* with DMSO and Triton X-100.

Introduction

Plant cell cultures have during decades been considered as potential sources for the production of natural compounds. The development has, however, been relatively slow and today only a couple of industrial processes have been reported (Yamada and Fujita 1983; Ushiyama 1986). Various technological and biological limitations are responsible for the limited industrial success with plant cell cultures as a source for biochemicals.

Some years ago, we initiated studies on the immobilization of cultivated plant cells to investigate if the advantages of immobilized biocatalysts also hold true for plant cells (Brodelius et al. 1979). One of the major advantages of such a biocatalyst is a continuous operation of a process. However, plant cells most often store secondary product(s) in the vacuoles which makes a continuous operation of a process involving immobilized plant cells impossible. Attempts are now being made to develop methods for the permeabilization of plant cells for release of intracellularly stored products. The cells should remain viable after the treatment to be fully biosynthetically active. This would allow the reutilization of the expensive biomass for biosynthetic purposes.

Various substances may be employed for the permeabilization of microbial and plant cells (Felix et al. 1981; Felix 1982). Dimethylsulfoxide (DMSO)[1] has been used for the reversible permeabilization of immobilized cells of *Catharanthus roseus* (Brodelius and Nilsson 1983). Extended studies on the permeabilization of *C. roseus, Thalictrum rugosum* and *Chenopodium rubrum* suspension cultures with different permeabilizing substances are now reported. These cell lines were selected as model systems for these studies since they have been maintained as stable suspension cultures for a few years. Furthermore, they were selected while the determinations of released vacuolar compounds are relatively uncomplicated.

Materials and methods

Chemicals. Hexadecyltrimethylammonium bromide (HDTMAB)[1], phenethyl alcohol (PEA), chloroform, isocitrate, $NADP^+$ and berberine were obtained from Fluka (Switzerland). Dimethylsulfoxide (DMSO) and Triton X-100 were purchased from Merck (FRG). ^{32}P orthophosphate was supplied by Amersham (UK).

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Abbreviations: DMSO, dimethylsulfoxide; PEA, phenethylalcohol; HDTMAB, hexadecyltrimethylammonium bromide; ICDH, isocitrate dehydrogenase

All other chemicals were of analytical grade and were obtained from commercial sources.

Cultivation of cells. Stock suspension cultures were cultivated on gyratory shakers (120 rpm) at 26 °C in the following manner:

C. roseus: LS-medium (Linsmaier and Skoog 1965) supplemented with 10 μM 2'4-D and 10 μM NAA in the dark.

T. rugosum (kindly supplied by J. Berlin, Stöckheim, FRG): MS-medium (Murashige and Skoog 1962) supplemented with 2 μM 2'4-D in the dark.

C. rubrum (kindly supplied by J. Berlin, Stöckheim, FRG): MS-medium supplemented with 2 μM 2'4-D in daylight (16 h).

Permeabilization. For the permeabilization experiments cells in early stationary phase were used. This corresponds to incubation times of 7, 10 and 12 days for *Catharanthus roseus, T. rugosum* and *Chenopodium rubrum,* respectively. The standard procedure used for the permeabilization of cells was as follows:

Cells were collected by filtration. An aliquote (0.5 g fresh weight) was suspended in appropriate permeabilizing agent (10 ml) shaken for 30 min on a gyratory shaker (80 rpm) at room temperature in a 25 ml Erlenmeyer flask. The cells were collected by centrifugation (500 × g; 2 min) and washed with the appropriate medium (2 × 5 ml). The supernatants were combined and analyzed for released product.

Analytical procedures. Isocitrate dehydrogenase activity was determined as previously reported (Felix et al. 1981).

Berberine was quantitated by TLC as previously described (Funk et al. 1987) and betanin was quantitated by OD measurements at 450 nm.

The vacuoles of *Catharanthus roseus* cells were loaded with inorganic phosphate as described elsewhere (Lundberg et al. 1986).

Results and discussion

In order to release products from vacuoles of cultivated plant cells two membrane barriers have to be penetrated (i.e. the plasma membrane and the tonoplast surrounding the vacuole). Various chemical may be employed to make these membranes permeable to different compounds. The permeability after treatment may be monitored in various ways.

Permeabilization assays

The activity of cytoplasmic enzymes may be employed for monitoring the permeability of plasma membrane. Enzymes requiring nucleotide coenzymes such as NADP(H), ATP or CoA are particularly convenient to use (Felix et al. 1981). These coenzymes cannot penetrate an intact plasmalemma and therefore no enzyme activity is expressed unless the membrane has been made permeable to these cofactors.

The release of small molecular weight compounds from the vacuoles may be used as a measure on the permeability of the tonoplast. However, the tonoplast is not permeabilized unless the plasma membrane has been made permeable. Therefore, the response of the tonoplast to various treatments may not reflect the sensitivity of this membrane but rather that of the plasma membrane.

In this study isocitrate dehydrogenase (ICDH) is used to monitor the permeability of the plasma membranes as previously described for cells of *Catharanthus roseus* (Brodelius and Nilsson 1983). The release of berberine and betanin from cells of *T. rugosum* and *Chenopodium rubrum,* respectively, is employed to measure the permeability of the tonoplasts of these two cultures. For tonoplasts of *Catharanthus roseus* another procedure is used. In vivo ^{31}P-NMR studies have shown that these cells quickly take up inorganic phosphate from the medium and store it within the vacuoles (Brodelius and Vogel 1985). Furthermore, it was recently reported that ^{31}P-NMR may be used to study the permeabilization of the tonoplast (Lundberg et al. 1986). Here radioactive inorganic phosphate (^{32}P) is used to study the permeability of tonoplasts of *C. roseus* cells after treatment with various permeabilizing agents. Before treatment the cells are "loaded" with inorganic phosphate (Lundberg et al. 1986).

For each system studied a dependence between membrane permeability and concentration of permeabilizing agent can be established resulting in dose-response curves. At a sufficient high concentration of permeabilizing agent the cell population is fully permeabilized. At this point (100% permeabilization) no further increase in enzyme activity or release from the vacuoles is seen upon an increase in the concentration of permeabilizing agent. In this manner the lowest concentration of permeabilizing agent resulting in total permeabilization is determined ($[P]_{100}$).

Permeabilization as function of cell concentration

The response (degree of permeabilization) is highly dependent on cell concentration at a specific concentration of permeabilizing agent as exemplified in Fig. 1. The amount of berberine released from cells of *T. rugosum* is linear to about 50 mg cells (fresh weight) per ml suspension. At higher cell densities a comparatively lower amount of berberine is released indicating a saturation effect. The amount of permeabilizing agent

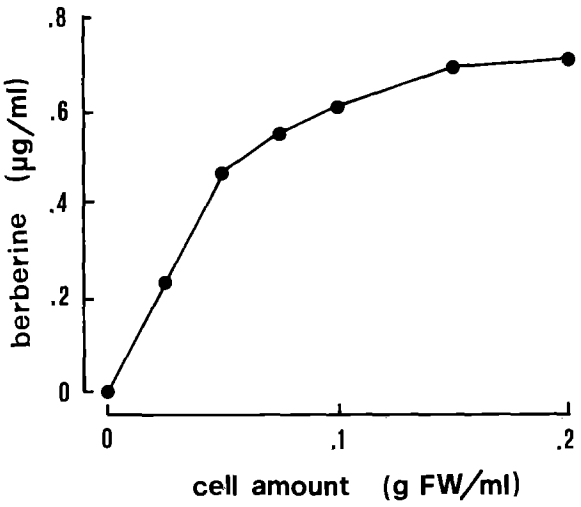

Fig. 1. Release of berberine from cells of *T. rugosum* as a function of cell concentration after treatment with 30 ppm HDTMAB

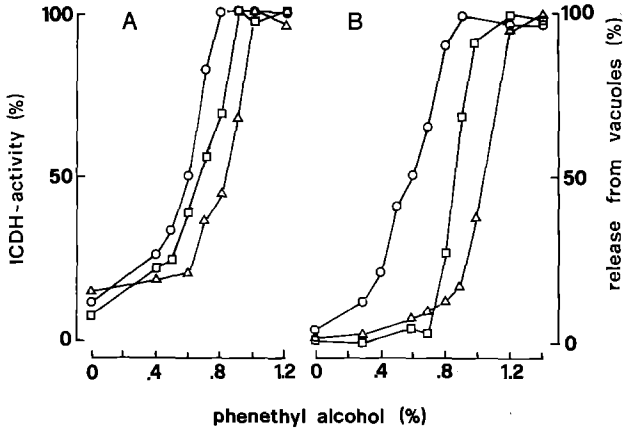

Fig. 3. Relative ICDH activity within cells (**A**) and relative release of products from vacuoles (**B**) as a function of phenethyl alcohol concentration. (—△—) *Catharanthus roseus;* (—□—) *Chenopodium rubrum;* (—○—) *T. rugosum*

per unit area of membrane decreases with increasing cell concentration resulting in a lower absolute degree of permeabilization.

Permeabilization as function of incubation time

Figure 2 demonstrates the time course of permeabilization. At high concentrations of permeabilizing agent the cells are rapidly permeabilized, while at lower concentrations the process is somewhat slower. The standard conditions used in the subsequent studies are: 50 mg cells (fresh weight) per ml and treatment for 30 min.

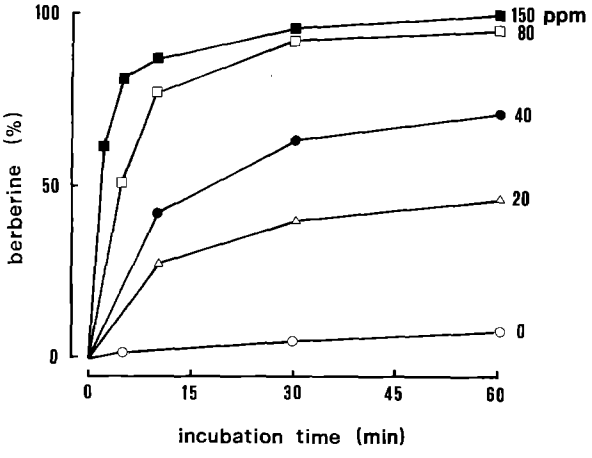

Fig. 2. Release of berberine from cells of *T. rugosum* as a function of incubation time after treatment with various concentrations of HDTMAB as indicated in the figure

Permeabilization as function of concentration of permeabilizing agent

In this study five different permeabilizing agents have been used. These were randomly selected from a large number of compounds that have been employed for the permeabilization of microbial cells (Felix 1982).

Figure 3 shows as an example the effects of phenethyl alcohol on the permeability of membranes of the three cell lines used in this study. Similar curves may be established for other permeabilizing agents. From these curves the concentrations required for 50% and 90% permeabilization (i.e. $[P]_{50}$ and $[P]_{90}$) have been determined. The results are summarized in Table 1 (for plasma membranes) and Table 2 (for tonoplasts). The values given in Tables 1 and 2 are the average of two separate experiments with 9 double assays per curve. The diviations were less than 5% within one experiment and less than 10% between experiments. The three cell lines studied respond differently to the various permeabilizing agents. For instance, *C. roseus* ($[DMSO]_{50}=3\%$) is more sensitive to DMSO than *T. rugosum* ($[DMSO]_{50}=13\%$), while the latter cell line is more sensitive to PEA ($[PEA]_{50}=1.0\%$ and 0.6% for *C. roseus* and *T. rugosum,* respectively). These and other differences in the response to various permeabilizing agents may be due to different membrane compositions and/or cell morphology. However, further studies are required to establish the reasons for the differences observed.

Table 1. Concentration of various permeabilizing agents required for expression of 50 and 90% of maximum isocitrate dehydrogenase activity in cultivated plant cells

Permeabilizing agent	*Catharanthus roseus*		*Chenopodium rubrum*		*Thalictrum rugosum*	
	50%	90%	50%	90%	50%	90%
DMSO (% v/v)	2	5	5	30	7	15
PEA (% v/v)	0.82	0.96	0.66	0.86	0.60	0.72
Chloroform (% sat.)	58	68	42	53	55	70
Triton X-100 (ppm)	140	190	120	145	115	200
HDTMAB (ppm)	12	28	12	24	13	28

Cell viability after permeabilization

Cell viability after treatment is of fundamental importance for the development of a process involving intermittant release of intracellularly stored products by permeabilization. The influence of permeabilization on cell viability has been investigated by measuring cell growth after treatment of the cells. The cell cultures were treated for 30 min with permeabilizing agents resulting in around 10%, 50% or 90% permeabilization of the tonoplasts and subsequently the cells were transferred to fresh growth medium. After treatment with chloroform, PEA or HDTMAB no cell growth could be observed for any of the three treated cell cultures.

In a previous study it was shown that cells of *C. roseus* could grow after treatment with various concentrations of DMSO (Brodelius and Nilsson 1983). Cells of *T. rugosum* or *Chenopodium rubrum* require considerably higher concentrations of DMSO for permeabilization (Table 2) and they do not grow after the DMSO-treatment. Likewise, after treatment with Triton X-100 *C. roseus* cells can grow as illustrated in Fig. 4. The treatment resulting in 50% release of vacuolar products results in a relatively short lag phase. *T. rugosum* and *C. rubrum* cells are more sensitive to Triton X-100. Only cells of the former type treated with a low Triton X-100 concentration (0.01%) resulting in release of 10% of the stored berberine showed some growth (data not shown).

Cells of *Chenopodium rubrum* appear to be the most sensitive of the three cell lines tested. They did not survive any of the treatments. *Catharanthus* cells are more tolerable to permeabilization that the other two species. The reasons for these differences are not known at present. However, it has been demonstrated that cell cultures of *C. roseus* contain different cell types with special functions (Neumann et al. 1983). A small part of the cell population is specialized in storage of alkaloids. These "storage" cells show a vacuolar pH of 3 as compared to pH 5—6 for normal cells. This low pH is consistant with the ion-trap hypothesis for transport of alkaloids into the vacuoles. If these "storage" cells also take up phosphate at a higher rate than other cells the studies on the effects of the various permeabilization agents may have been restricted to this subpopulation of cells. Consequently, if these cells are more sensitive to permeabilization than other cells it may be expected that under certain permeabilization conditions survival of the culture is obtained even when quantitative release of product is achieved. Thus, the growth observed in a few instances after per-

Table 2. Concentration of various permeabilizing agents required for release of 50% and 90% of intracellularly stored products from cultivated plant cells

Permeabilizing agent	*Catharanthus roseus*		*Chenopodium rubrum*		*Thalictrum rugosum*	
	50%	90%	50%	90%	50%	90%
DMSO (% v/v)	3	7	10	35	13	30
PEA (% v/v)	1.04	1.16	0.86	0.98	0.60	0.80
Chloroform (% sat.)	53	66	54	64	50	67
Triton X-100 (ppm)	140	185	185	230	140	210
HDTMAB (ppm)	44	72	22	84	24	60

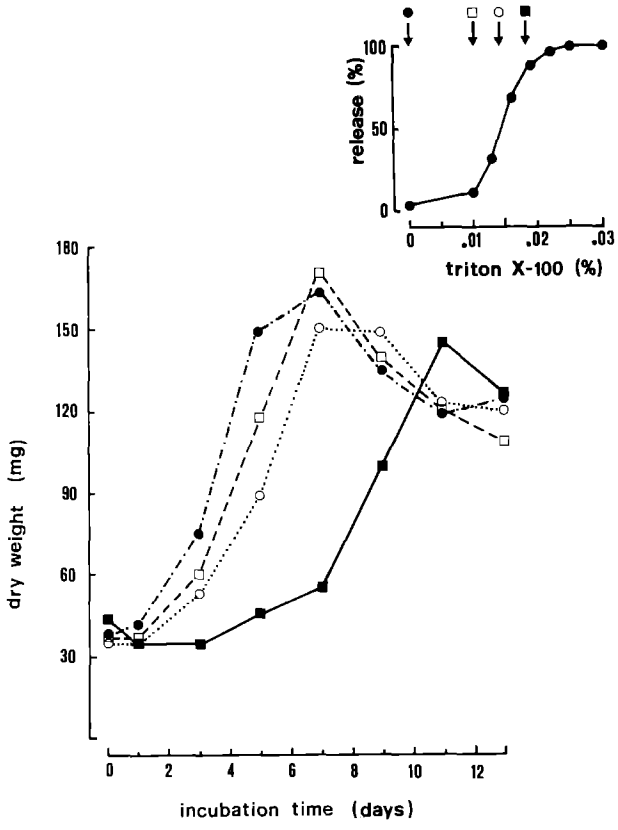

Fig. 4. Dry weight increase of freely suspended cells of *C. roseus* as function of incubation time. The cells were treated for 30 min with Triton X-100 at concentrations corresponding to 0%, 10%, 50%, and 90% release of vacuolar substances as indicated in the inserted figures. (—●—) 0; (—□—) 0.010; (—○—) 0.015; (—■—) 0.020% Triton X-100

meabilization may be due to the fact that a fraction of the cell population is resistant to the conditions used during treatment.

Other similar attempts to chemically permeabilize cultivated plant cells have also resulted in non-viable cell populations (Parr et al. 1984; Rueffer 1985; Wichers 1985). Relatively high DMSO concentrations were required to release intracellularly stored quinoline alkaloids from cells of *Cinchona ledgeriana* (Parr et al. 1984). Very limited release of alkaloids from cells of *Berberis stolonifera* was observed after DMSO treatment (Rueffer 1985). Even though only 0.07% of the alkaloids stored within the cells was released, a non-viable cell preparation was obtained after DMSO treatment. Alginate-entrapped cells of *Mucuna pruriens* hydroxylating tyrosine to DOPA were permeabilized by treatment with 10% isopropanol (Wichers 1985). The hydroxylation capacity of the immobilized cells was improved three-fold by this treatment (most likely due to removal of substrate uptake barriers). However, already in a second batch most of the enzymic activity had been lost due to leakage of the enzyme from the permeabilized cells.

Conclusions

In conclusion, chemical permeabilization for the release of intracellularly stored products appears to be appropriate only in a very limited number of systems if sustained cell viability is required. It is not likely that other permeabilizing agents will improve the situation. The low viability after treatment is most likely not due to the permeabilizing agent as such but to destruction of cell compartmentation and to the release of toxic compouds and degradive enzymes (e.g. proteases). Other methods for the release of intracellularly stored products should be developed for the possible utilization of immobilized plant cells in a semi-continuous process. Recently, attempts were made to permeabilize plant cells by electroporation (Brodelius et al. 1987). However, also in this case the viability of the cells decreased as the amount of released product increased. More promising are the attempts to reverse the ion-trap mechanism by changing the extracellular pH (Renaudin and Guern 1982).

However, permeabilization may in the future find application in the downstream processing of cultured plant cells. The harvesting of water soluble intracellular products after a fermentation process may be readily achieved by a permeabilization procedure. Such a procedure should be more selective than extraction with organic solvents resulting in an easier purification of the product. Furthermore, permeabilized plant cells may be used for bioconversion of substrates that are not taken up by intact cells as illustrated by the glucosylation of warfarin by cells of *Populus alba* after treatment with cetyl trimethyl ammonium bromide (Fuller and Bartlett 1985).

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References

Brodelius P, Nilsson K (1983) Permeabilization of immobilized plant cells, resulting in release of intracellularly stored products with preserved cell viability. Eur J Appl Microbiol Biotechnol 17:275—280

Brodelius P, Vogel HJ (1985) A phosphorus-31 nuclear magnetic resonance study of phosphate uptake and storage in cultured *Catharanthus roseus* and *Daucus carota* plant cells. J Biol Chem 260:3556—3560

Brodelius P, Funk C, Shillito RD (1987) Permeabilization of cultivated plant cells by electroporation for release of intracellularly stored secondary products. Submitted

Brodelius P, Deus B, Mosbach K, Zenk MH (1979) Immobilized plant cells for the production and transformation of natural products. FEBS Lett 103:93—97

Felix H (1982) Permeabilized cells. Anal Biochem 120:211—234

Felix H, Brodelius P, Mosbach K (1981) Enzyme activities of the primary and secondary metabolism of simultaneously permeabilized and immobilized plant cells. Anal Biochem 116 :462—470

Fuller KW, Bartlett DJ (1985) The chemosynthetic potential of plants and its realisation by immobilized systems. In: Fuller KW and Gallon JR (eds.) Plant products and the new Technology. Oxford University Press, Oxford, UK, pp 229—247

Funk C, Gügler K, Brodelius P (1987) Increased secondary product formaton in plant cell suspension cultures after treatment with a yeast carbohydrate (elicitor). Phytochemistry 26:401—405

Linsmaier EM, Skoog F (1965) Organic growth factor requirements of tobacco tissue cultures. Physiol Plant 18:100—127

Lundberg P, Linsefors L, Vogel HJ, Brodelius P (1986) Permeabilization of plant cells: ^{31}P NMR studies of the permeability of the tonoplast. Plant Cell Reports 5:13—16

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassay with tobacco tissue culture. Physiol Plant 15:473—497

Neumann D, Krauss G, Hieke M, Gröger D (1983) Indole alkaloid formation and storage in cell suspension cultures of *Catharanthus roseus*. Planta Med 48:20—23

Parr AJ, Robins RJ, Rhodes MJC (1984) Permeabilization of *Cinchona ledgeriana* cells by dimethylsulfoxide. Effects on alkaloid release and long-term membrane integrity. Plant Cell Reports 3:262—265

Renaudin J-P, Guern J (1982) Compartmentation mechanisms of indole alkaloids in cell suspension cultures of *Catharanthus roseus*. Physiol Veg 20:533—547

Reuffer M (1985) The production of isoquinone alkaloids by plant cell cultures. In: Phillipson JD, Roberts NF and Zenk MH (eds.) The chemisty and biology of isoquinoline alkaloids. Springer-Verlag, Berlin Heidelberg, FRG, pp 265—280

Ushiyama K (1986) Mass production of plant tissue by tissue culture. Proceedings BIO FAIR TOKYO '86, October 15—18, Tokyo, Japan, pp 170—172

Wichers HJ (1985) Production of L-DOPA by suspension grown cells of *Mucuna pruriens*. PhD Thesis, Rijksuniversiteit te Groningen, Groningen, The Netherlands

Yamada Y, Fujita Y (1983) Production of Useful Compounds in Culture. In: Evans DA, Sharp WR, Ammirato PV and Yamada Y (eds.) Handbook of Plant Cell Culture, Vol. I, Macmillan Publishing Co, New York, pp 717—728

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