The Permeability Coefficients of the Plasmalemma and the Chloroplast Envelope of Spinach Mesophyll Cells for Phytohormones

H. Gimmler, B. Heilmann, B. Demmig, and W. Hartung

Lehrstuhl Botanik I, Universität Würzburg, Mittlerer Dallenbergweg 64, D-8700 Würzburg

Z. Naturforsch. **36 c,** 672–678 (1981); received March 9, 1981

Chloroplast Envelope, Permeability Coefficients, Phytohormones, Plasmalemma, Spinach

The uptake of auxin (IAA), abscisic acid (ABA) the synthetic cytokinin benzylaminopurine (BA), gibberellic acid (GA_3) and mevalonic acid (lactone) (MVA) into intact chloroplasts and protoplasts from spinach mesophyll cells was measured and the permeability coefficients P_s of the chloroplast envelope and the plasmalemma were calculated.

With all solutes tested uptake and P_s values were considerably higher in the chloroplast system than in the protoplast system. At an external pH of 7.0, rates of uptake exhibited the order BA ≥ IAA > MVA > ABA > GA_3 in both systems. However, the P_s values (corrected for the undissociated species of the solutes) exhibited the order IAA > GA_3 > (ABA or BA) > MVA. This corrected sequence indicates the theoretical capacity of penetration under the assumption that preferentially the protonated species of phytohormones are capable of readily penetrating membranes.

P_s values for phytohormones appeared largely to be determined by the distribution coefficient K_d and to a lesser extent by the molecular weight (M_r). In the Collander-plot the relation between the logarithm of P_s values for phytohormones and some other solutes such as acetate, glycerol, glucose, sorbitol and sucrose and the logarithm of K_d/M_r 1.5 approached linearity.

Introduction

Hormone transport through plant membranes is an important factor in determining intracellular hormone concentrations which control and direct plant development. Hormone transport may occur via passive diffusion or through carriers. Simple diffusion is facilitated by the lipid-solubility of the protonated species of the phytohormones [1–4, 24]. Most papers published so far describe permeation characteristics of individual phytohormones or the permeability of individual plant membranes (but *cf.* [5]). However, hormone-directed plant development is the result of a complex interaction of several phytohormones which are distributed between several cellular compartments. These compartments are surrounded by different biomembranes. In this investigation we compared the permeation of the phytohormones abscisic acid, auxin, gibberellic acid A_3 and the synthetic cytokinin benzylaminopurine into spinach mesophyll protoplasts and their isolated, intact chloroplasts. The uptake of these phytohormones were compared with the uptake of precursors of ABA-synthesis such as acetate or mevalonic acid and also with the uptake of non-electrolytes such as glycerol, glucose, sucrose and sorbitol. This allowed us to compare the permeability of two different plant membranes, the plasmalemma and the chloroplast envelope to various solutes of physiological interest.

Abbreviations: ABA, abscisic acid; AC, acetate; BA, benzylaminopurine; GA_3, gibberellic acid A_3; GLU, glucose; GLY, glycerol; IAA, β-indolyl acetic acid; MVA, mevalonic acid (lactone); SOR, sorbitol; SUC, sucrose.

Reprint requests to Dr. H. Gimmler.

0341-0382/81/0700-0672 $ 01.00/0

Materials and Methods

Materials

Both intact chloroplasts and protoplasts were isolated from freshly harvested, young leaves of greenhouse-grown *Spinacia oleracea. Type A chloroplasts* were isolated according to the method of Jensen and Bassham [6]. The percentage of intact chloroplasts varied between 70 and 95% and the rate of CO_2-assimilation was between 70 and 150 µmol $CO_2 \times mg^{-1}$ chlorophyll $\times h^{-1}$. *Protoplasts* were obtained from spinach leaves according to the method of Edwards *et al.* [7] as modified by Hartung *et al.* [8]. CO_2-dependent O_2-evolution by illuminated protoplasts was usually about 70–100 µmol $O_2 \times mg^{-1}$ chlorophyll $\times h^{-1}$. The assay medium for chloroplasts was the "C"-medium of Jensen and Bassham [6] (containing 0.33 M sorbitol), whereas the assay medium for protoplasts (8) contained 0.5 M sorbitol.

Methods

Protoplasts were *counted* and their *diameter* measured in a modified Thoma chamber. Protoplast *volume* and protoplast *surface* were calculated from the diameter assuming a spheric size of the protoplasts. In addition the surface was calculated also from volume measurements obtained by the silicone-oil-layer centrifugation technique (see below). *Volume* and *surface* of chloroplasts were obtained by similar techniques and in addition from Coulter Counter measurements [9, 10].

The *uptake* of *radioactive compounds* into protoplasts and chloroplasts was measured by the silicone-oil-layer centrifugation technique [11]. Data were corrected for the non-osmotic space. Tritiated water was taken as a rapidly penetrating compound and ^{14}C-labelled sorbitol as a very slowly penetrating solute. Reaction vessels contained with increasing density from bottom to top in the protoplast experiments: 20 µl 1.5 M sorbitol plus 20 mM Tris-buffer (pH 7.6), 70 µl silicone oil AR 200 (Wacker-Chemie, München), and 270 µl assay medium including protoplasts corresponding to 7–17 µg of chlorophyll and radioactive compounds as described in tables and figures. In chloroplast experiments additions were: 20 µl 1.5 M sorbitol plus 20 mM Tris-buffer (pH 7.6), 70 µl silicone-oil AR 200 + AR 20 4 + 1 (v + v) and 200 µl medium "C" [6], including chloroplasts corresponding to 20–30 µg of chlorophyll and radioactive compounds as described in tables and figures. Standard conditions for all uptake studies were pH 7.0 (minimal pH difference between the medium and the cytoplasm, respectively the stroma), 20 °C and darkness. The time of incubation varied between 15 sec and 20 min. Rates of uptake were calculated from the period of linear uptake. This range differed in chloroplasts and protoplasts and with the different solutes tested (*cf.* Fig. 1).

Distribution coefficients were determined by measuring the partition of radioactive compounds between equal volumes of octanol and the assay medium. Radioactive compounds were added to the two phase system and vigorously mixed for 1 min at 20 °C. After phase separation, aliquots of the lipophilic octanol phase and the aqueous medium phase were counted and counts were corrected for quenching. K_r was defined as $(S)_{octanol}/(S)_{medium}$. Since distribution coefficients are often determined from the partition between water and petrolether or olive oil, the K_r of our experiments deviates from the conventional distribution coefficient K_d by the constants α_k and β_k which both are characteristic for our partition system but are independent from the nature of the tested solutes [12]:

$$K_d = \alpha_k K_r^{-\beta_k}. \quad (1)$$

Normally distribution coefficients are measured for non-electrolytes only. Since most phytohormones are electrolytes, the distribution coefficients of such solutes between an organic solvent and an aqueous medium phase with a defined pH can be calculated in two ways. Either the ratio of the total radioactivities in the organic and aqueous phase is calculated or the ratio of the undissociated species of the electrolytes in both phases. We preferred the latter way and assumed that the anionic species in the organic phase can be neglected, whereas the concentration of the undissociated species in the aqueous phase can be calculated from the pH of the medium and the pK of the electrolytes (Table IV).

The *permeability coefficient* P_s was calculated from

$$J_s = -P_s (c_s^0 - c_s^i), \quad (2)$$

where J_s is the flux of a solute s in $mol \times cm^{-2} \times sec^{-1}$, c_s^0 the concentration of s in the medium and c_s^i the corresponding concentration in the protoplasts or the chloroplasts. Internal compartmentation and the effects of unstirred layers on external and internal concentration of s are neglected. Since at the beginning of the incubation protoplasts and chloroplasts did not contain radioactive material, c_s^i was neglected, too. Equation (2) is then simplified to

$$J_s/c_s^0 = -P_s, \quad (3)$$

P_s values were calculated for the undissociated species of electrolytes.

Radiochemicals ^{14}C-labelled ABA, IAA, BA, acetate, MVA (lactone), sucrose, glucose and sorbitol and ^{3}H-labelled water and glycerol were purchased from Amersham & Buchler (Braunschweig), ^{3}H-labelled gibberellic acid from NEN (Boston).

Results

Determination of the surface

For the calculation of the permeability coefficients P_s of the plasmalemma and the chloroplast envelope the size of the surface of the protoplasts, respectively

Table I. Surface and volume data of spinach protoplasts (suspended in a medium containing 0.5 M sorbitol) and isolated intact chloroplasts (suspended in a medium containing 0.33 M sorbitol) as obtained by three different methods (primary data = black numbers). Since data obtained by the silicone-oil-layer centrifugation technique are osmotic rather than total volumes, values were corrected by the addition of the non-osmotic volume, which is believed to be at least 20% of the total volume. Chloroplast data are also corrected for the integrity of the suspension (generalized values). Since chloroplasts in a medium containing 0.33 M sorbitol have spheroid size rather than the size of a true sphere, surface data for chloroplasts are slightly underestimated. In the average one protoplast contained 112×10^{-9} mg of chlorophyll and one chloroplast 1.1×10^{-9} mg of chlorophyll. Note that the data obtained by the microsomal method reflect data of a mixed population of smaller protoplasts from the *palisade parenchyma* and larger protoplasts from the *spongy parenchyma*. Therefore values of column 1 cannot be converted directly to each other.

Parameters	Dimension	Protoplasts		Chloroplasts	
		Microscopical method	Silicone-oil-layer centrifugation technique	Coulter Counter	Silicone-oil-layer centrifugation technique
Diameter	µm	**44**	36	4.0	4.1
Volume	µm³	44500	25300	**33**	37
Volume × mg^{-1} chlorophyll	µl	380	**226**	31	**34**
Surface	µm²	5890	4170	50	54
Surface × mg^{-1} chlorophyll	cm²	513	372	460	497

the chloroplasts is required. Table I shows the results of corresponding measurements and calculations. Surface values obtained by three different methods are in principal agreements with results of other investigators [9, 10, 13, 14]. Data of Table I are generalized: They are corrected for the percentage of broken chloroplasts in the suspersion. Values are restricted to protoplasts suspended in a medium containing 0.5 M sorbitol, respectively to chloroplasts suspended in a medium containing 0.33 M sorbitol. For the calculation of P_s values from the uptake experiments of this investigation the individual surface data of the experiments were used which take into account the varying percentage of integrity of the chloroplast suspension.

Rates of uptake

The time-dependent uptake of phytohormones and other solutes of interest into protoplasts and chloroplasts was measured by the silicone-oil-layer centrifugation technique. Fig. 1 gives an example for

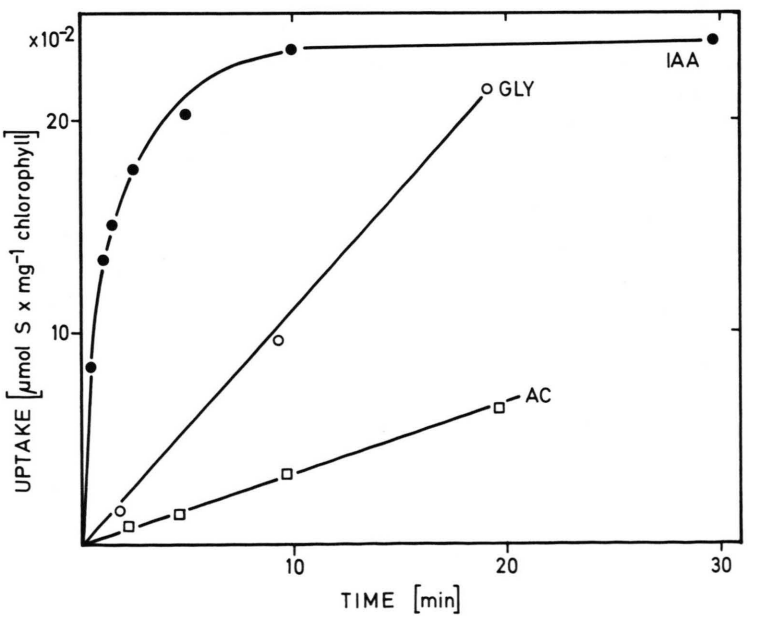

Fig. 1. The uptake of IAA, glycerol and acetate into protoplasts from spinach mesophyll cells. IAA and acetate were applied at external concentrations of 2.5×10^{-4} M, whereas the glycerol concentration was 2.5×10^{-3} M. From the period of linear uptake rates were calculated. Corresponding experiments were carried out for all solutes and with both the protoplast and the chloroplast system.

Table II. Uptake of phytohormones and related compounds into intact protoplasts and chloroplasts from spinach mesophyll cells (average of three independent experiments with two parallels each). The pH of the medium was 7.0 and the external concentration of the radioactive compounds was 2.6×10^{-4} M (20 °C, darkness). In column A rates of uptake are computed on the basis of the chlorophyll content, whereas in column B the uptake was related to the size of the surface of the system (*cf.* Table I).

Compound	Rate of uptake			
	A $\mu mol\ S \times mg^{-1}$ chlorophyll $\times h^{-1}$		B $pmol\ S \times cm^{-2} \times sec^{-1}$	
	Protoplasts	Chloroplasts	Protoplasts	Chloroplasts
BA	3.3	22.3	2.2	19
IAA	3.9	3.2	2.6	2.4
MVA	0.09	1.5	0.06	1.1
ABA	0.033	1.1	0.022	0.8
AC	0.16	1.3	0.11	0.9
GA_3	0.008	0.4	0.005	0.26

protoplasts and three different solutes. Rates of uptake were calculated from the period of linear uptake. This period differed in chloroplasts and protoplasts and with different solutes. With some compounds and chloroplasts uptake was too fast to be resolved at room temperature. In such cases minimal rates were computed. On the other hand penetration of compounds such as sucrose was so slow that reliable rates of uptake could not be measured within 10 or 20 min. In these cases maximal rates of penetration were calculated. An extension of the incubation time was not possible, because the integrity of chloroplasts decreases after longer times, if kept at room temperature. The results for the uptake of phytohormones and possible precursors of ABA-synthesis are summarized in Table II. With both the chloroplast and the protoplast system uptake exhibits the following order: BA ≥ IAA > MVA > ABA > GA_3. A direct comparison of the permeability of the chloroplast envelope with that of the plasmalemma can be made by comparing fluxes computed on the basis of μmol *S* per unit area and time instead on the basis of the chlorophyll content. The columns 3 and 4 of Table II indicate that the chloroplast envelope is much more permeable for most of the investigated solutes than the plasmalemma.

Permeability coefficients

From the rates of uptake (Table II) and the surface data of Table I permeability coefficients were calculated using Eqn. 3 (Table III). Phytohormone anions are in equilibrium with the undissociated phytohormones. P_s values were calculated for the undissociated species using the pK values listed in Table IV, because neutral solutes penetrate much faster than charged solutes of comparable structure [1–4, 24]. First of all Table III demonstrates again that the chloroplast envelope is much more permeable for most of the solutes than the plasmalemma. This is not only valid for phytohormones, but also for non-electrolytes such as glucose, glycerol, or sucrose. However, the order of the P_s values is very different from that of the uptake rates. P_s values exhibit the order IAA > GA_3 > (BA or ABA) > MVA, whereas the uptake rates showed the

Table III. Permeability coefficients P_s of the chloroplast envelope and the plasmalemma of spinach mesophyll cells for phytohormones (undissociated species) and some other solutes. During the uptake experiments the external concentration of phytohormones, MVA and acetate was 2.5×10^{-4} M, whereas the concentration of glycerol, glucose and sucrose was 2.5×10^{-3} M. The sorbitol concentration was 40 mM and in this case mannitol was used as osmoticum.

Compound	Permeability coefficient P_s $[m \times sec^{-1} \times 10^{-8}]$	
	Chloroplast envelope	Plasmalemma
BA	105	11
IAA	1733	1980
MVA	6.2	0.3
ABA	494	2
AC	810	91
GA_3	1314	41
GLY	10.2	0.2
GLU	4.2	0.2
SOR	< 0.2	0.01
SUC	< 0.3	0.003

Table IV. Physico-chemical data of investigated solutes. K_r for electrolytes was calculated for the undissociated species (pH 7.0). Since both the molecular weight and K_r do influence membrane penetration the ratio of K_r to M_r was calculated. M_r was inflated by using its 1.5 fold power (empirical factor).

Compound	Molecular weight	pK	Distribution coefficient K_r (Conc. of $S_{octanol}$/ Conc. of S_{medium})	$\frac{K_r}{M_r^{1.5}}$
BA	172	–	0.541	24×10^{-5}
IAA	175.2	4.7	30.6	13×10^{-3}
MVA	180.1	–	0.145	98×10^{-6}
ABA	264.3	4.8	27.6	64×10^{-4}
AC	60.1	4.75	1.83	39×10^{-4}
GA_3	346.4	3.8	3.79	58×10^{-5}
GLY	92.1	–	0.0154	17×10^{-6}
SOR	182.2	–	0.0015	61×10^{-8}
GLU	180.2	–	0.0014	60×10^{-8}
SUC	342.3	–	0.0009	14×10^{-8}

order BA ≥ IAA > MVA > ABA > GA_3. The sequence of P_s values reflects the theoretical capacity of permeation, but does not consider the existing proton concentrations in the cytoplasm and the organelles *in vivo*. The sequence of uptake rates is of more practical importance, since this order reflects the situation at physiological pH values around 7.0. Regarding the magnitude of P_s values for the plasmalemma and for solutes such as glycerol one may our data with those obtained for the plasmalemma of micro- and giant algae [15–17] or artificial bilayers [18]. They are at the upper limit of the reported broad range. Regarding the phytohormones, a P_s value of 1000 m × sec^{-1} × 10^{-8} was reported for the undissociated IAA for the plasmalemma of an alga [2] and 100–1000 m × sec^{-1} × 10^{-8} for artificial lipid bilayers [25]. This compares with a P_s value of 1980 m × sec^{-1} × 10^{-8} for the plasmalemma of spinach mesophyll cells (Table III). For the chloroplast envelope no P_s values for phytohormones have been reported, but a P_s-value of about 800 m × sec^{-1} × 10^{-8} can be calculated for ABA from the data of Heilmann *et al.* [3], which compares with a P_s-value of about 500 m × sec^{-1} × 10^{-8} reported in this paper (Table III).

Correlation between P_s values and physico-chemical data of the solutes

The permeability of a biomembrane is thought to be proportional to the distribution coefficient K_d and inversely proportional to the molecular weight, respectively the molecular volume of a compound:

$$P_s = f K_d / M_r . \quad (4)$$

As can be seen from the data of Table III and IV, no clear-cut correlation exists between P_s and either the

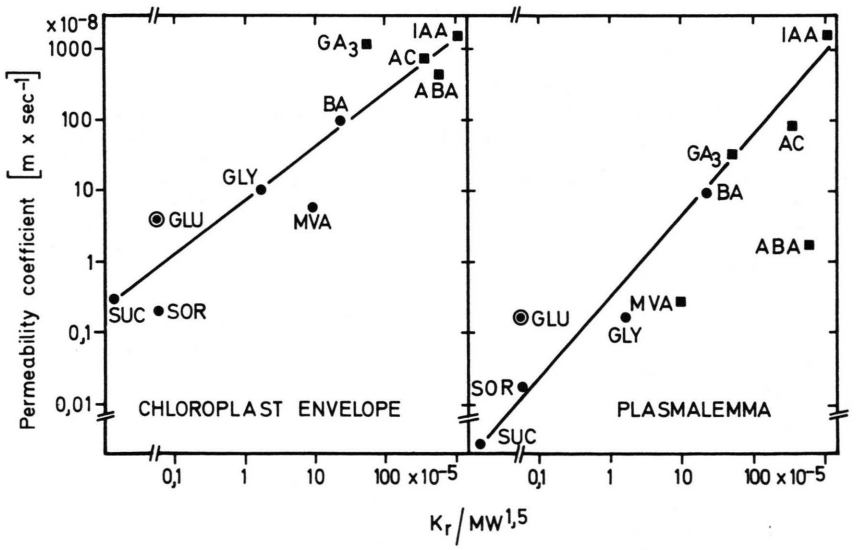

Fig. 2. The logarithm of the permeability coefficients P_s of the chloroplast envelope and the plasmalemma from spinach mesophyll cells for various non-electrolytical and electrolytical solutes as function of the logarithm of the ratio distribution coefficient K_r to the molecular weight, inflated by using its 1.5 fold power (Collander-plot) Electrolytes (■), non-electrolytes (●). For glucose (double circle) the involvement of facilitated diffusion cannot be excluded [23]. For electrolytes it was assumed that the undissociated species penetrate biomembranes much more rapidly than the anionic species.

distribution coefficient or the molecular weight alone. Both parameters have to be combined as shown in Eqn. (4). P_s values depend also on the chemical nature of the solutes (compare *e. g.* Overton's rules, [12]), but the effect of the chemical nature is surprisingly small compared to that of the ratio K_d/M_r.

For a given membrane and a special distribution coefficient K_r Eqns (1) and (4) can be combined to

$$P_s = f\,\alpha_k\,K_r^{-\beta_k}/M_r \cdot 1.5\,, \qquad (5)$$

in which f is a factor depending on the biochemical properties and the thickness of the membrane, whereas α_k and β_k depend on the special partition system used for the determination of K_r. Collander [15] demonstrated that in a double logarithmic plot P_s *versus* K_r/M_r resulted within certain limits in a straight line, if the molecular weight was inflated by using its 1.1–1.5 fold power. This factor was determined empirically and depends upon both the properties of the tested membranes and the partition system used. In Table IV we calculated from M_r and K_r the term $K_r/M_r \cdot 1.5$. K_r was calculated for the undissociated species of the electrolytes using the pK values shown in Table IV. The logarithm of this term was plotted as function of the logarithm of the P_s values ("Collander-plot", Fig. 2). It can be seen that a number of different solutes such as sugars and sugar alcohols can be grouped with the phytohormones around a straight line as predicted by Collander [15].

Discussion

The data of this investigation clearly establish:

1) The chloroplast envelope is much more permeable for most of the investigated phytohormones and possible precursors and also for sugars and sugar alcohols than the plasmalemma. Since the chloroplasts envelope consists of two unit membranes and the plasmalemma only of a single unit membrane this might be surprising. However the physiological role of the chloroplast is entirely different from that of the plasmalemma. Also the chemical composition of the chloroplast envelope is unusual if compared with other plant membranes, *e. g.* with that of the plasmalemma [13, 20]. Furthermore the outer membrane of the chloroplast envelope is much more permeable for many solutes of low and medium molecular weight, whereas the inner membrane functions as the physiological barrier of the chloroplasts [19]. Thus our results demonstrate a high permeability of the inner membrane of the chloroplast envelope and a low permeability of the plasmalemma for various solutes of different chemical nature.

2) The P_s values of the chloroplast envelope and the plasmalemma for the protonated species of phytohormones are significantly higher than those for glycerol. Glycerol is thought to be a rapidly penetrating solute for many plant and animal membranes. Thus phytohormones exhibit a high penetration capacity.

3) The order of uptake of phytohormones at an external pH of 7.0 is BA ≥ IAA > ABA > GA_3. This order is of practical importance. It should be considered during uptake studies with plant hormones *in vivo*. The order of P_s values, however, is IAA > GA_3 > (ABA or BA). This sequence reflects penetration of the protonated species. P_s values of the chloroplast envelope and the plasmalemma for phytohormones and also for sugars and sugar alcohols correlate with the ratio of the distribution coefficient K_r to the molecular weight, if the latter is inflated by using its 1.5 fold power. Thus phytohormones approximately follow the predictions of Collander [15].

It is not permissible to draw conclusions from the measured P_s values for the inter- or intracellular distribution of phytohormones. The steady-state distributions of phytohormones are governed by other factors, such as the pH-gradients with electrolytes [3, 21], lipophilicity of the compartment, and specific or unspecific binding [21]. The P_s values do however influence the kinetics of distribution changes caused *e. g.* by light-dark transients or by osmotic stress.

The results of this paper confirm also the good permeability of the chloroplast envelope for the mevalonic acid lactone [22]. In earlier papers it was questioned that MVA can be used as precursor of ABA-synthesis in chloroplasts because of its inability to penetrate the chloroplast envelope and to reach possible sites of ABA-synthesis in the stroma. This paper clearly demonstrates that this is not the case. At an external pH of 7.0 MVA enters the chloroplasts as fact as ABA or acetate do.

It should be noted that our results do not suggest that the uptake of any of the investigated phytohormones is facilitated by carriers (*e. g.* [4], but see also [5]). The P_s values follow the predictions of

Collander [15], which indicates that the phytohormones penetrate biomembranes preferentially by simple diffusion. If the uptake rates of phytohormones would be drastically enhanced by facilitated diffusion, P_s values of these solutes should be significantly higher than predicted by the Collander-plot. However, we cannot exclude the existence of phytohormone carriers in cell membranes of other plant tissues or membranes of other organelles. Furthermore we cannot exclude the existence of carriers with very low affinities to their substrates.

In further experiments an extension of this study is planed to obtain comparable data also for the tonoplast of spinach mesophyll cells.

Acknowledgements

This investigation was supported by the Deutsche Forschungsgemeinschaft and by a grant of the Stiftung Stipendien-Fonds des Verbandes der Chemischen Industrie. The technical assistance of Mrs. G. Hartmann and Mr. V. Kummer is acknowledged. We thank Prof. U. Heber for stimulating discussions.

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