

Protoplasts from Oat Primary Leaves as Tools for Experiments on the Compartmentation in Lipid and Flavonoid Metabolism

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Protoplast Yield and Stability, Lipid and Flavonoid Components, Organelle Isolation

Mesophyll protoplasts were isolated from defined stages of developing primary leaves of *Avena sativa* by a simple procedure in high and reproducible yield of about 50%. Rates of photosynthesis were measured by different techniques and turned out to be lower by a factor of 2 – 5 with protoplasts as compared to parent tissues, whereas dark respiration rates were about the same. Stability of protoplasts during storage in suspension medium at room temperature was checked by various methods. In contrast to protoplast numbers and accessibility of soluble enzymes, which suggested reasonable stability, photosynthetic oxygen evolution seems to be a more sensitive criterion. This rate dropped to about 50% of its original value during storage for 3 h. The protoplasts from different developmental stages did not show significant differences with respect to these various parameters. Mechanical rupture or chemical destruction of the plasma membrane by filipin resulted in release of chloroplasts, which according to activity profiles of soluble marker enzymes after gradient centrifugation were intact. Centrifugation in a vertical rotor separated mitochondria from chloroplasts. Irrespective of the developmental stage of the parent tissue each protoplast contained about 200 chloroplasts. In conjunction with chlorophyll determinations it was calculated that about 2×10^9 chloroplasts account for 1 mg of chlorophyll. Lipid mixtures from leaves, protoplasts and chloroplasts were compared. They did not contain compounds which usually indicate enzymatic alteration due to destruction of cellular compartmentation. Flavonoid profiles obtained from mesophyll protoplasts deviate significantly from epidermal patterns and prove the occurrence of flavonoids in photosynthetically active mesophyll cells.

Introduction

Various areas of plant physiology and biochemistry are investigating aspects of subcellular localization of enzymes, substrates and products. The availability of purified organelles is a prerequisite for these studies and the difficulty in using conventional tissue fractionation procedures increases with increasing fragility of the structures to be isolated. The resulting organelles often exhibit cross contamination and are obtained in low yields.

In addition, increasing interest is being directed towards elucidation of intracellular ways travelled by many compounds before reaching their final place of destination and function. To this group belong all those compounds which accumulate in organelles or membranes without being synthesized at these particular sites. Apart from macromolecular proteins and nucleic acids, many low molecular weight compounds such as lipids and flavonoids may behave in a similar way.

The obvious way to study such intracellular migration and cooperation at the level of organelles are

pulse-chase experiments combined with concomitant analyses of labelled products in various compartments [1]. Objects suitable for such experiments should incorporate and metabolize a wide variety of precursors and, more critical, should be easily dividable into a complete spectrum of intact organelles. At present most promising objects for such investigations seem to be mesophyll protoplasts. It has been shown repeatedly that they yield intact organelles after very mild desintegration [2, 3]. Therefore we included into our continuing programs on compartmentation and metabolism of membrane lipids and flavonoids mesophyll protoplasts as tools to study questions concerning subcellular distribution of enzymes and interorganellar transport of products.

Before starting these investigations we adapted and modified techniques required for experimentation with protoplasts from such plant species, which we use since several years for our investigations. In the following paper we summarize the results of our efforts to work out methods for isolation and purification of protoplasts and to find optimal conditions for their physiological performance and survival as measured by various parameters. In addition these experiments should show to what extent protoplasts reflect the metabolic activity of parent tissues. Finally, we

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also succeeded in releasing completely intact organelles from protoplasts.

Materials and Methods

1. Plants

Avena sativa L. cv. "Gelbhafer-Flämingskrone" was grown in a phytotron as described [4]. Developmental stages of primary leaves are characterized according to age as follows: stage 3 (5 days after sowing), stage 4 (6 days), stage 5 (7 days), stage 6 (8 days) [5]. Leaves were harvested at the beginning of a 13 h photoperiod when chloroplasts are free of starch [6].

2. Analytical procedures

Commonly used parameters and marker enzymes such as protein, chlorophyll, lipids, fatty acids, flavonoids, pyruvate kinase, cytochrome c oxidase and GAPDH were measured as before [4, 6, 7]. Malate dehydrogenase = MDH activity was determined in 0.6 M sorbitol buffered with 5 mM MES/KOH at pH 6.0 by following the decrease in extinction of NADH (0.06 mM) after addition of oxaloacetate = OAA (0.3 mM) in a total volume of 2.4 ml [8, 9]. Irreversible NADPH-dependent glyceraldehyde 3-phosphate dehydrogenase = GAPDH was assayed according to a published procedure [10]. For polarographic determination of oxygen evolution or consumption by protoplasts in suspension medium a Clark type electrode fitted to a reaction vessel of 1.5 ml was used which was illuminated with 60 000 lux of white light. Manometric measurements were carried out in conventional Warburg flasks. For photosynthetic measurements at 12 000 lux the center well contained 0.2 ml of 1 M $\text{NaHCO}_3/\text{Na}_2\text{CO}_3$ in a ratio of 85/15, whereas for respiration 0.3 ml of 10% KOH were used. In both cases 1.5 ml of protoplast suspension were assayed. Leaf pieces placed in the outer compartment were kept moistened by addition of water (0.5 ml). CO_2 -consumption was measured with an infrared gas analyzer (IRGA) in a closed circuit gas system [11].

3. Isolation of protoplasts from oat

Each leaf was cut transversely from the adaxial side about 1 cm below the tip, which then was removed together with as much as possible of the adhering abaxial epidermis of the remaining part of the leaf. After cutting off the basis, a leaf piece was obtained which was devoid of its abaxial epidermis. It

represents the middle portion of the leaf and increases in length with increasing stage number [5]. Due to their different size 10 – 30 of such pieces were floated for plasmolysis in a petri dish (diameter 9 cm) on 15 – 20 ml of osmoticum (0.6 M sorbitol in H_2O , resulting pH 5.0; buffering with 5 mM MES/KOH at pH 5.6 was also used), which then was replaced (water aspirator) by 15 – 20 ml of enzyme medium (cellulysin grade B from Calbiochem, 1% in 0.6 M sorbitol, adjusted with KOH to pH 5.6 – 5.8). After a vacuum infiltration of 1 – 3 min the petri dishes were kept at 25 °C for 4 h in the dark for release of protoplasts. Incubation with shaking or in dim light (1200 lux) did not improve the final yields. The resulting mixture was passed through a sieve of stainless steel to remove undigested tissues from the suspension of protoplasts, which then were collected by sedimentation (3 – 5 min at $55 \times g$). After removal of the supernatant solution (water aspirator) protoplasts were washed twice by resuspension in suspension medium (0.6 M sorbitol, 5 mM MES/KOH pH 5.6) and subsequent sedimentation. The resulting suspensions, almost free of contaminating chloroplasts and other cell debris, were adjusted to a protoplast titer of about $10^6/\text{ml}$. Protoplast numbers were counted microscopically using a hemocytometer.

4. Protoplasts from spinach and chervil

For this procedure only a general outline will be given, since details have been described elsewhere [12]. *Spinacia oleracea* L. cv. "vital" and *Anthriscus cerefolium* L. were grown in a phytotron for 3 and 5 weeks, respectively. Leaves were plasmolyzed in 0.5 M sorbitol and then cut into small pieces by using razor blades. After washing with 0.5 mM sorbitol, leaf pieces were vacuum infiltrated with the enzyme solution (0.5 – 0.7 M sorbitol, 0.1% bovine serum albumine, 1% macerozyme and 2% meicelase, both from Welling & Co., Hamburg, Germany, pH adjusted with KOH to 5.8). Protoplast release required incubation times of 4 – 7 h for *Anthriscus* and 12 – 16 h for *Spinacia*. After several washing steps protoplasts were freed from contaminating chloroplasts and other debris by sedimentation onto a cushion of 0.5 M sucrose.

5. Protoplast rupture and fractionation

Protoplasts (suspension of 2 ml, 300 μg chlorophyll) were ruptured mechanically [2, 3] by gentle

passage through a nylon net (mesh size 15 μm) closing the exit of a 20 ml-syringe. Microscopic observation showed that two passages resulted in complete disappearance of intact protoplasts without severe damage of chloroplasts and mitochondria. The suspensions of ruptured protoplasts were used for determination of chloroplast numbers per cell (Table I) and for gradient centrifugation (Figs 5–7). For chemical rupture protoplasts (1.5 ml of suspension, 120 μg chlorophyll) were incubated at room temperature for 10 min in the presence of filipin (100 μg dissolved in dimethyl formamide, Upjohn company, Kalamazoo, MI, USA) with occasional swirling.

The suspensions (1–2 ml) containing ruptured protoplasts were loaded onto a continuous gradient of sucrose (22–60%, w/w, 26 ml) made up in 10 mM Hepes, 5 mM MgCl_2 , pH 7.6. After centrifugation for 20 min at $3300 \times g$ (Hettich Rotixa/K centrifuge) in cellulose nitrate tubes, gradients were fractionated into fractions of 1.2 ml (Isco fractionator, model 640).

For separation of mitochondria identical gradients were centrifuged in a SV-288 vertical rotor [13] using the RC-5B Sorvall centrifuge.

Results and Discussion

Isolation and yields of protoplasts

Before concentrating our efforts on the isolation and characterization of *Avena* protoplasts we tried to obtain protoplasts from plants such as spinach, tobacco and chervil which due to the presence of hexadecatrienoic acid [14] are particularly interesting for investigations in the lipid field. We tried to reproduce several published procedures (ref. cited in [12]), but obtained yields which were far below those obtained with *Avena* (Fig. 1). Despite experimental variation of many parameters we were not able to standardize a procedure for our cultivars resulting in satisfactory and reproducible yields of protoplasts from these plants. Isolated protoplasts showed rates of light-dependent oxygen evolution of 18–75 (*Anthriscus*) or 15–35 (*Spinacia*) $\mu\text{mol}/\text{mg}$ chlorophyll/h as measured polarographically. Respiration rates were 5–10 $\mu\text{mol}/\text{mg}$ chlorophyll/h for both plants. Lipid compositions are presented together with those for *Avena* (see Fig. 8 and Table II).

Therefore we changed to *Avena*, which has been well characterized with respect to its flavonoid metabolism [4–6, 15–19]. Many publications describe

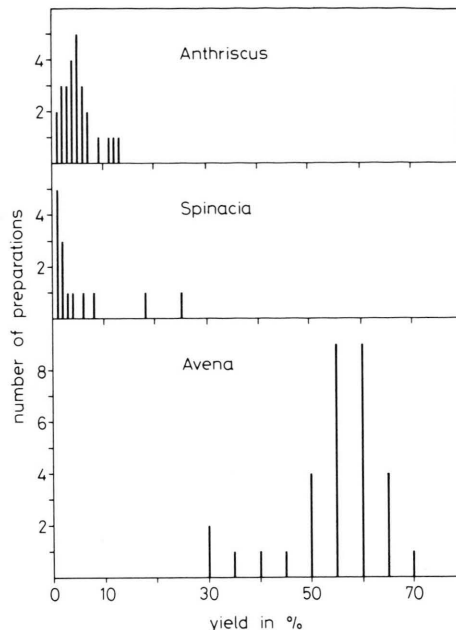


Fig. 1. Yields of protoplasts as measured by recovery of chlorophyll in purified protoplasts. The chlorophyll content of leaf pieces used for the isolation was set as 100%. Purification of spinach and chervil protoplasts required cushion centrifugation, whereas oat protoplasts were pure after differential sedimentation.

isolation procedures for protoplasts from the related cereal plants barely, wheat, rye, corn, sorghum and also oat applying various techniques of culture and leaf digestion [3, 20–43]. In addition we intended to isolate protoplasts from defined developmental stages of primary leaves from *Avena*, which have been characterized in our above mentioned publications.

It turned out that mesophyll protoplasts could be obtained in a pure state by the procedure described in the experimental part equally well from all stages in high and reproducible yields of about 50% based on chlorophyll recovery (Fig. 1). Yields depend strongly on a clean removal of the lower epidermis, which fact may explain the variation observed in Fig. 1. Similar observations have been made before by other authors [32, 36]. Variation of techniques including discontinuous gradients for purification of protoplasts released without prior removal of the epidermis [22] resulted in very low yields when carried out with our plants. Irrespective of leaf age we obtained $2–5 \times 10^6$ protoplasts/g fresh weight, which is in the same order as reported for other cereal plants [21, 36, 42].

Characterization of protoplasts

Since photosynthesis is one of the most important functions of leaf mesophyll, several methods were applied to measure this parameter for a comparison of isolated protoplasts with intact parent leaves of different ages as cultivated in the phytotron. The results are summarized in Fig. 2. With intact leaves CO_2 -fixation rates of about $150\text{--}170\text{ }\mu\text{mol}/\text{mg}$ chlorophyll/h were measured by recycling a gas stream in a closed system and keeping the concentration of CO_2 at a constant level. For these experiments only the middle parts of 5–6 leaves were fixed in a gas chamber excluding tips and basal parts of the intact seedlings from the measurement. The enclosed leaf portions were usually used for protoplast isolation. Therefore the resulting rates may be considered to be representative for *in vivo* performance of *Avena* mesophyll. In comparison, field grown oat leaves fixed $^{14}\text{CO}_2$ under different experimental conditions at a rate of about $150\text{ }\mu\text{mol}/\text{mg}$ chlorophyll/h [44]. When instead of intact organs leaf pieces were used in Warburg vessels to measure photosynthetic oxygen evolution, lower rates of $40\text{--}80\text{ }\mu\text{mol}/\text{mg}$ chlorophyll/h were obtained. This reduction may be ascribed to suboptimal conditions resulting from leaf injury and the particular environment in the reaction vessels. Therefore we consider rates of about $150\text{ }\mu\text{mol}/\text{mg}$ chlorophyll/h as more reliable in representing *in vivo* rates.

The photosynthetic activity of isolated protoplasts was measured by three methods using in addition to CO_2 -fixation and manometry also polarographic determination of oxygen evolution. Although different light intensities were used, all three methods resulted in comparable rates of $30\text{--}40\text{ }\mu\text{mol}/\text{mg}$ chlorophyll/h with polarographic values about 50% higher (Fig. 2). These lower values suggest that release of protoplasts from tissues and the aqueous surrounding in the suspension medium reduce the photosynthetic activity. At this point it may be mentioned that release of one protoplast necessitates cutting and re-sealing of $10^3\text{--}10^4$ protoplasmic connections with neighbouring cells. Photosynthetic rates of protoplasts as shown in Fig. 2 fit well into the range of CO_2 -fixation rates reported in the literature for protoplasts from various species, where rates as low as 6 and as high as $240\text{ }\mu\text{mol}/\text{mg}$ chlorophyll/h may be found [3, 37, 45–47].

Also included in Fig. 2 are respiration rates from leaf pieces and corresponding protoplasts. Manome-

tric and polarographic determination resulted in comparable values of $5\text{--}10\text{ }\mu\text{mol O}_2/\text{mg}$ chlorophyll/h for protoplasts, which compare well with manometrically measured rates from leaf pieces. These values result in $0.8\text{--}1.5\text{ }\mu\text{mol O}_2/\text{h}/10^6$ protoplasts, which may be compared to $0.7\text{ }\mu\text{mol}/\text{h}/10^6$ cells from barley aleuron [48]. Protoplasts from *Petunia* mesophyll displayed lower values of $0.1\text{--}0.2\text{ }\mu\text{mol O}_2/\text{h}/10^6$ protoplasts [50].

The investigations described above were conducted at pH 6.0. In experiments on pH-dependence of photosynthesis and respiration, variation of pH from 5.3 over 6.0, 6.7 to 7.6 did not result in any significant differences, whereas at pH values above 7.0 aggregation of protoplasts was observed. Therefore

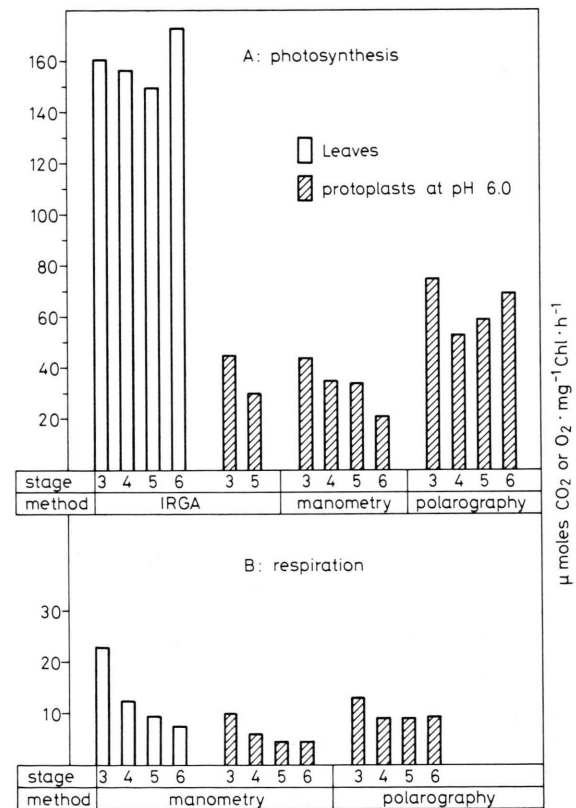


Fig. 2. Comparison of leaves and protoplasts from oat with respect to photosynthesis (A) and respiration (B), which were measured by several methods. IRGA: Fixation of CO_2 from a gas stream circulating in a closed system. Manometry: Oxygen evolution or consumption measured in Warburg flasks. Polarography: Oxygen evolution or consumption determined with a Clark type electrode. Each block represents a different stage in leaf development, which is indicated by its number.

pH 6.0 was used for all experiments, which is close to pH-values of 5.7 and 6.5 found to be favourable for preserving physiological activity of other cereal protoplasts [32, 36, 42].

Integrity and survival of protoplasts

For future experiments on the uptake and subcellular transport of precursors and metabolites it is important to know the integrity or permeability of the protoplast membrane. Therefore we tried to establish an enzymatic assay to measure plasmalemma integrity. This search was based on the possibility that compounds such as pyridine nucleotides may not be able to permeate this cell-enclosing membrane barrier. On the other hand, not all biomembranes are impermeable for these compounds. It is well known that from the membranes surrounding chloroplasts and mitochondria only the inner ones are impermeable to these nucleotides. Accordingly an integrity assay for chloroplasts is based on the impermeability of their inner membranes for NADPH and includes measurements of GAPDH activity in lysed and unlysed samples [51]. Assuming a similar NADH impermeability of the plasmamembrane, cytoplasmically located enzymes should be suitable for measuring intactness of this membrane in protoplast suspensions.

Candidates for an assay of this kind should be pyruvate kinase and irreversible NADPH-dependent GAPDH, both of which are found nearly exclusively in the cytoplasm [10, 52, 53]. But these activities were too low to be measured reproducibly at pH-values, which are optimal for oat protoplast survival. At present this excludes their use as integrity markers. Therefore we examined two other enzymes, which are also, but unfortunately not exclusively, localized in the cytoplasm [2, 54, 55]. These two NAD(P)H-dependent dehydrogenases, oxidizing malate or glyceraldehyde 3-phosphate, behaved in the expected way. In suspensions of purified protoplasts only low activities were observed, whereas sonication or addition of Triton X-100 raised these rates dramatically as shown in Fig. 3. The effect of Triton is due to destruction of membranes and does not result from an enhancement of the enzymatic reaction itself, since Triton had no effect when using sonicated samples. The Triton-dependent rate comprises activities from cytoplasm as well as from other compartments, whereas the contribution of individual compartments to the rate observed before addi-

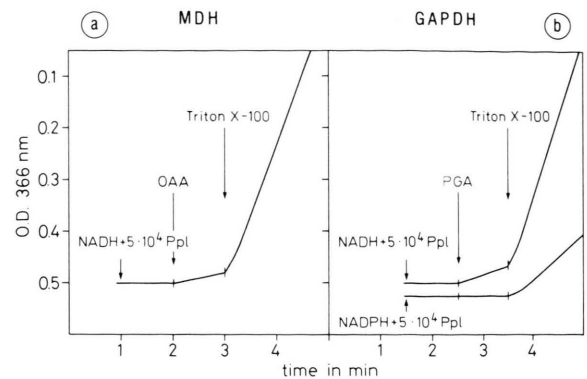


Fig. 3. Accessibility of dehydrogenases in oat protoplast suspensions. a: MDH. NADH absorbance as affected by the addition of oxaloacetate (OAA = oxaloacetate to start the reaction of accessible MDH) and of Triton X-100 (final concentration 0.1% to disrupt plasma and organelle membranes). b: GAPDH. NAD(P)H absorbance as affected by the addition of 3-phosphoglycerate (PGA = phosphoglycerate to start the GAPDH reaction in the presence of previously added ATP and phosphoglycerate kinase) and of Triton X-100 (to disrupt membranes). Ppl = protoplasts. All solutions were made up in suspension medium containing 0.6 M sorbitol to prevent damage of protoplasts by osmotic shock.

tion of Triton is not clear and depends on the stability of organelles released into the suspension medium. On the other hand, NADPH-dependent GAPDH activity, which is localized entirely or nearly entirely in chloroplasts [2, 54], is barely detectable in protoplast suspensions (Fig. 3 b).

This indicates, as is confirmed below by gradient centrifugation (Fig. 5) that chloroplasts, if present at all in protoplast suspensions, survive release into suspension medium after rupture of plasma membranes.

The release of NADH-accessible MDH was used in the experiments described below (Fig. 4 c) as a parameter for stability. MDH may occur in up to nine isoenzymes in plant cells [55] and is present in most compartments including cytoplasm, microbodies, mitochondria and chloroplasts [2], which in spinach contain 20 – 30% of the cellular activity [2, 52].

Apart from these considerations it is not clear at all, whether it is justified or not to assume that the plasma membrane, as expected from its function as cell envelope, is absolutely impermeable to metabolites such as 1,3-diphosphoglycerate and nucleotides, which at first glance is also suggested by the rate differences shown in Fig. 3. From experiments on the

recovery of poisoned CO_2 -fixation in plant protoplasts [47, 56] it has been concluded that the substrates involved in the MDH reaction (NADH, OAA, malate) can indeed pass plasma membranes, although it is not clear, if they can permeate freely without restriction. Therefore, measurement of rate differences of MDH or GAPDH activity before and after addition of Triton may not be used to calculate the absolute proportion of intact protoplasts as can be done with chloroplasts. But when these rate differences are determined at two different times of incubation they should reflect the decrease of plasma membrane integrity in the case of complete or partial impermeability. On the other hand, if one assumes free permeability of the plasmalemma, then the only slightly increased accessibility of MDH in protoplast suspensions during storage (Fig. 4 c) demonstrates that only chloroplasts and mitochondria retain intact membranes.

Next we tried to apply various techniques to measure protoplast stability (Fig. 4). The number of intact protoplasts as counted by microscope dropped by only about 10–15% during incubation for 3 h (Fig. 4 a). A decrease of similar magnitude results from enzymatic determination of MDH accessibility

in protoplast suspensions (GAPDH gave similar results). Protoplast numbers and MDH accessibility (Fig. 4 a and 4 c) suggest similar stability of protoplasts irrespective of the age of leaves, from which they were isolated. In contrast to these results, photosynthetic activity as measured by polarography dropped to about 50% of the original value after an incubation for 3 h at room temperature (Fig. 4 b). Therefore this parameter seems to be more sensitive to physiological variation and deterioration and does not seem to be directly related to the physical integrity of the protoplasts. Therefore photosynthetic activity may be a suitable indicator of physiological performance of protoplasts.

A loss of 50% activity is high compared to the stability reported for protoplasts from other sources, which sometimes showed hardly any decrease in photosynthetic activity even after 20 h when kept at 4 °C [3]. On the other hand, 3 h may be sufficient for labelling experiments, also in view of problems resulting from contamination by microorganisms [32]. Preliminary experiments with [^{14}C]cinnamic acid showed that this compound was taken up and metabolized by oat protoplasts during an incubation for 3 h. This incorporation was dependent on the presence of intact protoplasts, since protoplast disruption destroyed this activity completely. Staining with Evan's Blue [57] and fluorescein diacetate [26] indicated the absence of non-viable protoplasts obtained from the different developmental stages.

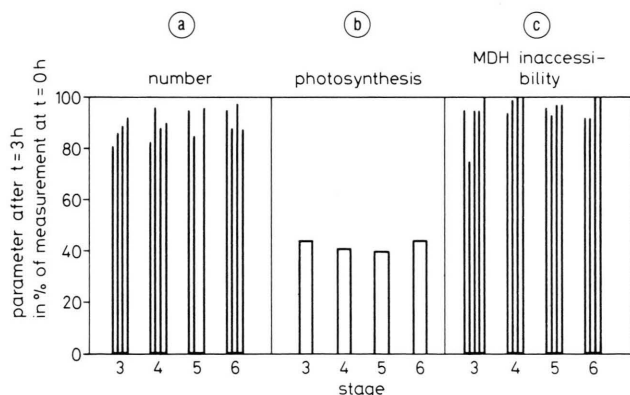


Fig. 4. Stability of oat protoplasts as measured by three parameters. In each case the value of the first measurement at $t = 0$ h was set as 100%, whereas the second determination carried out after 3 h of incubation at room temperature is expressed in % of the first value. a: decrease in protoplast numbers as counted in a hemocytometer. b: decrease of photosynthetic oxygen evolution measured polarographically. c: decrease of inaccessibility of substrates to cellular MDH (see text for explanation). Assays were carried out in 0.6 M sorbitol buffered with 5 mM MES/KOH to pH 6.0. During storage of protoplasts, enzymatic inaccessibility (differences between OAA- and Triton-enhanced rates, see Fig. 3 a) decreased, which effect was used as a stability criterion.

Release of organelles

The suitability of protoplasts as sources of intact organelles has first been demonstrated in experiments with several plant species [2, 3]. We applied the method of passing protoplast suspensions through nylon nets and obtained similar results with spinach, chervil and oat. Sucrose density gradient centrifugation of mechanically ruptured oat protoplasts at low speed resulted in nearly all cases in only one green band representing intact chloroplasts as shown by the localization of NADPH-dependent GAPDH in these fractions (Fig. 5 b), whereas only negligible activity was present in the supernatant fractions. On the other hand, protoplasts cannot be ruptured by simple centrifugation (Fig. 5 a), even not by applying higher accelerations, as are possible with the ultracentrifuge. In addition, the gradient shown in Fig. 5 a demonstrates that free chloroplasts are practically absent from our protoplast suspen-

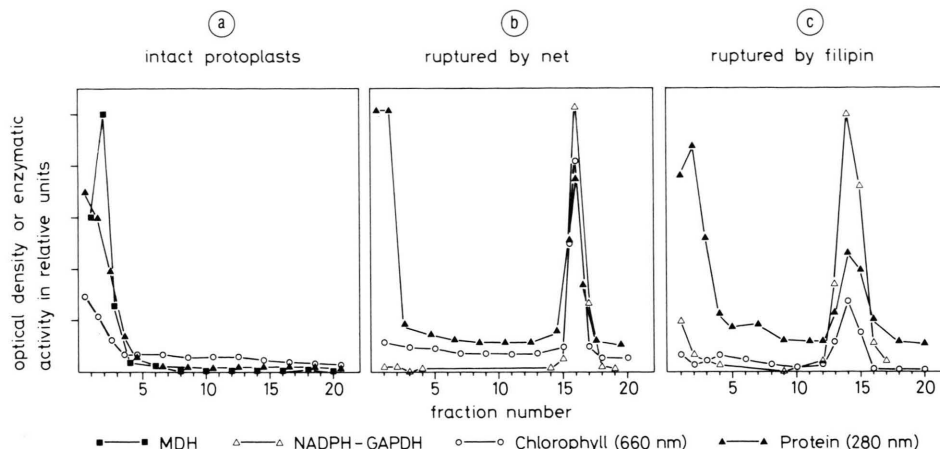


Fig. 5. Sucrose density gradient centrifugation at $3300 \times g$ of a: 1.5 ml oat protoplast suspension, b: 1.5 ml of protoplast suspension after two passages through a nylon net, c: 1.5 ml of protoplast suspension after 10 min incubation in the presence of filipin (100 μ g). Linear gradients of sucrose (60–22%) in 10 mM HEPES, pH 7.6, 5 mM $MgCl_2$ were used. The different curves in each panel have been spaced vertically to avoid superimposed baselines of different parameters. The zero baseline positions are represented by the ends at the right. Assays were carried out in solutions without sorbitol to release enzymes by osmotic shock.

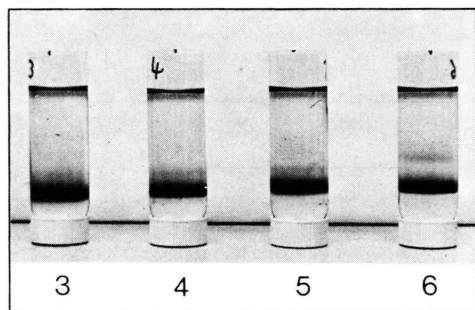


Fig. 6. Comparison of chloroplast release from oat protoplasts, which were isolated from different leaf stages as indicated by numbers. Protoplasts were ruptured by two passages through a nylon net. Sucrose gradient centrifugation was carried out as in Fig. 5. Dark bands correspond to intact chloroplasts.

sions (see above). The suitability of this method to isolate intact chloroplasts from different developmental stages of leaves is demonstrated in Fig. 6, where comparable gradients as described above are shown.

To isolate mitochondria, isopycnic centrifugation in sucrose gradients using swing-out rotors in the ultracentrifuge at $50\,000 \times g$ for 2–3 h could not be applied. Oat chloroplasts do not withstand this procedure without damage. After such centrifugations broken chloroplasts, mainly thylakoids of lower densities are found as contaminants of mitochondria.

Therefore we used shorter centrifugation times as are possible with vertical rotors and obtained satisfactory separation of mitochondria from intact chloroplasts (Fig. 7). The profile of MDH activity indicates major proportions to be present in chloroplasts, mitochondria and cytoplasm. This distribution should also be looked at in view of our preceding discussion of the use of cytoplasmic MDH as marker for plasmalemma integrity. Subcellular proportions of MDH activity, particularly the mitochondrial fraction, varied to some extent in different experiments. The proportion of NADH-dependent GAPDH activity was low in the cytoplasm compared to results from spinach [2, 58, 59].

In addition to mechanical release of cell organelles another way may be chemical destruction of plasma membranes by using polyene antibiotics which intercalate specifically into sterol containing membranes [60]. To this group belong amphotericin B and nystatin, which have been used before with plant protoplasts, but apparently without significant success [61]. It is known that these compounds intercalate into lipid bilayers forming permanent pores with hydrophilic pores, through which only small molecules such as water and ions can freely pass. Therefore these two compounds would not be expected to be suitable for release of organelles. In contrast to these two, filipin not only induced release of potassium ions but also of proteins such as glucose 6-phosphate

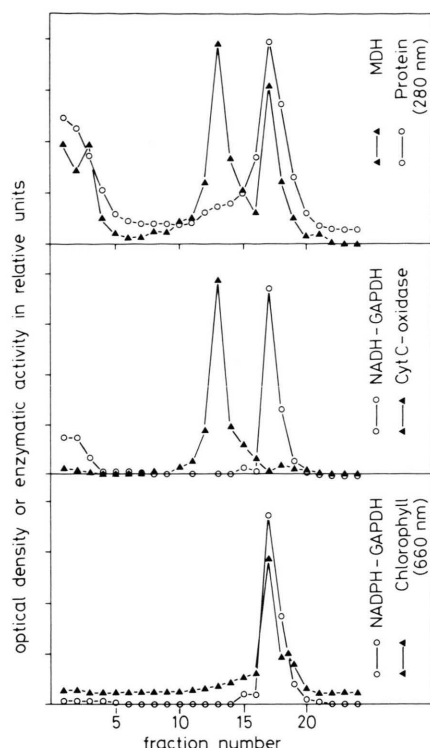


Fig. 7. Separation of mitochondria and chloroplasts after mechanical release from oat protoplasts. The sucrose gradient was centrifuged at 20 000 rpm for 15 min in a vertical rotor (see experimental part). Baseline adjustment as described in the legend of Fig. 5.

dehydrogenase from liposomes [60]. This observation together with a presumably elevated sterol level also in plant plasma membranes [62] suggested the experiment shown in Fig. 5 c. 10 min incubation of protoplasts in the presence of filipin followed by gradient centrifugation showed that intact protoplasts had completely disappeared. The chlorophyll

was concentrated in the band of intact chloroplasts, which again contained most of the GAPDH, although a small proportion was present in the supernatant fractions. This experiment confirms the effects of filipin observed in previous studies with liposomes and may demonstrate differences in the sterol content of plasma membrane and chloroplast envelopes, which in contrast to the plasmalemma survived this treatment.

The rupture of protoplasts without desintegration of chloroplasts enables an accurate determination of the number of chloroplasts per cell. It is obtained by first counting the protoplast number per volume of protoplast suspension and by a second counting of chloroplast numbers after passage through a nylon net. The results are included in Table I and demonstrate that irrespective of leaf age each cell contains about 200 chloroplasts. This indicates that the phase of plastid multiplication is completed in the tissues used for our experiments. Mesophyll cells from spinach contain similar numbers of chloroplasts [63].

Because chlorophyll and protein determinations were carried out with the same samples, the above countings allowed the calculation of the amount of chlorophyll or protein per cell and the number of chloroplasts or cells per mg of chlorophyll. Again these values and also those for lipid components are in the same order of magnitude as reported for other plants [64–67]. Table I summarizes quantitative data and recalculations.

Lipids and fatty acids

We also determined the lipids and fatty acids from chloroplasts, protoplasts and leaves with the results shown in Table II and Fig. 8. The normal fatty acids and membrane lipids known from plant cells

Table I. Quantitative determination of various components in *Avena* protoplasts and chloroplasts released from protoplasts. The numbers in the last column represent independent preparations and determinations used to calculate means and standard deviations.

protoplasts/g fresh weight	$2 - 5 \times 10^6$	
chloroplasts/protoplast	216 ± 37	20
chlorophyll/protoplast	$9.0 \pm 2.6 \times 10^{-11}$ g	23
chlorophyll/chloroplast	$4.4 \pm 1.4 \times 10^{-13}$ g	20
protein/protoplast	$1.8 \pm 0.5 \times 10^{-9}$ g	17
protein/chlorophyll in protoplasts	20	
protoplasts/mg protein	$5.5 \cdot 10^5$	
protoplasts/mg chlorophyll	$1.1 \cdot 10^7$	
chloroplasts/mg chlorophyll	$2.4 \cdot 10^9$	
fatty acid methyl esters/protoplasts	$1.8 \pm 0.5 \times 10^{-10}$ g	16
fatty acid methyl esters/chloroplast	$6.1 \pm 1.5 \times 10^{-13}$ g	15
monogalactosyl diacylglycerol/protoplast	$8.5 \pm 1.5 \times 10^{-11}$ g	4
monogalactosyl diacylglycerol/chloroplast	$3.4 \pm 1.0 \times 10^{-13}$ g	5
phosphatidyl choline/protoplast	$3.3 \pm 0.7 \times 10^{-11}$ g	4
phosphatidyl choline/chloroplast	$4.4 \pm 1.6 \times 10^{-14}$ g	5
total flavonoid/protoplast	$3.3 \pm 0.4 \times 10^{-11}$ g	18

Table II. Comparison of fatty acid mixtures (in mol %) from various sources as indicated. Fatty acids are characterized by number of carbon atoms and double bonds. 16 : 1-*trans* = 3-*trans*-C_{16:1}. The numbers of independent preparations and analyses are indicated in the last column. Components below 0.5% have been omitted.

Sample	Fatty acids								n
	16 : 0	16 : 1	16 : 1- <i>trans</i>	16 : 3	18 : 0	18 : 1	18 : 2	18 : 3	
<i>Spinacia</i> protoplasts	18.9	—	1.9	7.8	—	7.3	11.9	52.1	3
<i>Anthriscus</i> leaves	15.0	2.6	3.2	15.7	0.8	1.5	21.0	40.3	2
protoplasts	14.4	2.4	3.6	15.5	1.5	2.1	20.1	42.6	4
chloroplasts	13.7	2.6	4.6	21.2	1.7	2.6	15.7	38.1	1
<i>Avena</i> leaves	18.1	—	2.3	—	1.2	2.8	18.4	56.4	3
protoplasts	13.9	—	2.9	—	0.5	1.7	14.2	66.5	5
chloroplasts	15.0	—	4.0	—	1.0	2.2	11.5	67.9	6

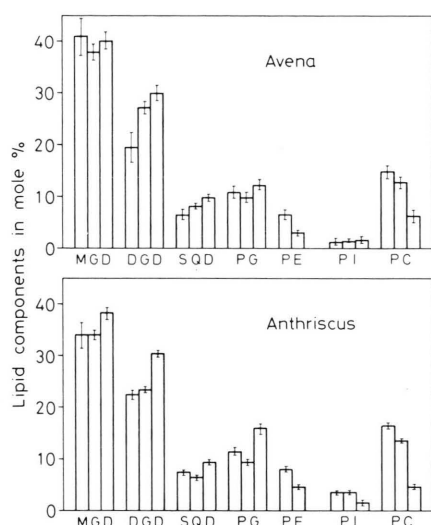


Fig. 8. Comparison of acyl lipid mixtures from leaves (left columns), protoplasts (middle) and protoplast-derived chloroplasts (right) from *Avena* and *Anthriscus*. Standard deviations are indicated by vertical bars and were calculated from several independent preparations (5 in the case of all *Avena* samples, 5 for *Anthriscus* protoplasts, 3 for leaves and 2 for chloroplasts). MGD, DGD, SQD = monogalactosyl-, digalactosyl-, sulfoquinovosyl diacylglycerol; PG, PE, PI, PC = phosphatidyl glycerol, -ethanolamine, -inositol, -choline.

are also found in very similar proportions in the protoplasts. This indicates that major alterations of these mixtures have not occurred during removal of cell walls, as in contrast has been observed during protoplast formation from bacterial cells [68]. In addition, phosphatidic acid, acylated galactolipids and free fatty acids, all of which may result from enzymatic alteration of membrane lipids after destruction of cellular compartmentation, were not detected [69]. Chloro-

plast lipids did not contain phosphatidyl ethanolamine which indicates the purity of these organelles after release from protoplasts and recovery from sucrose gradients. Since the lipid composition as presented in Fig. 8 was determined *via* acyl ester content of individual components, we were also able to compare fatty acid profiles from individual lipids extracted from leaves, protoplasts and chloroplasts (data not shown). There were no major or significant differences between the samples of different origin, so that we can be sure, that lipids from protoplasts resemble lipids from leaf mesophyll.

Flavonoids

Flavonoid analyses carried out with whole oat leaves [4] and isolated tissues such as epidermis and mesophyll have shown specific patterns for these two

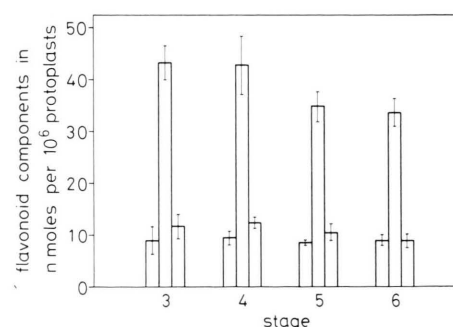


Fig. 9. Major flavonoids in mesophyll protoplasts obtained from different leaf stages of oat. In each stage the left column represents 7-methoxy-vitexin 2''-rhamnoside, middle column = isovitexin 2''-arabinoside and right column = vitexin 2''-rhamnoside [71]. Isovitexin is present as minor component. Standard deviations are indicated by vertical bars and were calculated from analyses of 5 independent preparations.

parts of the leaf [15]. Without use of protoplasts a further differentiation into the various types of cells and tissues, which make up the mesophyll as interior part of the leaf, was not possible. The availability of protoplasts derived exclusively from green mesophyll tissues enable an investigation of the flavonoids present in photosynthetically active mesophyll cells, which after isolation lack cell walls and are enclosed only by their plasma membrane. As shown in Fig. 9 the major flavone of this cell type is isovitexin 2''-arabinoside representing about 65% of the flavonoid mixture irrespective of the leaf stage. The components 7-methoxy-vitexin 2''-rhamnoside and vitexin 2''-rhamnoside are present in lower levels. A possible decrease of the absolute content of flavones per cell with increasing leaf age has not yet been established.

Compared to our previously analyzed patterns for mesophyll and epidermis, protoplasts resemble me-

sophyll profiles. However, at present we can not decide whether or not flavonoids occur also in other than photosynthetically active tissues of the mesophyll or even in cell walls. In addition, it is not sure that cell populations present in protoplast suspensions are homogenous with regard to flavonoid content [70].

In future experiments the flavonoid patterns of subcellular compartments will be investigated to extend our previous studies on flavonoid localization in chloroplasts [6, 19].

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