High-yield isolation of grape leaf protoplasts as an instrument in physiological research[1]

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Summary. Protoplasts were isolated from young grape leaves in yields permitting reliable quantitative determination of cell components. Comparisons of the analytical data with results from intact tissue indicate that the isolates reflect the content of organic acids as well as the photosynthetic carbon flow of the source material.

Isolated protoplasts from plant tissues are widely used as experimental systems, with the interest of most scientists focusing on the prospects of somatic hybridisation and cell wall regeneration. Nevertheless, the potential of protoplasts is not confined to these 2 areas. The homogeneity of protoplast suspensions, combined with a ready accessibility to exogenous substances, make them ideal for the investigation of precursor-product relationships. This type of research, however, presupposes a thorough knowledge of the metabolic state of the protoplasts as well as that of the source tissue, thus permitting a general interpretation of data from protoplast experiments[2].

Material and methods. Several attempts have been made to isolate protoplasts from grape berries[3] and leaves[4]. However, it has proved difficult to obtain reproducible yields, the physiological condition of the source tissue being critical[5]. This problem was greatly reduced by the use of young leaves from sprouting hardwood cuttings of *Vitis vinifera* (cv. Riesling × Silvaner), which were grown without direct sunlight in a 0.3% Luwasa nutrient solution. The laminae were surface sterilized in 80% ethanol and then sliced into thin segments in a 0.35 M mannitol solution. About 1 g of the slices was transferred to a petri dish containing 13 ml of the following solution: 2% cellulase (Onozuka R-10); 0.35 M mannitol; 0.25 mM morpholinoethanesulfonate; 2 mM $CaCl_2$; 2 mM $MgCl_2$; 5 mM KCl; 10 mM cysteamine and 0.4% polyethylenglycol[3]. The mixture was brought to pH 5.8 with NaOH. The material was infiltrated under reduced pressure for 2 min and then incubated at 27 °C on a gyratory shaker. At the end of the digestive period, released protoplasts were decanted and filtered through a cotton wool pad, and the leaf slices gently rinsed twice with incubation solution without enzyme. The combined filtrates were pipetted onto 3 ml Ficoll 400 (20% by weight) in a centrifugation tube and spun at 1500 × g for 15 min. The intact protoplasts remain at the Ficoll interphase. Non-volatile organic acids in aqueous leaf and protoplast extracts were analyzed by gas-liquid chromatography of the trimethylsilyl derivatives of the respective anionic fraction[6]. For photosynthetic studies, a preparation containing approx. $4 \cdot 10^6$ protoplasts was incubated in $NaH^{14}CO_3$ (0.125 mM; 370 kBq) for 5 h in a waterbath at 25 °C and illuminated (Philipps HPL-N, 400 W). The experiments were terminated by centrifugation, and the pellet was subsequently freeze-killed and extracted[7].

Results and discussion. The isolation procedure yields approximately $50 \cdot 10^6$ protoplasts per g leaf tissue (fresh wt), during an incubation period of 5 h, which amounts to a release of 25%, based on the total chlorophyll content of the starting material and that of the isolates. The preparations were routinely checked by microscopic inspection of protoplast size and shape (cyclosis) and the integrity of the plasma membrane established by confirming the exclusion of an isotonic 2.5% trypan blue solution[8]. The stability of the tonoplast was demonstrated by its ability to retain neutral red in the vacuole after administration of the stain (0.1% in

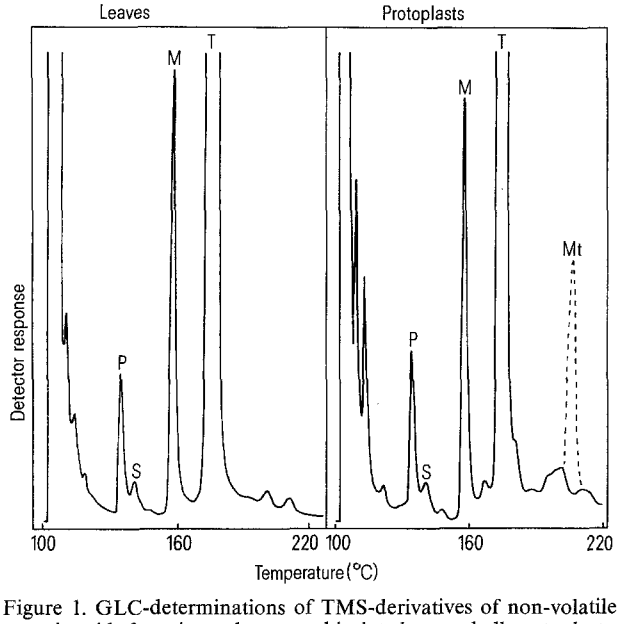

Figure 1. GLC-determinations of TMS-derivatives of non-volatile organic acids from intact leaves and isolated mesophyll protoplasts. P: phosphoric, S: succinic, M: malic, and T: tartaric acids. The protoplast extract still contains mannitol (Mt) used in very high concentrations (0.35 M) during the isolation procedure.

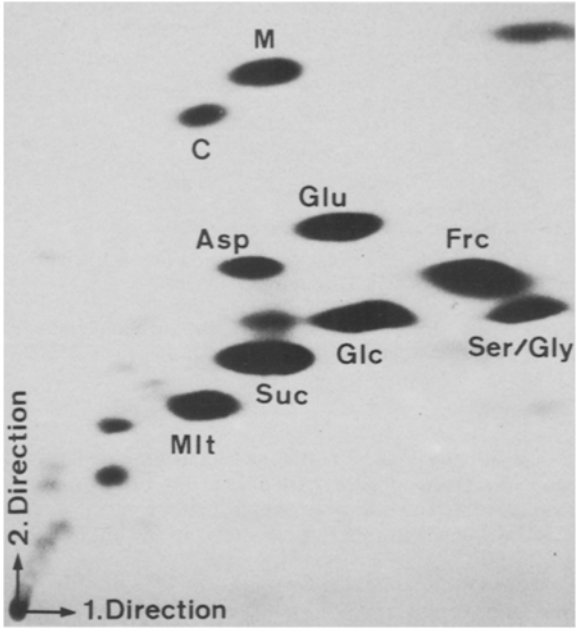

Figure 2. TLC-separation of labelled products from grape leaf protoplasts after administering $NaH^{14}CO_3$ for 5 h. Solvents: 1. direction: Isobutyric acid/n-butanol/isopropanol/n-propanol/water/ammonia aq. – 2. direction: sec-butanol/formic acid/water[7]. M: malic, C: citric, Asp: aspartic, and Glu: glutamic acid, Mlt: maltose, Suc: sucrose, Glc: glucose, Frc: fructose, Ser/Gly: serine/glycine.

0.35 M mannitol) to sedimented protoplasts[9]. Freshly isolated protoplasts did not have any remnants of cell walls as indicated by their failure to stain with 0.1% Calcofluor White M2R.

Our main interest being focused on grape acid metabolism and photosynthetic carbon flow, we first compared the fraction of non-volatile organic acids in protoplasts with that of intact leaves. The 2 extracts were found to be indistinguishable (fig. 1), which implies that the protoplasts in this respect precisely represent the situation within the intact mesophyll. The viability of the isolated protoplasts was controlled by determining their ability to assimilate CO_2 from $NaH^{14}CO_3$. Total $^{14}CO_2$ incorporation was found to be 18.25 µmoles/h and mg Chl in protoplast suspensions, which compares favourably with values of 4–30 µmoles/h and mg Chl in intact leaves[7] and leaf discs[10], depending on the experimental conditions. Similar fixation rates were observed in experiments with mesophyll protoplasts from tobacco and *Antirrhinum*[11]. Qualitative analyses of the $^{14}CO_2$ fixation products in intact tissues[7,10] and grape leaf protoplasts (fig. 2) show that the distribution of radioactive label is virtually the same, with the bulk of the radioactivity appearing in sucrose, glucose, fructose, malic acid and glycine/serine. The low amount of label in the phosphorylated intermediates must be attributed to the relatively long incubation time. This also seems to account for the high labelling of citrate and glutamate, both of which were also found to accumulate radiocarbon after several hours of metabolism[12,13] in experiments with intact leaves.

Consequently, we believe our protoplast preparations reflect the metabolic state of the source tissue and thus provide a new and useful method for physiological and biochemical research on grape metabolism.

1 Acknowledgment. Financial support by the 'Jubiläumsspende für die Universität Zürich' is gratefully acknowledged.
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Effect of ascorbic acid on biotransformation and modification of the toxicity of mercurials in goldfish *(Carassius auratus)*[1]

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Summary. Ascorbic acid mediated a small but significant degradation of methylmercury to inorganic mercury in goldfish *(Carassius auratus)* and reduced the toxicity of mercuric chloride despite its substantial conversion into organic form.

Because ascorbic acid is a strong reducing agent it is supposed to have potent detoxifying properties and has been used in cases of intoxication by heavy metals, including mercury[3]. Recently, Gage[4] demonstrated biodegradation of organic mercury compounds by ascorbate in vitro. This study examines the effect of ascorbic acid on biotransformation and modification of the toxicity of mercuric and methylmercuric chlorides in vivo in goldfish.

Material and methods. Details about the goldfish *(Carassius auratus)*, aquaria, and radioactive mercuric chloride and methylmercuric chloride used for this work have been described previously[5]. The modification of acute toxicity of these mercury compounds by L-ascorbic acid (BDH, Analar) was determined in a series of experiments in various dosage and application regimes as before[6,7] and the results were assessed both on the basis of acute toxicity, as determined by 24-h survival rates, and on the effect of treatment on the uptake of mercury, which was measured by counting the fish for gamma activity due to ^{203}Hg in a Packard Autogamma Scintillation Spectrometer Model 5130 as was done earlier[6].

In each experiment 10 goldfish were kept in an all-glass aquarium containing 10 l of water. Different batches of goldfish were used in different experiments. The average weight of the goldfish was 1.726 g. The lid was placed on each tank in all the experiments, except experiment III, in order to minimize the oxidation of ascorbic acid by air. Methylmercuric chloride (80 ng Hg/ml) or mercuric chloride (400 ng Hg/ml) was added together with ascorbic acid at 10, 100, or 1000 times the Hg level on a molar basis. The simultaneous uptake of Hg and vitamin C was allowed to take place for 24 h. In another mode of application regimen, the fish were first exposed to ascorbic acid for 24 h and then to mercurial for the next 24 h using the same concentration. At the end of 24 h of Hg uptake the fish were taken out of the aquarium, washed, divided into 2 groups of survivors and non-survivors, and counted for ^{203}Hg activity, then kept frozen.

The frozen fish were thawed and a homogenate of the whole fish was prepared in 5.0 ml of 0.5 M NaOH and 0.05 M L-cysteine in an Ultra Turrax homogenizer using 2 drops of antifoam tri-n-butyl phosphate (BDH). 1.0 ml of this crude homogenate was used for the study of biotransformation of mercury compounds by specific determination of inorganic mercury[8] using a Conway microdiffusion unit at 20 °C for 24 h. Our batch of methylmercuric chloride was found to contain $1.40 \pm 0.20\%$ of inorganic Hg on 5 different determinations on different days.

Results and discussion. The results of the effect of ascorbic acid treatment on the survival of goldfish, the uptake of mercury and the percentage of it found in the inorganic fraction in the fish exposed to mercuric and methylmercuric chlorides is shown in the table. The effect of pretreatment with ascorbic acid in reducing methylmercury toxicity was not consistent, nor was it always statistically significant. However, the percentage of the inorganic fraction of Hg in fish was always more than in the original methylmercuric chloride preparation which indicated that the conversion of