The Influence of Ficoll and Sorbitol on the Separation of Plant Cell Organelles

By

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Introduction

We have been concerned in our laboratory with the isolation and characterization of plant organelles. In particular we have studied vacuoles (lysosomes) and peroxisomes. Both of these organelles are sensitive to classical isolation procedures and we have sought new and more effective systems with which to isolate them. This lecture describes a system developed primarily for the isolation of plant peroxisomes, but which has proved useful as a general isolating system.

The rationale for isolating organelles may be divided into three distinct parts:

1. Homogenization of tissue.
2. Concentration of desired organelle in one fraction, most conveniently by differential centrifugation.
3. Separation of desired organelles from other organelles or contaminating material of the enriched fraction, usually with density gradient centrifugation.

Clearly, it is vital that the organelles remain intact through all stages and this may be influenced by choice of homogenizing medium as well as homogenizing technique. A density must also be selected that not only maintains organelle integrity but also permits maximum separation from contaminating material and other organelles.

The electron microscope may be used to check organelle integrity throughout the isolation procedure.

In this lecture the isolation of organelles from two plant tissues is described; namely leaves of spinach beet *(Beta vulgaris L.)* and appendices of Arum lily *(Arum italicum)* flowers (see PARISH, 1971 a, b).

Results with Classical Sucrose Media

We commenced with media commonly used for organelle isolation, and in particular for the isolation of glyoxysomes from castor bean endosperm (BRIEDENBACH and BEEVERS, 1967) and of leaf peroxisomes (TOLBERT et al., 1968). These media consist of 0.5 M sucrose in 0.02 M glycylglycine buffer (pH 7.5) for the latter and 0.4 M sucrose, 0.1 mM $MgCl_2$, 10 mM KCl, 1 mM EDTA, 10 mM dithiothreotal in 0.05 M Tris-HCl buffer (pH 7.5) for the former.

Various homogenizing techniques were tried but in no case could more than 20% of total catalase activity be sedimented from *Beta vulgaris* leaves.

The whole chloroplasts were removed by centrifugation at 500 g for 20 min. and the supernatant centrifuged at 6000 g for 20 min. According to TOLBERT et al. (1968) the majority of peroxisomes are in the 6000 g sediment and this was layered onto step gradients. The steps were 4.5 ml of 70% (w/v) sucrose, 8 ml 61% sucrose, 8 ml 51% sucrose, 6 ml 44% sucrose, overlayered with 6 ml of extract. The gradients were spun at 25 000 rpm for 5 hours in a SW 25 head using a Spinco L 50 Ultracentrifuge.

These media and gradients have provided a satisfactory separation of peroxisomes from leaves of spinach and sunflower but not for example from corn, sugarcane, tobacco, pea, wheat and spinach beet (TOLBERT et al., 1969; ROCHA and TING, 1970). Glyoxysomes have also been isolated by similar techniques, but were unstable during the first five days of germination (GERHARDT and BEEVERS, 1970).

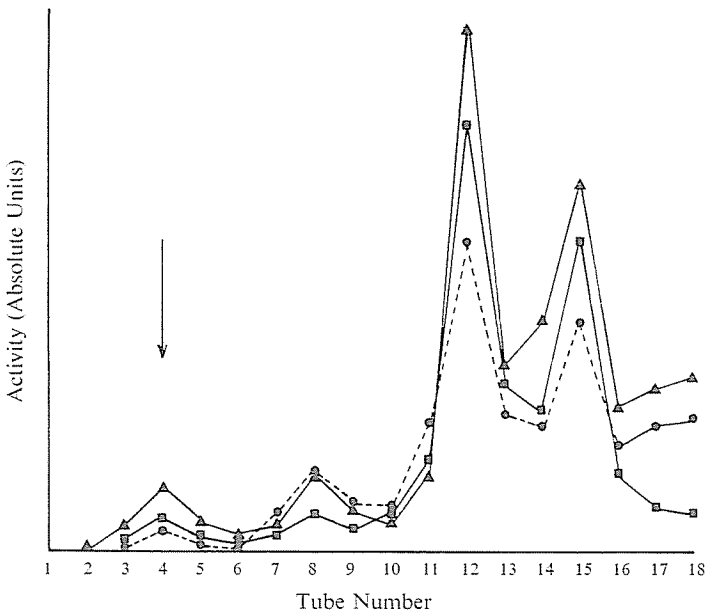

Fig. 1. Distribution of catalase (●), protein (▲) and chlorophyll (▩) among fractions from a sucrose step gradient after centrifugation of the 6000 g fraction isolated in sucrose medium. The arrow indicates the fraction in which peroxisomes should be concentrated (from PARISH, 1971a).

The distributions of protein, chlorophyll and catalase (peroxisomal marker enzyme) in the gradient are shown in Fig. 1. There is virtually no separation of catalase from chlorophyll and only very low catalase activity is present at the density of peroxisomes (ρ 1.25).

Choice of Isolating Medium

We decided to replace the sucrose in the isolating medium with isotonic sorbitol and Ficoll (Pharmacia; a sucrose polymer, mol. wt. 400 000). A number of workers have reported that organelles are disrupted by isotonic sucrose whereas they remain intact in sorbitol (eg. yeast mitochondria, SZABO and AVERS, 1969). The reason is unknown. Ficoll was included because of its high molecular weight and low osmotic activity which would be expected to stabilize particles.

The optimal medium was 0.5 M sorbitol and 2.5% Ficoll in 0.05 M Tris-HCl buffer pH 7.5 (SF medium). When working with tissues high in phenols the addition of reducing agent (eg. 10 mM dithiothreotal) was necessary.

In the case of *Beta vulgaris* leaves the sedimentable catalase was increased by a factor of three to more than 50% when SF medium was used.

Homogenization of Tissue and Resuspension of Sediments

The use of a pestle and mortar with abrasive (eg. washed sand) gave rather variable results. Most consistantly good results were achieved using a Sorvall Omnimixer and, after cutting the tissue into small slices, homogenizing for 5 seconds at full speed. The homogenate was passed through three nylon filters with diameters of 475, 200 and 63 μ respectively. The filtrate was then free of all cell walls and nuclei.

Resuspension of sediment in SF medium after differential centrifugation demanded great care. The use of a standard teflon homogenizer broke many organelles and a loosely fitting pestle was substituted. Many sediments could be simply resuspended with a Vortex tube vibrator. A slow speed centrifugation after resuspension removed aggregates which can give erroneous gradient results.

Differential Centrifugation

The selection of an organelle-enriched fraction is a matter of trial and error. Even when the most active fraction has been discovered it may, in its entirety, be unsuitable for gradient centrifugation. In the case of *Beta vulgaris* leaves the peroxisome-rich fraction sedimented between 500 g (15 min.) and 10 000 g (20 min.). The fraction giving best separation of organelles on density gradients was that sedimenting between 1000 g (15 min.) and 6500 g (20 min.). The fraction between 500 g and 1000 g contained peroxisomes, but the concentration of chloroplast membranes was so high that they hindered a clear separation of organelles by trapping (breaking?)

mitochondria and peroxisomes. The 6500–10 000 g (20 min.) fraction contained light mitochondria which were difficult to separate from chloroplast membranes, even using relatively "flat" gradients.

Density Gradients

The gradients of choice must not only assure particle integrity but also provide optimal separation. STECK et al. (1970) have postulated a theoretical model that correctly predicts optimal conditions for the fractionation of membranous vesicles. They show that resolution as a function of sequestered charges is favoured when ionic charge, osmotic activity and fixed charge density are minimized. A lower fixed anion concentration (Z) in the vesicles favours separation when charge per membrane mass (Q) is in the lower range (eg. up to 0.06 m equiv/g). In contrast to sucrose, Ficoll gradients have a low Z value. Sucrose will favour separation of vesicles with high Q values, but has the disadvantage of disrupting particles.

Gradients consisting entirely of sorbitol and Ficoll were impractical for the separation of particles as at the densities required (peroxisomes: ρ 1.25) the gradients were extremely viscous and unmanageable. We achieved best separation with a mix-

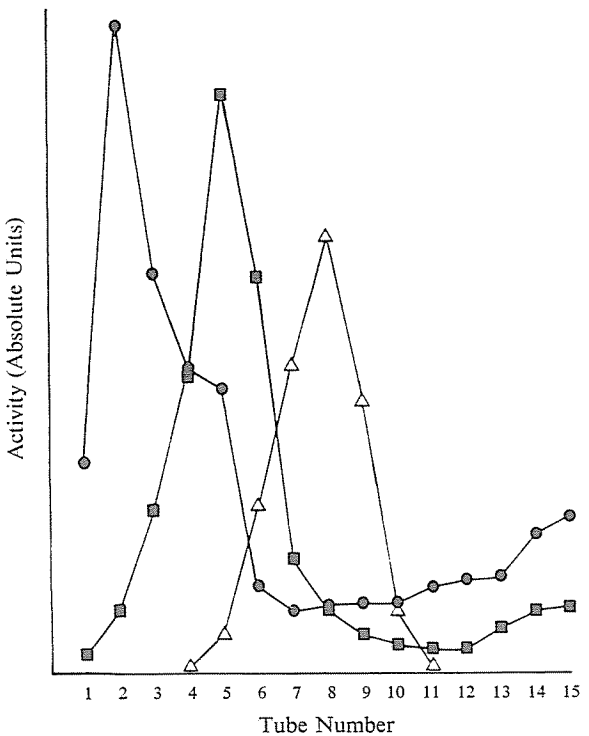

Fig. 2. Distribution of catalase (●) citrate synthetase (■) and chlorophyll (△) among fractions from an SFS gradient after centrifugation of the P 6500 fraction (from PARISH, 1971a).

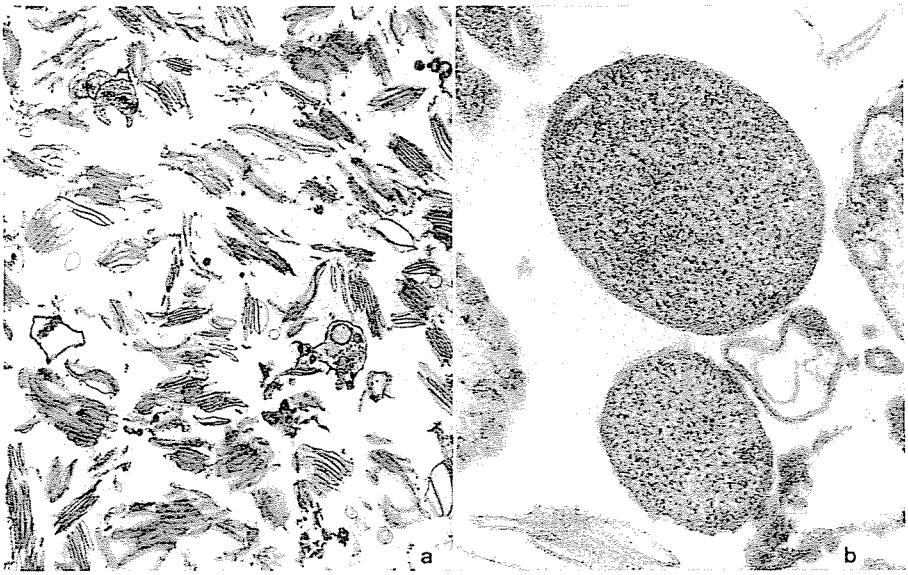

Fig. 3. a) Chlorophyll rich fraction isolated from an SFS gradient and showing chloroplast thylakoids (× 26 000). b) Peroxisomes isolated from the peroxisome-rich fraction of an SFS gradient (× 92 000). (From PARISH, 1971a.)

ture of sorbitol, Ficoll and sucrose (so-called SFS gradients). Sucrose concentration was varied according to the tissue examined, but the general rule was to generate gradients from equal volumes of SF medium plus x % sucrose and y % sucrose plus 2.5% Ficoll.

Results

The *Beta vulgaris* P 6500 fraction was layered onto linear gradients made from equal volumes (2.2 ml) of SF medium and 64% sucrose plus 2.5% Ficoll over a 0.3 ml cushion of 70% sucrose (w/v).

A fraction sedimenting between 1000 g (15 min.) and 10 000 g (20 min.) was prepared from *Arum italicum* appendices and layered onto a gradient made from equal volumes of SF medium plus 32% sucrose and 55% sucrose plus 2.5% Ficoll over a 0.3 ml cushion of 70% sucrose.

All gradients were spun in a SW 39 rotor in a Spinco L 50 Ultracentrifuge for 3 hours and fractions collected after puncturing a hole in the bottom of the tubes.

The results with *Beta vulgaris* leaves are shown in Fig. 2. A good separation of mitochondria (citrate synthetase), chloroplast membranes (chlorophyll) and peroxisomes (catalase) was achieved. The respective fractions were examined with the electron microscope. The chloroplast membranes contained no contaminating material (Fig. 3a) and the peroxisomes were intact (Fig. 3b). The peroxisome fraction contained some mitochondria, even though mitochondrial enzyme activity was

Fig. 4. Peroxisome, mitochondrion and chloroplast in a mesophyll cell of spinach beet (× 46 000). The peroxisome has been stained with diaminobenzidine and the section was not postcontrasted. (From PARISH, 1971a.)

relatively low, which underlines the microscopic evidence of a high mitochondria: peroxisome ratio in the tissue. The isolated peroxisomes were identical in shape and structure to those seen *in vivo* (Fig. 4).

The gradients also gave good separation of mitochondria (malate dehydrogenase, succinate dehydrogenase) and peroxisomes (urate oxidase) from Arum lily appendices (Fig. 5). When viewed with the electon microscope, isolated mitochondria (Fig. 6a) and isolated peroxisomes (Fig. 6b) were intact and identical in structure to their *in vivo* counterparts (Fig. 7).

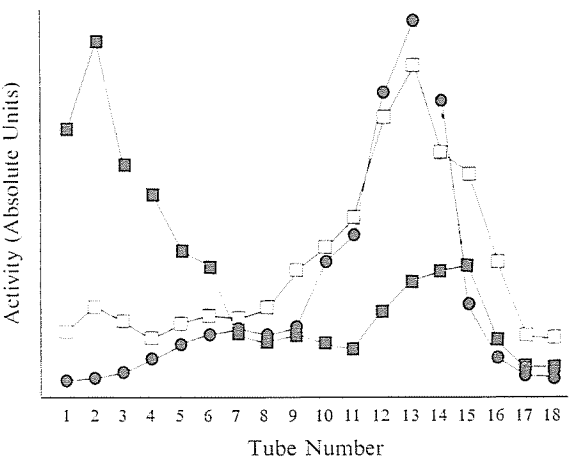

Fig. 5. Distribution of succinate dehydrogenase (●), malate dehydrogenase (□) and urate oxidase (■) among fractions from a linear SFS gradient after centrifugation of the P 10 000 fraction. (From PARISH, 1971b).

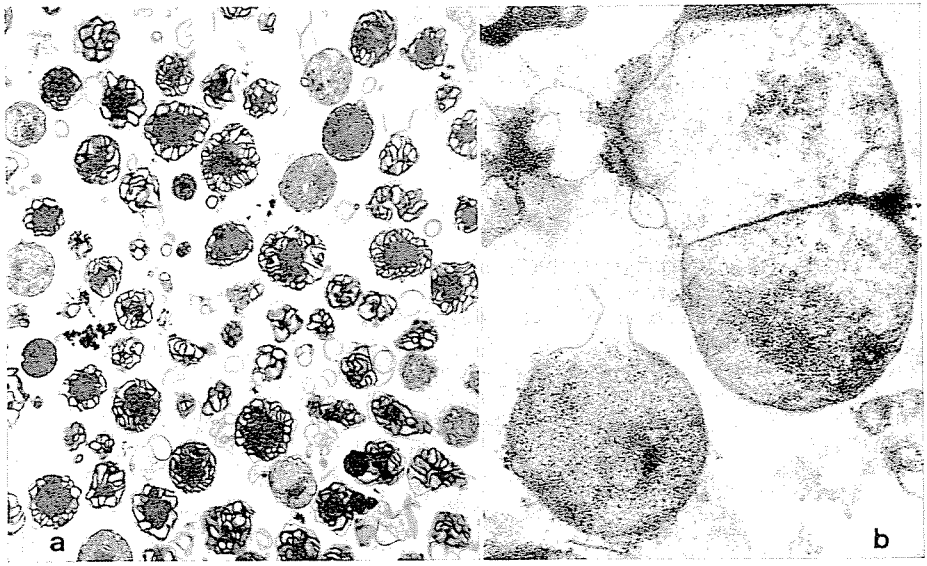

Fig. 6. a) Mitochondrial fraction isolated from an SFS gradient (× 11 200). b) Peroxisome fraction isolated from an SFS gradient (× 42 000). (From PARISH, 1971 b).

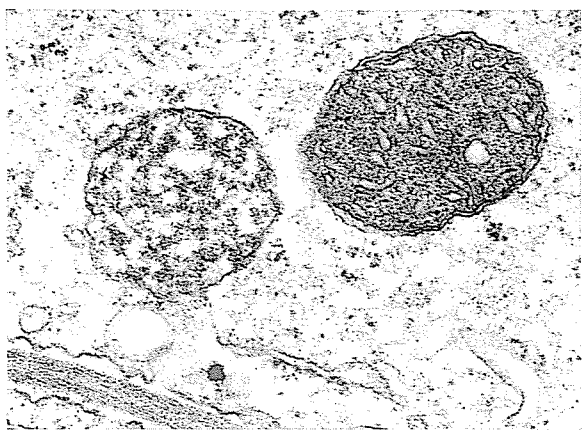

Fig. 7. A mitochondrion (right) and a peroxisome in *Arum italicum* appendix (× 36 000). (From PARISH, 1971 b).

Conclusions

By following the general scheme outlined in this lecture and using SF medium and SFS density gradients we are now able to isolate intact organelles from plant tissues where previous attempts had been unsuccessful.

Acknowledgements

The author is grateful to Miss R. RICKENBACHER for excellent technical assistance. The work was supported in part by a grant from the firm BURRUS, manufacture for tobacco, Boncourt.

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