The Preservation and Investigation of Plant Mitochondria

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SUMMARY

A method for making squash preparations of plant root tip cells is described. This entails the use of an inert preserving substance, polyvinyl alcohol. The advantages of the method are the preservation of the cytoplasmic bodies, including the mitochondria; the retention of fat droplets inside the cells; the fact that the nucleus remains optically homogeneous; and the fixing of free aldehyde groups and of diffusible substances in the cytoplasm. It is a relatively rapid and simple technique, and the cells produced by it resemble closely those produced by the method for making living squash preparations of such cells.

INTRODUCTION

METHODS of fixing plant cells in such a manner as to preserve the cytoplasmic components, in particular the mitochondria, are few and complicated. This is due to the nature of the mitochondria, which are destroyed by acids, by dichromate mixtures of more acid pH than about 4.2–5.2 (Zirkle, 1929), and by alcohol and other fat solvents. Squash preparations are usually produced by acid hydrolysis of the intercellular cement, e.g. as in the standard aceto-carmine method and when the Feulgen hydrolysis (6 minutes in N.HCl at 60° C.) is used to separate the cells. Such methods destroy plant mitochondria. The tissue must therefore be fixed in such a way that the mitochondria are rendered resistant to fat solvents and then embedded and sectioned. Probably the Newcomer-Zirkle technique (see, for example, Newcomer, 1940) based on the work of Zirkle on dichromate fixatives (Zirkle, 1929; 1931) is one of the most useful. The roots may be prefixed to improve nuclear fixation, but in our own studies this step has been omitted and the roots are placed for 24 hours in a chromium fixative at the correct pH (1.25 g. potassium dichromate, 1.25 g. ammonium dichromate, 1.0 g. copper sulphate, made up to 100 ml. with distilled water). They are then washed in running tap water for 24 hours, after which they are immersed in 2 per cent. osmium tetroxide for 6 days and again washed in running water for 24 hours; they are then embedded by standard techniques. The sections are bleached in 1 per cent. potassium permanganate for 5 minutes and then treated with 3 per cent. oxalic acid either for 3 minutes or until they are decolorized. This method, however, has many drawbacks. It is lengthy, and fixation of root tips of the broad bean, Vicia faba, is variable and patchy, while results obtained with onion root tips have been unsatisfactory; the technique was originally designed

for maize roots. The enzymatic digestion of the intercellular cement (for references see Chayen, 1949; see also Chayen, Davies, and Miles, in press) is rendered more difficult by this method, so that the cells cannot be studied easily in squash preparations. Moreover, it interferes with the fluorescence of the cells, perhaps partly by washing fluorescent material out of the cytoplasm and partly by its mode of fixation, which makes them useless for primary fluorescence microscopy; it also affects the ultraviolet absorption characteristics of the tissue, probably because of the presence of chromium in the sections.

Freezing-drying of root tips was attempted for us by Dr. L. G. E. Bell, but only very occasionally were satisfactory results obtained. When successful, excellent preservation of cytoplasmic detail was observed, but in most of the roots all such details were obliterated. This was believed to be due either to bad impregnation with wax after the freezing and drying, or to premature warming of the specimens before drying, owing to the small bulk of the root tips (Bell, unpublished).

Thus, it was necessary to devise a new technique. Mitochondria are so easily destroyed that it seemed as if the ideal fixative would be some inert substance that could mummify the cells. It had previously been discovered (Chayen, unpublished) that polyvinyl alcohol (PVA) preserves insect blood cells, which normally disintegrate within 15 minutes of being placed on a slide. It was therefore decided to test whether this substance would also preserve the delicate mitochondria of plants. Polyvinyl alcohol had been used by Downs (1943) as a medium for mounting and clearing biological specimens and for slowing the movements of living mosquito larvae and motile microorganisms, and by Lubkin and Carsten (1942) as an embedding agent for microtome sectioning.

In the present method, roots or root tips are placed for 1 day in an approximately 5 per cent. aqueous solution of polyvinyl alcohol. They are then transferred to an aqueous solution of a pectinase preparation to remove the intercellular cement. After a suitable period (one to several hours depending on the tissue and the enzyme solution) the tissue can be macerated very readily by gently tapping it with a needle. At this stage stain may be added, such as a very dilute aqueous solution of Janus green, and the cells are then mounted in a more viscous polyvinyl alcohol solution which has been plasticized with glycerine to prevent cracking of the PVA on drying. A more complex mounting agent, containing PVA as the major constituent, has been described by Gray and Wess (1950); it has the advantage that it sets to a cement having a constant and known refractive index.

Solutions

1. Stock solution. Polyvinyl alcohol (PVA) powder is troublesome to dissolve. It was more convenient to prepare a stock solution from which samples could readily be diluted as required. To make this stock solution 30 g. of PVA powder (obtained from Vinyl Products Limited) are mixed carefully
with sufficient glass-distilled water to form a stiff paste. This is best done in a large pestle and mortar in order to break up any lumps. More water is then added to bring the final volume of water in the solution to 100 ml. and is worked well into the paste. This solution is heated on a water bath, the temperature of which reaches about 90°C, until it is completely cleared of foam; this process usually takes about 30 minutes. Since the cleared solution usually sets to a weak jelly after a few days, it is stored in a wide-necked jar with ground-glass stopper, thus reducing further evaporation and facilitating the removal of samples of the jelly.

2. Preserving (fixing) solution. Five g. of the jellied stock solution are added to 20 g. of glass-distilled water and warmed until all the jelly has dissolved. This gives an approximately 5 per cent. PVA solution in water. Unfortunately micro-organisms grow in this solution so that it has to be made up freshly from the stock solution just before it is to be used. Some roots are especially liable to attack by micro-organisms and it has been found that better preservation of such roots is obtained in a mixture of 4 volumes of approximately 5 per cent. PVA to 1 volume of absolute ethanol. A milky appearance, noticeable at the meniscus when this mixture is prepared, disappears on shaking.

II. For mounting preparations

An aqueous solution of PVA is prepared by dissolving 4 g. of PVA powder in 20 ml. of water in a manner similar to that described for preparing the stock preserving solution. To the cooling solution is added 20 per cent. (by weight) of glycerol. The mixture is well stirred and is ready for use when cool. It may be left in a jar, and keeps for two or three days. Lubkin and Carsten (1942) give a similar formula for an embedding medium.

III. Pectinase preparation

The pectinase is extracted, as required, from samples of *Penicillium digitatum* which has been grown on bran and subsequently dried (see Chayen, 1949). The samples used in the present studies were different from those in previous investigations (e.g. Chayen, Davies, and Miles, in press; Chayen 1949) and gave suitable maceration in a very much shorter time. The enzyme is extracted by shaking 1 volume of bran with 4 volumes of glass-distilled water and leaving the suspension to stand for 1 hour. It is then centrifuged and the supernatant, diluted twice, is used. This extract appears to be roughly equivalent to a 5 per cent. solution of the pectinase powder sold by Nutritional Biochemicals Corporation (Cleveland, Ohio), a sample of which was kindly given us by Dr. R. Brown of Leeds.

METHODS

1. Squash preparations of onion root-tip cells

The pectinase technique for living root-tip cells described by Chayen (1949) could not be used to separate the living meristematic cells of onion
roots. When so treated, the onion roots degenerated and produced offensive odours. The apical 4–5 mm. of onion roots were therefore placed in the PVA-ethanol preserving mixture for 24 hours. For convenience, and to exclude the growth of micro-organisms, this was done in two stages: roots were placed in the solution at 11 a.m. and transferred to a fresh, similar sample at about 5:30 p.m. It was desirable to use about 15 ml. of preserving solution to 10 root tips. After a total of 24 hours in the preservative they were transferred to the pectinase solution for 1½ hours, after which the meristem cells separated very readily when squashed in water with a broad-bladed scalpel or with a spear-shaped needle.

When PVA-glycerol was used as the mountant, the cells were macerated in a small drop of water and stained, if required, with a very dilute, aqueous solution of Janus green; the mountant was dropped on to the cells, allowed to mix with the water, and the coverslip was then placed in position.

2. Squash preparations of bean root-tip cells

The apical 3–4 mm. of lateral roots of *Vicia faba* were immersed in the approximately 5 per cent. PVA solution for 24 hours and were then treated with the pectinase solution. Satisfactory maceration was only obtained after about 8 hours' treatment. When living lateral root tips were treated with a similar concentration of pectinase to produce living squash preparations, adequate separation of the cells was obtained only after a similar period (i.e. about 8 hours), although bean root tips which were fixed in acetic-alcohol required treatment for only about 1 hour.

Bean root tips were also well preserved by fixation in a mixture of 9 volumes of the approximately 5 per cent. PVA solution to 1 volume of absolute ethanol.

**Results**

Cells of onion root tips prepared by this technique were very different from similar cells prepared by such methods as the aceto-carmine squash method, acetic alcohol fixation followed by Feulgen hydrolysis, and fixation in acetic-alcohol followed by maceration by a pectinase. The cytoplasm of cells prepared by these three techniques was not homogeneous; it showed coarse precipitation of the cytoplasmic protein by the fixative, but was free from large granules and similar separate bodies. When cells which had been fixed with acetic acid, alcohol, or a mixture of the two, were treated, without hydrolysis, with the Feulgen reagent (as is frequently done as a control for true Feulgen staining), no colour was developed. On the other hand, the cytoplasm of meristematic cells of onion roots prepared by the PVA method appeared homogeneous but contained granules of various sizes, large droplets probably of lipoidal matter, and, when tested without hydrolysis with the Feulgen reagent, it stained pink, the colour being mainly associated with the mitochondria-like granules. It is probable that the colour is due to free aldehyde or similar groups present in lipoidal material. Such material is lost from the cells on
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treatment with fat solvents and so this reaction is not observed in tissues fixed with acetic acid and alcohol.

A very dilute aqueous solution of Janus green was used to differentiate the mitochondria from droplets and other bodies. The appearance of the cytoplasmic bodies and the general form of the cells resembled fairly closely what had been observed in living squash preparations and in the best frozen-dried material. When, however, a root which had been fixed with PVA was heated in normal hydrochloric acid for 6 minutes at 60° C., stained with the Feulgen reagent, macerated and examined directly (or made permanent by mounting in PVA-glycerine), it was found that the cells resembled those of roots fixed in, for example, acetic-alcohol, and stained by the Feulgen reaction. Whereas the unhydrolysed nuclei prepared by the PVA method were optically homogeneous structures containing nucleoli, after the Feulgen hydrolysis and staining they were granular and well stained, with the nucleoli and cytoplasm colourless. No discrete bodies were observed in the cytoplasm of meristematic cells after such treatment.

In studies of living squash preparations of bean root tips it was found that some ultraviolet-absorbing material was lost from the cytoplasm during the course of preparation. Such cells also emitted little primary fluorescence (see, for example, Chayen, 1952). PVA-fixed cells, however, showed intense primary fluorescence both in the cytoplasm and in the granules which surrounded the nuclei. Nucleoli also fluoresced. The fluorescence was not due to the presence of PVA, which does not emit visible light when excited at the wavelength used in this study, namely 3,650 A. Fluorescence was not observed in frozen-dried material.

DISCUSSION

The polyvinyl alcohol method, used in conjunction with enzymatic degradation of the intercellular cement, provides a very simple and fairly rapid method of preparing plant cells for cytological, as opposed to purely cyto-genetical, examination. Such preparations are superior to those produced by most standard cytological techniques in that the cytoplasmic particles and droplets are well preserved; there are no signs of coagulation or crude precipitation of cell proteins; the nucleus remains optically homogeneous with the nucleic acids apparently in the disperse phase (see Ris and Mirsky, 1949). Furthermore, not only are fat droplets when present, as in the onion, well-preserved, but free aldehyde and similar groups probably associated with lipoidal material, which are normally lost on fixation, have been demonstrated by the recoloration of Schiff's reagent (the Feulgen reagent) without prior hydrolysis (see Danielli, 1949; Cain, 1949). The method is so easy and so reproducible that it has been used successfully by an undergraduate class. An additional advantage is that soluble substances diffusely distributed in the cytoplasm do not appear to be lost from the cells.

On the other hand, if such cells are treated with the reagents used in the production of standard cytological preparations, all the cytoplasmic organiza-
Chayen and Miles—The Preservation and optical homogeneity are lost and the cells resemble those prepared by standard techniques alone.

This raises the question of the mode of action of the PVA. Baker (1945) defines fixatives as 'solutions that prevent decay and autolysis, render the substances of the cell insoluble, and lessen its subsequent shrinkage and distortion'. He considers one of their functions is to prevent subsequent treatment from causing unwanted changes. In this respect PVA is not acting as a true fixative in that the structures preserved will not withstand further treatment with, for example, xylene in the dehydrating, clearing and mounting schedule ordinarily used. It would appear to act more as a preservative or, possibly, as a narcotic. This might have been its action when used by Downs (1943) to stop the movement of micro-organisms and of mosquito larvae; if they were exposed to PVA for short periods and were then washed thoroughly they recovered and behaved normally. If the treatment, especially with the micro-organisms, was prolonged, however, death ensued. It may be that root-tip cells react in a similar way.

Brown (1951), using a pectinase method for isolating root-tip nuclei, found that they broke down unless some long molecule such as pectin or peptone was added. Such molecules appear to influence active surface groups and so stabilize the system. It is possible that short treatment with PVA has a similar effect on the whole cell, apparently inhibiting active surface groups and so retaining cytoplasmic material which may be lost even from cells produced by the method for living squash preparations. It may be, however, that it acts by entering the cytoplasm; since it is an inert substance it would thus 'mummify' the cell.

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