Isolation of Plant Nuclei

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A method for isolating nuclei from tobacco leaves is described. Preliminary results indicate that the method may be generally applicable for isolating nuclei from plant tissues.

Introduction

In contrast to the great number of papers dealing with the isolation of nuclei from animal tissue, references to the isolation of nuclei from higher plants are few. Nuclei have been isolated from plant embryos in both nonaqueous and aqueous media, and in aqueous media, from roots, endosperm, apical sections of seedlings, and exponentially growing plant cells in tissue culture. One of these methods is only suitable for preparation of small numbers of nuclei; another is, by virtue of the unusual tissue required, of limited applicability; and others require specially constructed apparatuses to liberate the nuclei from the cells. Yields of nuclei obtained have, with several exceptions, not been indicated; photomicrographs of the isolated nuclei, with few exceptions, not been published. In some instances the isolated nuclei have been shown to retain certain of their normal biochemical functions. Media used in the isolations were, in all cases, similar to those previously employed for isolation of animal nuclei.

In an attempt to isolate nuclei from young tobacco leaves, we tried several of the above methods, including the two previously used to obtain tobacco leaf nuclei. In all cases the leaf homogenates contained few nuclei (less than 10% of the number in the starting material), most of which were badly distorted or broken. In addition, some of the purification procedures yielded preparations grossly contaminated with cytoplasmic debris. Further preliminary experiments, in which attempts were made to apply methods used successfully for isolation of animal nuclei to tobacco leaves, made it clear that, in agreement with the observations of Accola, the isolation of plant leaf nuclei presents two problems not encountered in the isolation of nuclei from animal tissues: 1. Any treatment which breaks open the plant cell tends to deform or disrupt the nucleus. 2. Each green leaf cell contains several hundred chloroplasts, similar enough in size, shape, and density to the nucleus to present a serious separation problem. These difficulties have now been overcome, the first through development of a new procedure for preparing leaf homogenates, the second through minor modifications of existing methods for purifying nuclei.

Materials and Methods

Counting and Scoring Nuclei

To determine the concentration of nuclei in a given suspension, a measured volume of the suspension was mixed with an equal volume of 6% acetic acid — 0.002 M calcium chloride — 0.04% methyl green. The nuclei, which are stained blue by the dye, were then counted in a cell-counting chamber.

Each suspension of interest was scored for total nuclei and undamaged nuclei. Total nuclei included all nuclei which had been released from the cells, except that fragments judged to represent less than half a nucleus were not scored. Undamaged nuclei included only nuclei which were free from large adhering cytoplasmic fragments, were approximately spherical in shape, had an unbroken perimeter, were about the same size as in the living cells (approx. 6 μ), and which stained well with methyl green.

Cell Counts

The total numbers of cells in tobacco leaves of various lengths were determined by a slight modification of the procedures described by Maksymowych and Sunderland. The leaves were incubated 28 hrs at 38° in 120 times their weight of 5% chromic acid. The chromic acid was then removed, water containing 0.05% Tween 20 (polyoxyethylene sorbitan monolaureate) was added, and the tissue was broken into individual cells by several passages through a Dounce homogenizer with a clearance of 0.09 mm. The resulting cell suspension was made up to a known volume with water containing 0.05% Tween. Aliquots were counted in a counting chamber after dilution with an equal volume of a 0.01% solution of light green.
Isolation Medium

The medium developed for isolation of the nuclei has the following composition: 0.004 M n-octyl alcohol — 4% gum arabic — 0.10 M sucrose — 0.002 M calcium chloride — 0.02 M trishydroxymethylaminomethane (tris). The pH of the medium is adjusted to 7.6 with acetic acid.

The gum arabic used in the isolation medium was purified as follows: One kg of commercial gum arabic was dissolved in 4 l of water. The resulting solution was centrifuged 1 hr at 36,000 g, and the sediment was discarded. To the supernatant fluid, adjusted to pH 4.5, was added an equal volume of 95% ethanol. The resulting precipitate was allowed to settle, and the supernatant liquid was decanted. The precipitate was washed several times with 95% ethanol, once with 100% ethanol, and finally with diethyl ether, then dried in vacuo.

Preparation of Leaf Homogenates

Leaves, 25 to 55 mm long, are harvested from 30 to 60 cm tall tobacco plants (Nicotiana tabacum L., var. Samsun) which have been held in darkness for 24 to 36 hours. The leaves are immersed in isolation medium and infiltrated by placing them briefly under vacuum. After being incubated at 0°C for 16 to 20 hours, the leaves are transferred to fresh isolation medium (2 to 5 ml per leaf) and homogenized for 30 seconds in a high speed blender (Bühler Homogenizer, Edmund Bühler, Tübingen, Germany) consisting of a four bladed cutting knife rotating in a fluted glass container. The homogenizer is operated at top speed (56,000 rev/min).

In experiments to be described under Results, the effects of various modifications in the homogenization procedure, including modifications in the composition of the isolation medium, were investigated. In these experiments each homogenate was prepared from two leaves (usually about 30 mm long) and 10 ml of isolation medium. Pairs of leaves for the homogenates in a given experiment were matched as to size and shape and were harvested from a group of plants of the same age and size.

Purification of Nuclei by Differential Centrifugation

A leaf homogenate is prepared as described above from 10 leaves and 20 ml of isolation medium. The homogenate is filtered through one layer of cheesecloth (150 — 200 openings/cm²), then through two layers of cheesecloth, then four layers of cheesecloth, and, finally, through two layers of flannel. Residues from the filters are suspended in approx. 10 ml of isolation medium, and the resulting suspension is filtered as described above. The combined filtrates are centrifuged 10 min at 350 g. The supernatant liquid is carefully decanted and discarded, and the sediment is resuspended in 12 ml of the isolation medium by vigorous shaking. The nuclei are sedimented (10 min at 350 g) and resuspended in isolation medium (12 ml portions with vigorous shaking) two more times. The resulting suspension is centrifuged for 15 min at 25 g, and the sediment is discarded. The nuclei are then sedimented (10 min at 350 g) and resuspended in isolation medium (12 ml portions with vigorous shaking) three more times. The resulting suspension is centrifuged 5 min at 25 g, and the sediment is discarded.

Purification of Nuclei by Combined Differential and Density Gradient Centrifugation

The procedure for preparing nuclei described above is followed through the first centrifugation at 25 g. The resulting suspension is centrifuged 10 min at 350 g, and the sediment is suspended in a small volume of isolation medium. To each 1.00 ml of this suspension is added 7.72 ml of a supersaturated sucrose solution prepared by dissolving 245 gm of sucrose in 100 ml of water and making the resulting solution 0.002 M with respect to calcium chloride, 0.002 M with respect to n-octyl alcohol, and 0.02 M with respect to tris-acetate buffer, pH 7.6. The resulting suspension of nuclei, which should have a density of 1.318 ± 0.005, is layered over a portion of the supersaturated sucrose solution and centrifuged 10 min at 10,000 g (10,000 rev/min in Spinco Rotor SW 25) then, without stopping the centrifuge, 45 min at 72,000 g (25,000 rev/min in Spinco Rotor SW 25). During this time the nuclei collect at the interface between the two sucrose solutions. Upon completion of centrifugation, the bottom of the centrifuge tube is punctured, and the nuclei-containing fraction is collected, diluted with 4 times its volume of isolation medium, and centrifuged 10 min at 500 g. The sedimented nuclei are resuspended in a convenient volume of isolation medium. The method of density gradient centrifugation described here is a slight modification of that used by BRINZTIEL et al. for purification of nuclei from the apical sections of pea seedlings.

Experimental and Results

Influence of Various Factors on the Yield and Quality of Nuclei in Leaf Homogenates

Plant Size

When leaves of a given length were compared, those from older plants consistently gave better yields of nuclei than those from younger ones. The experiments in the present paper were all made with leaves from 30 — 60 cm tall plants.

Leaf Size

The number of nuclei in the homogenate prepared from a given leaf is, for leaves 2 to 16 cm in length, directly proportional to the length (Fig. I). The total number of cells in a leaf is, except for very large leaves, also proportional to the length (Fig. I), and, as a consequence, the yield of nuclei, expressed as
percentage of the total nuclei, is nearly independent of leaf length; about 50% of the total number of nuclei (= total number of cells) appear free in the homogenate.

**Octyl Alcohol**

The effect of varying the concentration of n-octyl alcohol in the isolation medium is illustrated in Fig. II. The yield of nuclei increases with increasing alcohol concentration, reaching a maximum at about 0.004 M. As illustrated in Fig. III, the yield of nuclei in a leaf homogenate increases upon incu-

Microscopic examination of a leaf homogenate reveals the presence of some unbroken cells. These have been found (for leaves 35 mm long) to represent about 15% of the original cells. About two thirds of the nuclei originally present in the leaf can, therefore, be accounted for. The remaining third may have been destroyed during homogenization.

**Method of Homogenization**

In developing the method for preparation of leaf homogenates, various methods of disrupting the cells were investigated. Of the methods tested, treatment of the leaves in a high-speed blender gave the best results. In Table I the yields and quality of nuclei found in homogenates prepared by several different homogenization methods are compared.

<table>
<thead>
<tr>
<th>Homogenizer</th>
<th>Speed rev/min</th>
<th>Homogenization time</th>
<th>Nuclei/leaf × 10^{-6}</th>
<th>Undamaged nuclei [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>BÜHLER</td>
<td>56,000</td>
<td>30 sec</td>
<td>9.2</td>
<td>77</td>
</tr>
<tr>
<td>BÜHLER</td>
<td>41,000</td>
<td>30 sec</td>
<td>6.9</td>
<td>73</td>
</tr>
<tr>
<td>POTTER-ELVEJEM 26</td>
<td>1,000</td>
<td>2 min</td>
<td>7.1</td>
<td>19</td>
</tr>
<tr>
<td>MORTAR and PESTLE</td>
<td>—</td>
<td>3 min</td>
<td>6.0</td>
<td>24</td>
</tr>
<tr>
<td>MORTAR and PESTLE</td>
<td>—</td>
<td>3 min</td>
<td>5.0</td>
<td>25</td>
</tr>
<tr>
<td>+ 1 g quartz sand</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table I. Effect of Homogenization Method on Yield and Quality of Nuclei. Leaf homogenates were prepared as described under Materials and Methods with modifications as indicated.
Table II. Incubation in Presence and Absence of n-Octyl Alcohol. Leaf homogenates were prepared as described under Materials and Methods with modifications as indicated. Values are the average of those obtained in two separate experiments.

<table>
<thead>
<tr>
<th>Incubated with</th>
<th>Homogenized with</th>
<th>Nuclei/leaf $\cdot 10^{-6}$</th>
<th>Undamaged nuclei [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>isolation medium</td>
<td>isolation medium</td>
<td>7.1</td>
<td>58</td>
</tr>
<tr>
<td>0.004 M octyl alcohol</td>
<td>isolation medium</td>
<td>5.1</td>
<td>58</td>
</tr>
<tr>
<td>isolation medium minus octyl alcohol</td>
<td>isolation medium</td>
<td>0.9</td>
<td>25</td>
</tr>
<tr>
<td>isolation medium</td>
<td>isolation medium minus octyl alcohol</td>
<td>4.2</td>
<td>49</td>
</tr>
</tbody>
</table>

bation of the leaves with the isolation medium at $0^\circ$C prior to homogenization, reaching a maximum at about 20 hours. As demonstrated in Table II, octyl alcohol is the component of the isolation medium active during incubation. Increasing the temperature at which the incubation was performed was found to decrease the time required for the yield of nuclei to reach a maximum, but had no effect on the height of the maximum attained. Incubation was routinely performed at $0^\circ$, at which temperature the yield of nuclei reached a maximum in 16 to 24 hours. Leaves were infiltrated with the isolation medium prior to incubation to bring the medium in as close contact as possible with the leaf cells.

**Gum Arabic**

The effect of varying the concentration of gum arabic in the isolation medium is illustrated in Fig. IV. The yield of nuclei increases with increasing gum arabic concentration, reaching a maximum at a concentration of about 3 per cent. A number of high polymer substances including gum arabic, dextran, polyethylene glycol, methylcellulose, pectin, alginic acid, polyvinylpyrrolidone, and bovine serum albumin were found to provide some degree of protection to the nuclei during homogenization. At low concentrations, however, all of these substances caused the cytoplasm of the disrupted cells to aggregate with the nuclei. In the case of the negatively charged polymers (gum arabic, pectin, and alginic acid), but not of the other substances, this effect disappeared upon raising the polymer concentration. Of the negatively charged polymers investigated, gum arabic had the most suitable solubility and viscosity properties and was, therefore, selected for inclusion in the isolation medium. Dounce and Litt have previously employed gum arabic in a medium for isolation of rat liver nuclei.

**Sucrose**

Sucrose solutions have found wide use in the preparation of animal nuclei. In the presence of sucrose, morphological features otherwise destroyed during isolation are retained. In addition, certain constituents which are normally leached from the nuclei in aqueous solutions are retained in the presence of isotonic concentrations of sucrose. It was, therefore, thought advisable to include sucrose in the isolation medium. The effect of varying the

Fig. IV. Variation in Yield of Nuclei with Gum Arabic Concentration. Leaf homogenates were made with isolation medium containing varying concentrations of gum arabic. In other respects preparation of the homogenates was as described under Materials and Methods. Each point represents the average of two independent determinations.

Fig. V. Variation in Yield of Nuclei with Sucrose Concentration. Leaf homogenates were made with isolation medium containing varying concentrations of sucrose. In other respects preparation of the homogenates was as described under Materials and Methods. Each point represents the average of two independent determinations.
sucrose concentration is illustrated in Fig. V. Up to a concentration of 0.1 M, sucrose seems to improve the yield of nuclei; at higher concentrations, the yield is depressed.

Calcium Ion

In 1950 Schneider and Petermann reported that the presence of calcium ion (0.0018 M) prevented the clumping and distortion which otherwise occurred during the isolation of nuclei from mouse spleen. Since that time, calcium ion has routinely been added to media used for the isolation of nuclei. The presence or absence of calcium chloride in the isolation medium was found to have little effect on the yield or quality (judged morphologically) of nuclei in leaf homogenates. Since the vacuole present in each mature leaf cell contains relatively large amounts of calcium salts, this is not surprising. When nuclei were washed repeatedly with calcium-free isolation medium, they exhibited a tendency to clump which increased with successive washings. Homogenates made with a modified medium in which the calcium chloride was replaced by 0.001 M ethylenediaminetetraacetic acid were nearly devoid of nuclei.

Hydrogen Ion Concentration

The cytoplasm of most plant cells is thought to have a \( p_H \) near neutrality. Because the contents of the cell vacuoles are acidic, however, a tobacco leaf homogenate prepared in an unbuffered solution has a \( p_H \) of about 6. Several hydrolytic enzymes in the tobacco leaf have been found to have \( p_H \) optima near 5. In order to maintain the \( p_H \) of the leaf homogenates at a value similar to that found in the cytoplasm and removed from the \( p_H \) optima of these enzymes, the isolation medium was buffered with 0.02 M tris and adjusted to \( p_H 7.6 \).

The yield and quality (judged morphologically) of the nuclei in tobacco leaf homogenates was found to be little influenced by \( p_H \) over the range investigated (\( p_H 5 - 8 \)).

Sulphydryl Compounds

The inclusion of sulphydryl compounds in the isolation medium had no detectable effect on the yield or morphology of the nuclei. DNA-dependent RNA polymerase activity, however, was greatly enhanced when 0.005 to 0.020 M 2-mercaptoethanol was added to the isolation medium. Unless otherwise indicated, experiments described in the present paper were made with isolation medium containing no sulphydryl compounds.

Diethyl Ether

Immersion of tobacco leaves for 30 seconds in diethyl ether at 0° followed immediately by homogenization in isolation medium containing no octyl alcohol yielded a homogenate containing nuclei in yields comparable to those obtained by the standard procedure. Many of the nuclei were, however, contaminated with adhering cytoplasmic fragments.

Reproducibility of the Homogenization Method

The method for preparing leaf homogenizes described under Materials and Methods has been in routine use in this laboratory for about a year and a half. Good results have consistently been obtained except for a three-month period during which the yields of nuclei declined to about one fifth of normal then rose again. This period occurred during the winter months and may have reflected the physiological state of the plants at that time. During this period it proved possible to bring the yield back to normal by increasing the time of incubation in isolation medium from 16 – 20 to 32 – 40 hrs or by increasing the incubation temperature from 0° to 12°.

Separation of Nuclei from Other Cell Constituents

The presence in leaf homogenates of large numbers of starch grains, which were difficult to separate from the nuclei by centrifugation, could easily be avoided by holding the plants in darkness for 24 to 36 hrs prior to harvesting the leaves. Cellulose fibers could be removed nearly quantitatively from the homogenates by filtration through cheesecloth and flannel. Nuclei could be recovered in high yields in the filtrate if care was taken that the filters did not become stopped and if the filter residues were washed with isolation medium as described under Materials and Methods. If these precautions were not observed, losses at this stage were considerable. A photomicrograph of a leaf homogenate after filtration through cheesecloth and flannel is presented in Fig. VI.

* Fig. VI, VIII, IX see p. 532 a and b.
The main part of the cytoplasmic constituents could be separated from the nuclei by alternating three cycles of low speed centrifugation (350 g) with one cycle of very low speed centrifugation (25 g) as described under Materials and Methods. The results of such a centrifugation schedule are shown in Fig. VII. It is evident that upon repeated centrifugation a point is reached (after about six cycles at 350 g) where further centrifugation no longer reduces the ratio of contaminating particles (mainly chloroplasts) to nuclei. The contaminating particles : nuclei ratios at this stage usually fell within the range 0.25 to 1.5 if small leaves (25 – 35 mm long) had been used as starting material. Preparations from larger leaves were more heavily contaminated. Yields ranged from 30 to 60% of the number of nuclei present in the homogenate. A photomicrograph of isolated nuclei is presented in Figs. VIII and IX. Nucleoli may be discerned in some nuclei. If the nuclei are suspended in concentrated salt solution, in detergent solution, or in a medium with a high pH, they swell somewhat, and the nucleoli become more conspicuous. The diameter of isolated tobacco leaf nuclei suspended in isolation medium ranges from about 4 to 8 μ with an average value of 6.0 μ.

**Characteristics of the Isolated Nuclei**

**Microscopic Appearance**

Phase contrast micrographs of isolated nuclei suspended in isolation medium are presented in Figs. VIII and IX. Nucleoli may be discerned in some nuclei. If the nuclei are suspended in concentrated salt solution, in detergent solution, or in a medium with a high pH, they swell somewhat, and the nucleoli become more conspicuous. The diameter of isolated tobacco leaf nuclei suspended in isolation medium ranges from about 4 to 8 μ with an average value of 6.0 μ.

**Fine Structure**

An electron micrograph of an isolated nucleus is presented in Fig. X. The homogeneous, densely-staining nucleolus is readily discerned. Except for some vacuolization, the extranucleolar region of the nucleus is also homogeneous. The vacuolization may be a result of damage occurring during isolation. Similar vacuolization – perhaps to an even greater degree – is evident in other published electron micrographs of plant nuclei. The nucleus is surrounded by a characteristic double membrane, the presence of which may protect the nucleus from damage during homogenization. Nuclei which had lost part or all of the membrane were frequently distorted or broken.
Gel-Forming Ability

If nuclei isolated by differential centrifugation are suspended in concentrated salt solution (e.g. 0.4 M ammonium sulfate) and allowed to incubate a few minutes at 30°, a viscous gel forms. Formation of such gels is characteristic of nuclei which contain highly polymerized DNA and indicates that the nuclei have escaped the action of certain cytoplesmic enzymes during isolation.

Enzymatic Activity

It is now generally recognized that isolation of nuclei in aqueous media may result in loss of soluble nuclear enzymes. Loss of such enzymes is not inevitable, however; thymus nuclei isolated in aqueous sucrose solutions have been demonstrated to retain not only soluble proteins, including several soluble enzymes, but even such low molecular weight compounds as mononucleotides. Nuclei isolated in aqueous media have retained such complex functions as the ability to accumulate amino acids and nucleosides by active transport and the ability to synthesize protein and RNA.

The extent to which nuclei isolated as described in the foregoing sections have retained their characteristic enzymatic activities is currently under investigation. Preliminary results indicate that nuclei isolated by differential centrifugation retain a DNA-dependent RNA-synthesizing system.

Range of Applicability of the Isolation Method

Results described so far have been obtained with tobacco leaves. Preliminary experiments indicate, however, that the methods described are generally applicable to isolation of nuclei from higher plant tissues. Good yields of intact nuclei were obtained in leaf homogenates of the pea, Pisum sativum L., and onion, Allium cepa L., and fair yields were obtained in homogenates of the stems and roots of tobacco using the normal homogenization procedure. To obtain nuclei containing homogenates from the leaves of spinach, Spinacia oleracea L., it was found necessary to increase the calcium chloride concentration in the isolation medium to 0.03 M, presumably due to the high oxalic acid content in spinach leaves. With the needles of a pine, Pinus nigra Arnold, difficulty was experienced; nuclei in homogenates prepared by the standard procedure proved to be unstable and gradually disintegrated during purification. With this exception it proved possible to purify, to various extents, the nuclei from all the above homogenates using the technique of differential centrifugation described under Materials and Methods. Modifications in the centrifugation schedule would certainly have improved the results in some instances, but this line of investigation was not pursued further.

Discussion and Conclusions

That plant leaf nuclei are destroyed by procedures used with wide success to prepare nuclei-containing homogenates from animal tissues might reasonably be explained in the following alternative ways: 1. Each plant leaf cell is surrounded by a tough wall composed of cellulose and various other polysaccharides; any treatment vigorous enough to disrupt this wall disrupts the nucleus also. 2. Each mature plant cell contains a vacuole filled with a solution of various inorganic and organic solutes, some in relatively high concentrations; release of the contents of this vacuole during homogenization results in destruction of the nucleus. The observation that the yield of nuclei from younger leaves, which have a much smaller vacuole and a less highly developed cellulose wall, is no better than that from older ones speaks against these hypotheses. The finding of Accola that the yield of nuclei from onion roots pretreated with enzymes to partially digest the cell wall was no greater than that from untreated tissue provides further evidence against the first of these hypotheses. One must consider the possibility that the fragility of plant nuclei results from some fundamental structural difference between plant and animal nuclei.

The modes of action of the various components of the isolation medium in protecting plant cell nuclei can, at present, only be surmised. Octyl alcohol, each molecule of which has both a hydrophilic and a hydrophobic portion, may well exert its protective effect via an interaction with the lipoprotein of the nuclear membrane. Chloroplasts, which are presumably surrounded by a similar membrane, seem also to be protected by octyl alcohol from destruction during homogenization. Sucrose and gum arabic may owe part of their effectiveness to their effect on the tonicity of the medium. Gum arabic, in addition, prevents aggregation of the cell constituents in the homogenate, an effect which may
be attributed to its polyanionic nature. The role played by calcium ion is unclear.

That high speed homogenization is more suitable than various "milder" methods for preparing leaf homogenates, although surprising, has a precedent in the work of CHAYEN and BENFIELD and of that of ACCOLA.

Although isolation of nuclei from animal tissues can, in most cases, be satisfactorily accomplished by homogenates might be tried to advantage.

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* Died December 16, 1962 in a mountain climbing accident.
Fig. VI. Phase Contrast Micrograph of Filtrate.

Fig. VIII. Phase Contrast Micrograph of Nuclei Purified by Differential Centrifugation.
Fig. IX. Phase Contrast Micrograph of Nuclei Purified by Combined Differential and Density Gradient Centrifugation.

Fig. X. Electron Micrograph of an Isolated Nucleus. Nuclei prepared by differential centrifugation in isolation medium modified to contain 0.005 M 2-mercaptoethanol were fixed with buffered 2% osmium tetroxide, stained with phosphotungstic acid, and embedded in Vestopal W. The length bar on the micrograph is approximate. The micrograph was made in the laboratory of Dr. H. Frank, Max Planck Institut für Biologie, Abteilung Weidel, Tübingen, Germany.