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Mass Isolation of Pea Nuclei

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ABSTRACT

Improvements in conventional filtration and centrifugation procedures made it possible to increase the yield of intact cytoplasm-free pea (*Pisum sativum*) nuclei from the usual 3 to 10% to 32% (6×10^8 nuclei per 7 grams fresh weight of pea apices) and to complete the isolation in 80 to 90 minutes. The isolated nuclei appeared to retain their structural integrity as revealed in electron photomicrographs, and remained intact for at least 5 hours at 20 Celsius.

The DNA:RNA:protein ratio of isolated pea nuclei was found to be 3.1:1:9.9. Their RNA polymerase activity, monitored by incorporation of ^{14}C into RNA from ^{14}C-UTP, was linear for about 10 minutes, and then gradually declined over the next 15 to 20 minutes.

Mass isolation of nuclei, whether from plants or animals, requires three basic steps: homogenization, filtration, and differential centrifugation. Although each step is essential, none has yet been perfected. I report here a rapid, reproducible procedure for isolating nuclei from plants. It represents a substantial modification and improvement of a previously described technique (9), and leads to reduction of the time required for isolation as well as an increased yield of undamaged nuclei.

MATERIALS AND METHODS

Growing Seedlings. Seeds of *Pisum sativum* L., cv. 'Alaska' (Asgrow Seed Co., Orange, Conn.) were soaked for 3 or 4 hr at 20 to 22 C in tap water containing 0.5 g of Phygon per liter. After rinsing and draining, the seeds were sown 1 to 2 cm deep in prewashed No. 4 vermiculite (Zonolite Corp., East Hampton, Mass.) for growing in darkness, and in No. 1 vermiculite for growing in continuous light. The temperature in the dark growth chamber (Hotpack Corp., Philadelphia, Pa.) was kept at 26 C, and the humidity, at 73%. In the light growth chamber (Environmental Growth Chambers, Chagrin Falls, Ohio) the plants were exposed to continuous illumination at about 2000 ft-c, a temperature of 24 C, and a humidity of about 55%. Dark-grown pea seedlings were watered once on the 4th day, and their apical buds were plucked on the 7th day after planting. Light-grown pea seedlings were automatically subirrigated once a day with a nutrient solution (120 g/100 liters of Hyponex, Hydroponic Chemical Co., Inc., Copley, Ohio), and their apical buds were plucked on the 8th day after planting.

Isolating Nuclei. Both gum arabic solutions (GAS)[3] and a resuspension solution (RS) were used in the isolation procedure. Both contained sucrose, 0.15 M; magnesium acetate, 4 mM; 2-mercaptoethanol, 5 mM; MES buffer, 5 mM; and double glass-distilled water. The GAS but not the RS also contained 2-ethyl-1-hexanol (0.1%) and gum arabic (4, 8, 10, or 12%). The pH of both solutions was adjusted to 6.0 to 6.1 with KOH. The solutions were stored at −2 C and freshly prepared every 3rd or 4th day.

Gum arabic (Fisher Scientific Co.) was purified before use by centrifugation of a 12% solution at 13,000*g* for 1 hr in a Sorvall GSA rotor, and then by filtration of the supernatant liquid first through two layers of Miracloth, then through washed glass-fiber filters (APO 2012450, Millipore Corp.), and finally through two layers of Whatman No. 42 filter paper. The other ingredients of GAS were added after the filtration step because some of them, especially Mg^{2+}, were adsorbed by the Whatman No. 42 filter. The RS was filtered through HA filters (Millipore Corp.).

To isolate nuclei, 7 g of apical buds were infiltrated with 4% GAS under vacuum for 12 min. The suspension of apices was next placed in an incubator at 10 C and allowed to steep for 14 hr (see Kuehl, 9). After steeping, the buds were rinsed twice with cold 4% GAS, were drained and placed in 10 ml of 4% GAS, and were homogenized in a Sorvall Omni-mix for 30 sec at top speed in the presence of 3 drops of 2-ethyl-1-hexanol to reduce foaming. Next the homogenate was rinsed out with 10 ml of 4% GAS and was filtered through a series of nylon screens (Nitex; Tobler, Ernst and Traber, Inc., Elmsford, N. Y.) with pore diameters of 375, 100, 25, and finally 10 μ. In order to speed up the filtration and to keep the solution volumes to a minimum, the nylon screens were stacked vertically in removable, 6-cm-high holders made by cutting off the bottoms of 250-ml Nalgene beakers. The residue on the top screen (375 μ) was resuspended in 15 ml of 4% GAS and was poured through the screens again to release trapped nuclei. Then, each screen was removed, in turn, after rinsing the residue on each with 10 ml of 4% GAS. The 10-μ screen was not rinsed. The final filtrate was diluted with 12% GAS to raise the concentration of gum arabic in the filtrate to 6.5%. The filtration procedure was carried out in a cold room at 3 C.

While the filtration was in progress, four discontinuous gradients, each having a layer of 8, 10, and 12% GAS, were prepared. Thirty milliliters of 12% GAS were poured into a 250-ml flat-bottom Nalgene centrifuge bottle, the constricted

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[3] Abbreviations: GAS: gum arabic solution; RS: resuspension solution; MES: morpholinoethane sulfonate; PS: plain incubation solution; CS: complete incubation solution.

top of which had been cut off. Forty milliliters of 10% GAS and 50 ml of 8% GAS were layered next on the 12% GAS by pouring through 100-ml funnels having narrow outlets to ensure a slow flow. About 20 ml of the filtrate were layered on each gradient and centrifuged for 12.5 min at 2000 rpm (about 900*g*) in an International refrigerated centrifuge equipped with a swing-out bucket rotor (No. 259). The nuclei were pelleted, whereas most of the debris remained above the 12% GAS layer. The supernatant solutions were aspirated off, leaving 2 to 3 ml of the 12% GAS above each pellet. The pellets were resuspended and pooled in RS to give a final volume of about 20 ml and a gum arabic concentration of 6.5%. About 10 ml of the nuclear suspension was layered next on each of two gradients, prepared as before and then centrifuged at 2000 rpm for 10.5 min. The supernatant solutions were aspirated off completely, and the pelleted nuclei were resuspended in 20 ml of RS. After a determination of the nuclear concentration with a Spencer hemacytometer, aliquots of the suspension were taken for experimentation. The procedure lasted 80 to 90 min, but because the filtrate was layered on a density gradient and centrifuged immediately after the rapid filtration, the nuclei were freed from the homogenate within 15 min after homogenization.

RNA Synthesis. A plain (PS) and a complete (CS) incubation solution were used for experiments on RNA synthesis in isolated nuclei. The ingredients of PS were: sucrose, 0.15 M; N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer, 20 mM; potassium acetate, 60 mM; magnesium acetate, 4 mM; 2-mercaptoethanol, 5 mM; and double distilled water. The PS was filtered through HA Millipore filters; its pH was adjusted to 6.9 with KOH, and then it was stored frozen at −20 C until needed. The CS was prepared from the PS by adding the desired amounts of ATP, GTP, CTP (Sigma Chemical Co.), and ^{14}C-UTP (Schwarz BioResearch, Inc.). A CS, lacking only ^{14}C-UTP, was prepared in advance and was stored frozen in several small aliquots at −20 C until needed. Radioactive UTP was dissolved in this CS not more than 2 hr before starting the reaction.

Incubation conditions for RNA synthesis were as follows. Aliquots of a nuclear suspension, each containing about 5.0×10^7 nuclei, were transferred to 10- × 75-mm culture tubes, and the nuclei were pelleted by centrifuging for 3 to 4 min at 1000*g* in an International clinical centrifuge at 3 C. Next, the supernatant solutions were decanted, the pellets drained briefly, and the nuclei resuspended in 0.15 ml of PS. After 5 min of equilibration in a water bath at 20 C, the reaction was started by the addition of 0.15 ml of CS to each tube. Because of this doubling in volume, the concentrations of the four nucleoside triphosphates in the CS were twice the concentrations desired in the final 0.3 ml of the reaction mixture. When pretreatment of the nuclei with inhibitors or stimulants was necessary, the substance in question was added to both the PS and CS at the desired final concentration.

The reaction was stopped by adding 2 ml of cold 5% trichloroacetic acid to each tube. After 30 min of precipitation at 3 C, the nuclei were pelleted, and the pellets were rinsed twice more with cold 5% trichloroacetic acid, twice with 80% ethanol saturated with sodium acetate, and once with 95% ethanol. The rinsing was performed in the reaction tubes by centrifuging for 3 to 4 min at 1000*g* in an International clinical centrifuge. In this way the background was reduced to 6 pmoles of UMP/5.0×10^7 nuclei. Next, the rinsed pellets were hydrolyzed in 0.2 ml of 0.5 N KOH at 45 C for 4 hr. Finally, the hydrolysates were transferred quantitatively with Bray's scintillation fluid to vials, and the radioactivity of the hydrolysates was determined with a scintillation counter. The counts per minute were converted to disintegrations per minute by the channels ratio method.

Miscellaneous Procedures. DNA in isolated nuclei and in apical buds was determined by the procedure of Webb and Levy (13), and protein was determined by the biuret method (7). RNA was estimated from the difference in absorption of sodium dodecyl sulfate-phenol extracted RNA at 257 and 290 nm (the 2% contribution of DNA to absorption was subtracted), which was then multiplied by the factor 52 μg of RNA/absorbance unit. The conversion factor was determined from analysis of anhydrous yeast RNA.

RESULTS

The appearance and degree of purification of pea nuclei during the isolation procedure are shown in Figures 1 and 2. The first gradient differential centrifugation is the major purification step, whereas the second centrifugation merely removes the debris dragged down with the sedimenting nuclei during the first centrifugation. Tables I and II provide some results on the purification procedure and the isolated nuclei. When the source of nuclei was buds only one-third of their normal size, or very large buds of 8- or 9-day-old dark-grown seedlings, the yield of nuclei was reduced. Similarly, the yield of nuclei from young, expanded leaves of light-grown seedlings (Table I) was small. In Table II, the diameters of each group represent the peaks in a continuous frequency distribution. The percentages, therefore, represent the relative numbers of nuclei in each group. DNA content and dry weight values agree with the data of Lyndon (11) who found a diploid (2C) DNA content of 9.6 pg per pea nucleus and a dry weight of 40 to 60 pg per pea nucleus. The DNA:RNA:protein ratio differs from the ratio 5:1:25 reported by Johri and Varner (8). This disagreement of results may be due to the different methods used for isolating nuclei and for determining the RNA and protein concentrations. Incubation of isolated nuclei in RS at 20 C for up to 5 hr produced no significant decrease in the number of nuclei.

RNA polymerase activity of the isolated nuclei was tested by determining the incorporation of ^{14}C-UMP, supplied as ^{14}C-UTP, into RNA. The reaction was found to be linear for 10 to 15 min, with a rate of about 12 pmoles of UMP incorporated per min per 5×10^7 nuclei, the rate gradually declining during the next 10 to 15 min. Other characteristics of the reaction are shown in Table III.

DISCUSSION

A survey of existing methods for isolating nuclei from plants showed that major improvements were needed in the procedures in order to obtain large numbers of structurally and functionally intact nuclei. Although Kuehl's isolation solutions (9) were the best available at the time, it was found in the present experiments that the yield of nuclei could be increased by adjusting the pH of the isolation solution to pH 6 to 6.1. Variation from this by ±0.5 pH units resulted in a 50% reduction in yield. These results are in agreement with the findings of Dounce (6) and Philpot and Stanier (12) who investigated the effects of pH and ionic strength of isolation solutions on the yield and appearance of animal nuclei.

The yield of nuclei was further increased by modifications of the filtration step which is especially necessary when isolating nuclei from plants. The large amount of debris left over from the homogenization step trapped many nuclei when the usual cheesecloth or Miracloth was used; furthermore, such cloth was ineffective in removing small pieces of debris such as cells and even clumps of cells. Changing to nylon screens (Nitex)

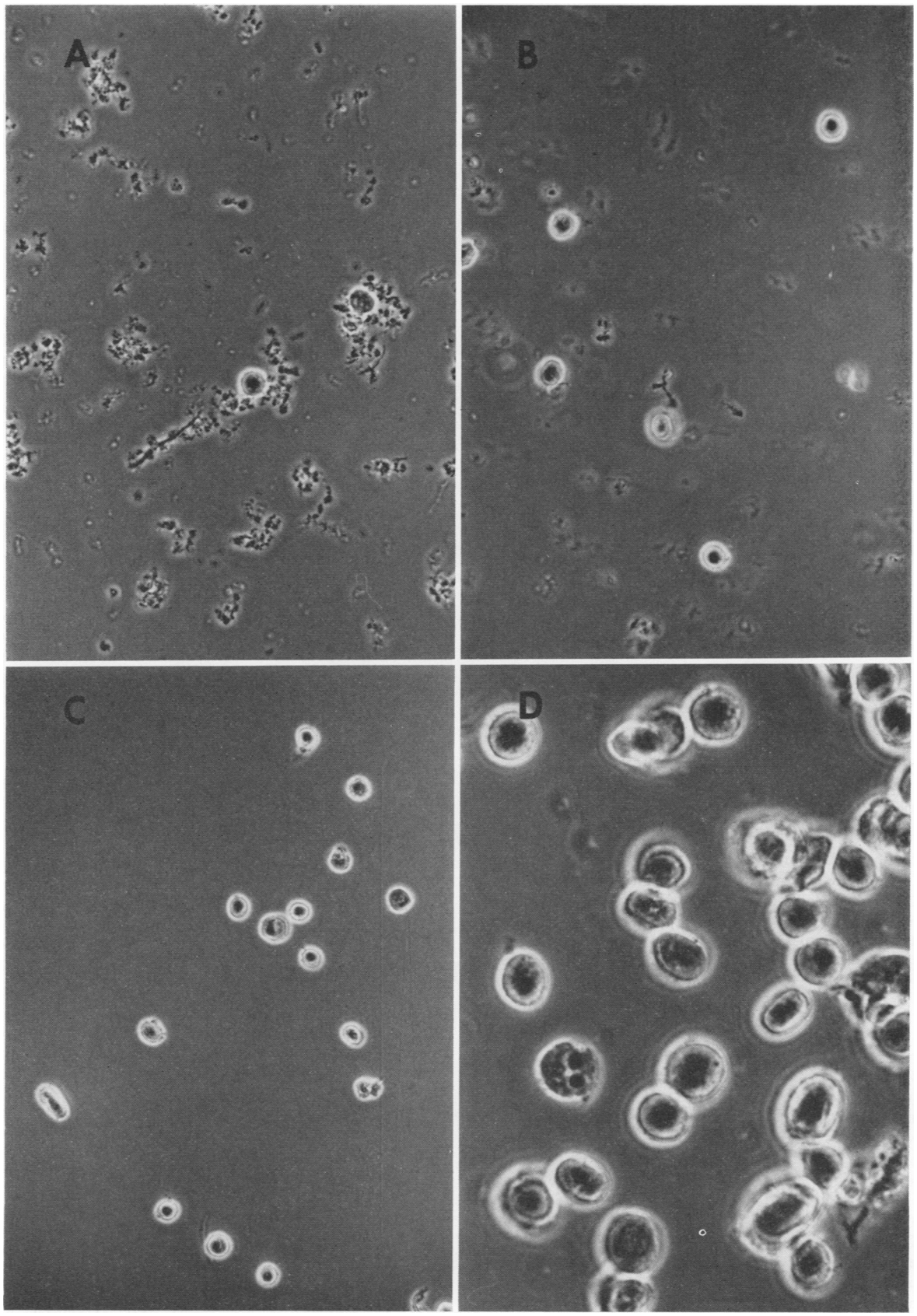

FIG. 1. Phase contrast photomicrographs of nuclear preparations at various stages of purification. The source of nuclei was apical buds of light-grown pea seedlings. A: Appearance of filtrate, 85-ml volume, × 480; B: nuclei after the first centrifugation resuspended in 20 ml, × 480; C: nuclei after the second (final) centrifugation resuspended in 20 ml, × 480; D: 5.0×10^7 nuclei resuspended in 0.3 ml of the plain incubation solution. Here the nuclear concentration is 25 times greater than in the filtrate, × 1200.

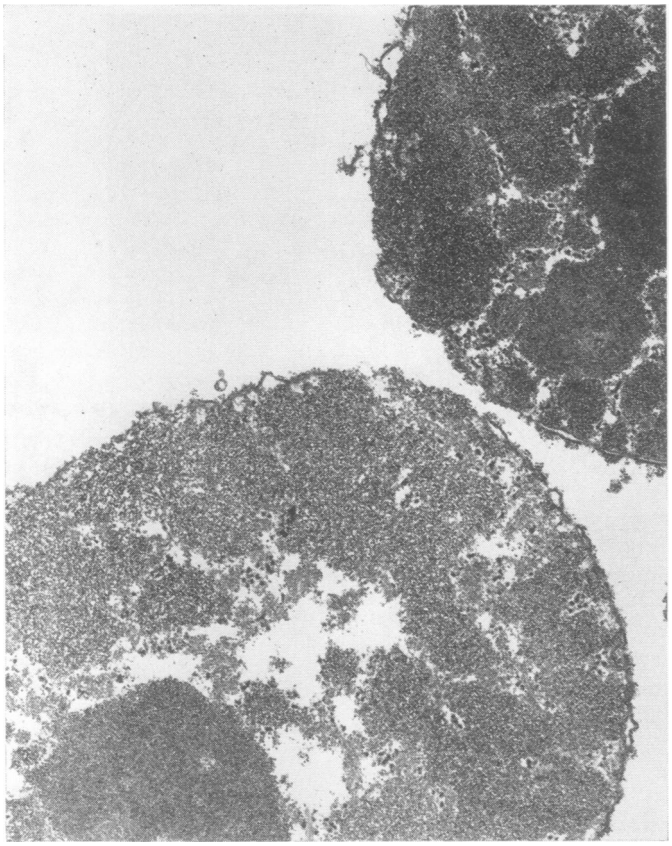

FIG. 2. Electron photomicrograph of isolated pea nuclei. Nuclei were fixed in 3% glutaraldehyde, stained with 1% OsO_4, dehydrated in acetone, embedded in Epon 812, and sectioned; the sections were then post-stained with uranyl acetate and Reynold's lead citrate. × 33,000.

Table I. *Results of the Isolation and Purification of Nuclei from Peas*

Recovery of nuclei from whole tissues is based on the DNA content of 7 g of dark-grown pea buds and the DNA content of purified nuclei recovered from 7 g of dark-grown pea buds. Other percentages are based on the number of particles present as determined with a Spencer hemacytometer.

Material	Yield of Nuclei per 7 g fr wt[1]	No. of Experiments Included in avg.
Dark-grown peas		
Normal apical buds	561 ± 34 × 10^6	25
Subnormal apical buds	329 ± 80 × 10^6	17
Light-grown peas		
Apical buds	599 ± 33 × 10^6	18
Young leaves	333 ± 10 × 10^6	3
Recovery from whole tissue	30 ± 7.2%	2[2]
Recovery from filtrate	74 ± 7.0%	7
Contamination with "cells"	<0.15%	3
Contamination with damaged nuclei	16 ± 2%	3
Contamination with chloroplasts (light-grown peas only)	12 ± 1.1%	7

[1] Results are expressed with the S.E.

[2] There were five replicates in each experiment.

arranged in a series of diminishing pore sizes resulted in several improvements: clumps of cells, single cells, and cell fragments larger than 13 μ in diameter were separated from the nuclei; the recovery of nuclei was increased 6-fold because the residue from the homogenate was distributed into several

Table II. *Characteristics of Isolated Pea Nuclei*

Measurements of nuclear diameters were accurate within the error limits of ±0.25 micrometer units (1.125 μ at a magnification of 240×).

Diameters of isolated nuclei	4.4– 5.8 μ (81.5%) 8.0–10.0 μ (12.7%) 11.5–14.5 μ (5.8%)
Dry wt of avg nucleus	53.0 ± 6.0 pg
DNA content of avg nucleus	10.5 ± 0.3 pg
RNA content of avg nucleus	3.4 ± 0.2 pg
Protein content of avg nucleus	33.6 ± 0.3 pg
Ratio of DNA:RNA:protein	3.1:1:9.9

Table III. *Characteristics of the Incorporation of ^{14}C-UMP (supplied as ^{14}C-UTP) into RNA in Isolated Pea Nuclei Incubated at 20 C*

(*a*) Nuclei isolated from dark-grown pea seedlings. Data taken at maximum incorporation.

(*b*) Nuclei isolated from light-grown pea seedlings. Data taken at 10 min after reaction (linear rate).

The concentrations of the additives were: actinomycin D, 20 μg/ml; spermine, 1 mM; and rifampicin, 10 μg/ml. The different levels of incorporation in some of the controls were due to differences in the concentrations of various ingredients in the complete incubation solutions, especially UTP and K^+. In (+) actinomycin D (*a*), the concentrations of ATP, GTP, and CTP were 0.6 mM each, and UTP was 0.08 mM. The specific radioactivity of ^{14}C-UTP in this case was 25 μc/μmole, and the K^+ concentration was 6 mM. In all other (*a*) experiments the concentrations of the nucleoside triphosphates were 1.2 mM each, except for UTP which was 0.55 mM. Here, the specific radioactivity of ^{14}C-UTP was 1.2 μc/μmole, and the K^+ concentration was the standard 60 mM. In the (*b*) experiments, the concentration of ATP was 3 mM, that of GTP and CTP was 1.5 mM, and that of UTP 0.4 mM. The specific radioactivity of ^{14}C-UTP was 1.64 μc/μmole, and the K^+ concentration was the standard 60 mM. All other ingredients of the reaction solutions were at the concentrations given in "Materials and Methods." Actinomycin D, spermine, and rifampicin were added to the nuclei resuspended in the plain incubation solution at the start of the equilibration period at 4 C. Each treatment was duplicated within an experiment, and when indicated by the standard error, the experiments were repeated twice, thus giving a total of six replications. These data represent incorporation above background (6 pmoles/5 × 10^7 nuclei).

Treatment	pmoles/5.0 × 10^7 Nuclei		Inhibition (↓) Stimulation (↑)
	Treated	Control	
(+) Actinomycin D (*a*)	2.5	16.4	(↓) 84.8 ± 2.3
(+) Actinomycin D (*b*)	10.5	119.0	(↓) 91.2
(−) ATP, GTP, CTP (*a*)	12.0	92.0	(↓) 86.9
(−) ATP, GTP, DTP (*b*)	9.7	119.0	(↓) 97.8
(−) GTP, CTP (*b*)	12.5	119.0	(↓) 89.5
(−) ATP (*b*)	9.4	119.0	(↓) 92.1
(+) Spermine (*a*)	203.0	78.0	(↑) 160.0 ± 16
(+) Rifampicin (*b*)	117.0	119.0	Not significant

size classes; the volume of the filtrate was reduced from approximately 200 to 55 ml. Although four nylon screens were used, the filtration step lasted only 5 to 8 min.

Recently, while I was developing a procedure for mass isolation of amoeba nuclei (to be published), I found that more nuclei can be recovered from the filtration step by gluing each screen to one end of a Nalgene screen holder with epoxy resin, and then setting one holder inside the other as before. When the screens are not glued in place, nuclei tend to slip under the rims of the holders, and float up the sides of the screens.

A common procedure in isolating nuclei from plant and animal tissues is to centrifuge the filtrate and then to resuspend the pellet for layering on a gradient. I learned that such differential centrifugation made it impossible to resuspend the pellet without destruction of at least 50% of the nuclei in the pellet. Furthermore, an initial differential centrifugation of the filtrate made subsequent purification of nuclei by mild procedures very difficult. The reason for this is that free chromatin and other nucleoproteins and lipoproteins present in the filtrate remain with the nuclei during a simple differential centrifugation, and glue nuclei together. On the other hand, by keeping the volume of the filtrate low, it was possible to layer the filtrate on gradients of gum arabic immediately after filtration and, thus, to achieve in the first differential density gradient centrifugation a degree of purity that allowed resuspension of the pelleted nuclei with negligible destruction. A second differential density gradient centrifugation was enough to remove the remaining contamination from the nuclei. Raising the concentration of gum arabic in the filtrate prior to layering on the gradients minimized the streaming phenomenon described by Anderson (1).

Gum arabic was used in the gradient instead of sucrose because there is some evidence of deleterious effects of high concentrations of sucrose on nuclear stability (9, 10). Electron photomicrographs of nuclei isolated in dense sucrose solutions (2–4, 14) show nuclei with broken outer membranes, abnormally expanded intermembrane spaces, and barely visible nucleoli. Because gum arabic even at 12% is rather viscous (mol wt around 240,000), and because sedimentation rates of particles are inversely proportional to the viscosity of the solutions, the purification achieved with gum arabic gradients was probably due to the viscosity of the solutions rather than to the density. Gum arabic at a concentration of 12% has a density of only 1.095 g/ml.

The changes in the isolation and purification procedures discussed above made it possible to recover more nuclei from the homogenate and, thus, to increase the yield of purified nuclei from the usual 3 to 10% to 32%. To increase further the yield of nuclei would require a major change in the present methods of homogenizing plant tissues such that more than 50% of the cell nuclei would be released into the homogenate undamaged. D'Allessio and Trim (5) have used, with great success, cell wall-digesting enzymes before homogenization. This treatment resulted in about a 3-fold increase in the number of nuclei released into the homogenate. However, these authors recovered only 51% of the nuclei before purification and only 26% of the nuclei after. By my estimates, the use of highly purified specific cell wall-digesting enzymes prior to a gentle homogenization (Dounce homogenizer) as well as my method of separating nuclei from the rest of the homogenate should give at least a 90% release of nuclei into the homogenate and a final recovery of undamaged nuclei as high as 63%.

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