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A method for enucleation of *Saccharomyces cerevisiae*

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Abstract: The first method for enucleation of yeast *Saccharomyces cerevisiae* is reported. Various strains, including some killer strain and respiratory-deficient mutants of *Saccharomyces cerevisiae* were enucleated after treatment with cytochalasin B. Removal of nuclei from protruding sphaeroplasts was induced by centrifugation in a Percoll density gradient. The enucleation yield (which averaged about 80%) and the quality of the cytoplasts were best when the yeast culture had been synchronized with nocodazole before the preparation. The presence of 1 mM $CaCl_2$ and ATP (10 μM) in the enucleation medium prevented the formation of fragile products or aggregation. Cytoplasts could be stored for at least 1 day without visible deterioration.

Key words: Saccharomyces cerevisiae; Cytochalasin B; Cytoplast; Nocodazole; Yeast

Introduction

The enucleation of yeast cells is of interest for the production (by fusion) of special industrial yeast hybrids, possessing mixed cytoplasms (cybrids) but only one nucleus. Such cybrids could be designed to have a modified phenotype without change of ploidy. However, no methods for the enucleation of yeast cells have yet been reported.

In animal cells, enucleation usually uses the cytochalasins [3,10,12,13,14,18,19]. In most cases, either cytochalasin B or D have been used to change the properties of the cytoskeleton in such a way as to give nuclear extrusion and, in a small number of species, enucleation [8]. However, only in the case of some mammalian systems (BSC-1 cells, chicken embryo fibroblasts, mouse L cells and human polymorphonuclear lymphocytes) have larger numbers of enucleated cells (cytoplasts) been obtained [10,12,13,14,19].

Yeast cells of *Saccharomyces cerevisiae* have many fundamental properties in common with cells of higher organisms [4,15]. In particular, the fact that the structure of tubulin and actin filaments is conserved between animal and yeast cells [5,6], makes it likely that cytochalasins should be effective enucleation agents for yeast.

In this work the use of cytochalasin B (in combination with synchronization) for the enucle-

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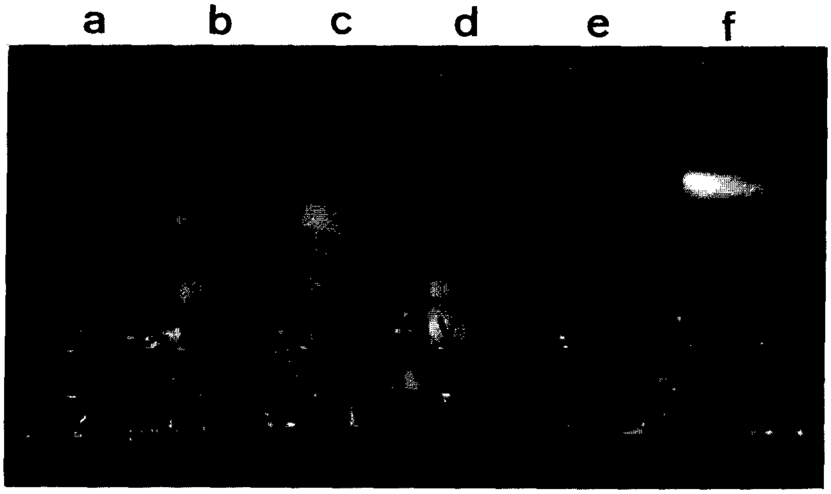

Fig. 1. A separation of enucleated protoplasts/sphaeroplasts from discontinuous density gradient of Percoll: (a) density marker beads, from bottom: violet (d = 1.142 g/ml), red (1.121 g/ml), green (1.098 g/ml), orange (1.087 g/ml), blue (1.075 g/ml); (b) separated sphaeroplast of *S. cerevisiae* 34, *rho*$^{+}$ strain, synchronized by nocodazole and treated with CB (enucleation medium II); cytoplasts were taken from a band (d = 1.11 g/ml) between the red and green density marker beads; (c) protoplasts of *S. cerevisiae* 34, *rho*$^{+}$ strain, treated by CB in enucleation medium I, w/o density marker beads; cytoplasts were taken from a band marked 'x'; (d) separated sphaeroplasts of *S. cerevisiae* H_3, *rho*$^{+}$ strain; cytoplasts were taken from the band marked 'y'; (e) the band of anucleated cells (big buds w/o nucleus) of *S. cerevisiae* 34, *rho*$^{-}$ strain, w/o density marker beads; cytoplasts were taken from a band marked 'z'.

ation of some strains of *Saccharomyces cerevisiae* is reported.

Materials and Methods

Strains

The following strains were used in this study: 1). *Saccharomyces cerevisiae* H_3 [*rho*$^{+}$] [K^+R^-], diploid (from Prof. Dr. O. Bendová, Charles University, Prague, Czech Republic); 2). *Saccharomyces cerevisiae* 34 (former name *Saccharomyces uvarum* var. *carlsbergensis*, brewery strain) [*rho*$^{+}$] [K^-R^-], aneuploid (from Prof. Dr. S. Donhauser, Technical University of Munich-Weihenstephan, FRG) and its derivative [*rho*$^{-}$], obtained earlier using ethidium bromide [17].

Media for yeasts and for enucleation

Strains of *S. cerevisiae* were grown in *YEPD* medium.

Enucleation medium I contained: 20 μg/ml cytochalasin B, 400 mM NaCl, 10 mM KCl, 55 mM $MgCl_2$, 10 mM $CaCl_2$, 15 mM Na-Hepes buffer pH 7.5, 0.6 M sorbitol; osmolality about 1500 mOsm.

Enucleation medium II contained: 20 μg/ml cytochalasin B, 1.2 M sorbitol, 10 μM ATP, 1 mM $CaCl_2$, 20 mM Tris-HCl, pH 8.2; osmolality 1500 mOsm.

Cytoplasts were stored in Storage medium, consisting of: 1 M sorbitol, 1% yeast extract, 2% peptone, 1 mM ATP, 1 mM $MgCl_2$, 10 mM $CaCl_2$, 100 mM KH_2PO_4, 50 mM Tris-HCl, pH 7.5 (final pH 7.0).

Chemicals and stock solutions

Cytochalasin B, and nocodazole (methyl-5-/2-thienylcarbonyl-2-phenyl-indole) were from Sigma Chemical Co., St. Louis, MO. Cytochalasin B was dissolved at 3 mg/ml in DMSO (dimethyl sulfoxide), and then diluted into a suitable medium (final concentration for enucleation 20 μg/ml). For synchronization of cultures, nocodazole from a freshly prepared stock solution (3.3 mg/ml in DMSO) was added to the YEPD-medium (final concentration 15 μg/ml), together with additional DMSO to reach a final concentration of 0.1%.

A stock solution of DAPI (5 mg/ml) was prepared in phosphate buffer pH 7.0. For fluorescence staining, the final concentration was 5 μg/ml (in 1.2 M sorbitol).

Percoll (sterile) and density marker beads were from Pharmacia LKB (Sweden).

Procedure of enucleation

Synchronization of yeast cultures. The following procedure was used before enucleation (with enucleation medium II). Nocodazole was added (to give a final concentration of 15 μg/ml) 2 or 3 h after the end of the logarithmic phase (after 10–11 h growth cell density 10^8/ml) in order to arrest the growth of yeast populations, and after 6–8 h gave almost exclusively large cells. This drug causes microtubule disassembly and blocks nuclear division, including loss of nuclear movement to buds [5].

Sphaeroplasting and protoplasting. Protoplasts were prepared as described previously [2]. For sphaeroplasts, the incubation with zymolase was reduced to 15 min or less (depending on strain).

Separation of nucleated sphaeroplasts or protoplasts. Sphaeroplasts or protoplasts were prepared from nocodazole-synchronized cultures. After centrifugation for 5 min at $1950 \times g$ in 1.2 M sorbitol, the pellet (either large sphaeroplasts or protoplasts) was resuspended in 1.2 M sorbitol, and centrifuged again (10 min at $1950 \times g$).

Enucleation. Sphaeroplasts or protoplasts of *S. cerevisiae* strains (prepared as above) were suspended (at 10^8/ml) in the required enucleation medium and shaken very slowly at 30°C for 24 h. Afterwards, the suspension was harvested (10 min at $670 \times g$) and suspended in Percoll (density d_3 1.10 g/ml, osmolality about 1500 mOsm). This suspension (2 ml) was layered onto a prewarmed (2 h at 30°C) Percoll (osmolality 1500 mOsm) density gradient, consisting of 1 ml of: $d_1 = 1.13$ g/ml, $d_2 = 1.11$ g/ml, d_3 with sphaeroplasts or protoplasts and $d_4 = 1.09$ g/ml. The discontinuous density gradient was centrifuged at $100\,000 \times g$ for 1 hour. After centrifugation, the bands that were produced (Fig. 1) were collected separately from the top of the tube, and diluted into isoosmotic sorbitol (1.2 M). The bands were examined for their content of enucleated spheroplasts or protoplasts by fluorescence staining with DAPI.

The preparation of good quality cytoplasts can be summarized as: Growth of cells → Synchronization with nocodazole → Harvest cells → Sphaeroplasting → Enucleation in medium II and gentle shaking → Harvest of sphaeroplasts → Discontinuous density gradient centrifugation → Collection of bands with enucleated cells (cytoplasts).

Fixation

Before fluorescence staining, sphaeroplasts or protoplasts were fixed by rapid heating (to 65°C for 10 min).

Nuclear staining

After fixation, sphaeroplasts or protoplasts were resuspended (up to 10^8 sphaeroplasts or protoplasts/ml) in 50 mM phosphate buffer (pH 7.0) with 1.2 M sorbitol. The position of the nucleus (i.e., inside or outside sphaeroplasts or protoplasts) was examined directly by fluorescence staining with the DNA-specific dye DAPI at a final concentration of 5 μg/ml [1] for 45 min at room temperature, washed with 0.85% saline in 1.2 M sorbitol, and then examined by fluorescence microscopy (Axiophot, Carl Zeiss, FRG). Micrographs were taken with a fast slide film (Kodak Ektachrome 800) followed by 'push processing' (EI 800, 1600, or 3200).

Results

Preliminary experiments attempted enucleation (with enucleation medium I) of whole cells from non-synchronized and synchronized cultures. In this medium these cells showed a general and sustained contraction, which usually resulted only in the formation of a protrusion: very few cells appeared to have enucleated (less than 1%). No significant differences in enucleation yield could be found between cultures synchronized by nocodazole (or by other synchronization methods, i.e. colchicine or killer toxin from *Kluyveromyces lactis*). Other methods of density-

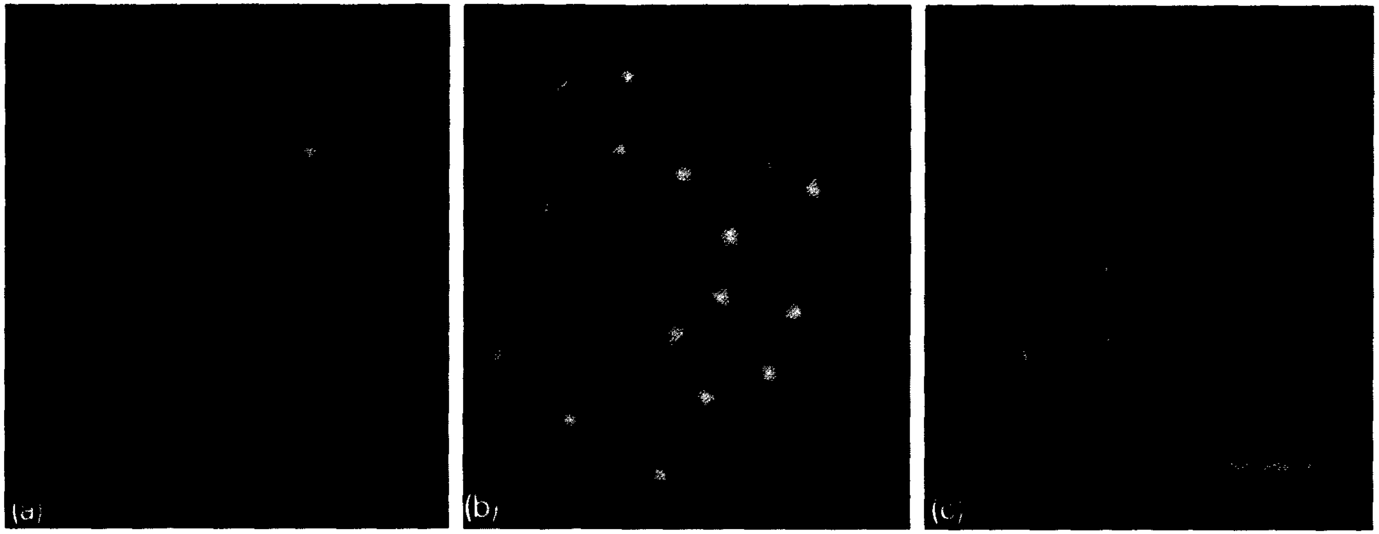

Fig. 2. Fluorescence micrographs of DAPI-stained sphaeroplasts (from non-synchronized culture) before enucleation: (a) *S. cerevisiae* 34 *rho*$^+$; (b) *S. cerevisiae* 34 *rho*$^-$; (c) *S. cerevisiae* H_3 *rho*$^+$. The large bright spots are cell nuclei, whereas the small spots visible only in a) and c) are mitochondria, b) was exposed under the same conditions as a) and c): the uninform intracellular background in b) is typical of other *rho*$^-$ strains (not shown). In all micrographs, the bar represents 8 μm.

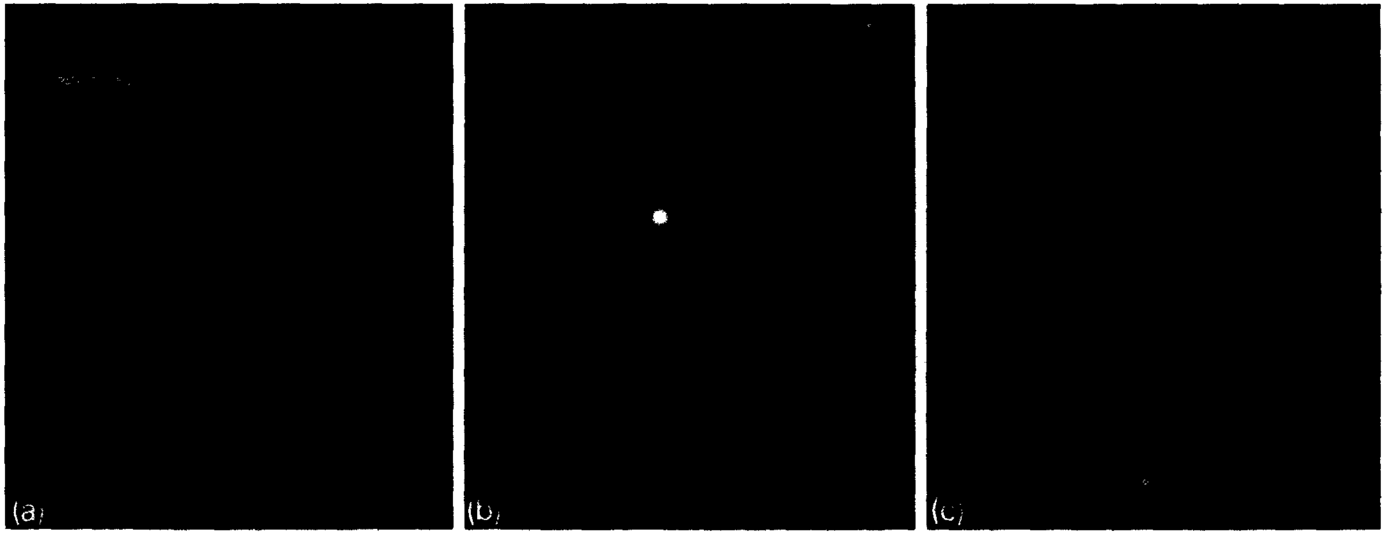

Fig. 3. Fluorescence micrographs of DAPI-stained sphaeroplasts after enucleation using enucleation medium II (including discontinuous density gradient centrifugation): (a) *S. cerevisiae* 34 *rho*$^+$; (b) *S. cerevisiae* 34 *rho*$^-$; (c) *S. cerevisiae* H_3 *rho*$^+$. Large bright spots visible in some cells are nuclei, but the small sports in *rho*$^+$ strains are mitochondria. In all micrographs, the bar represents 8 μm.

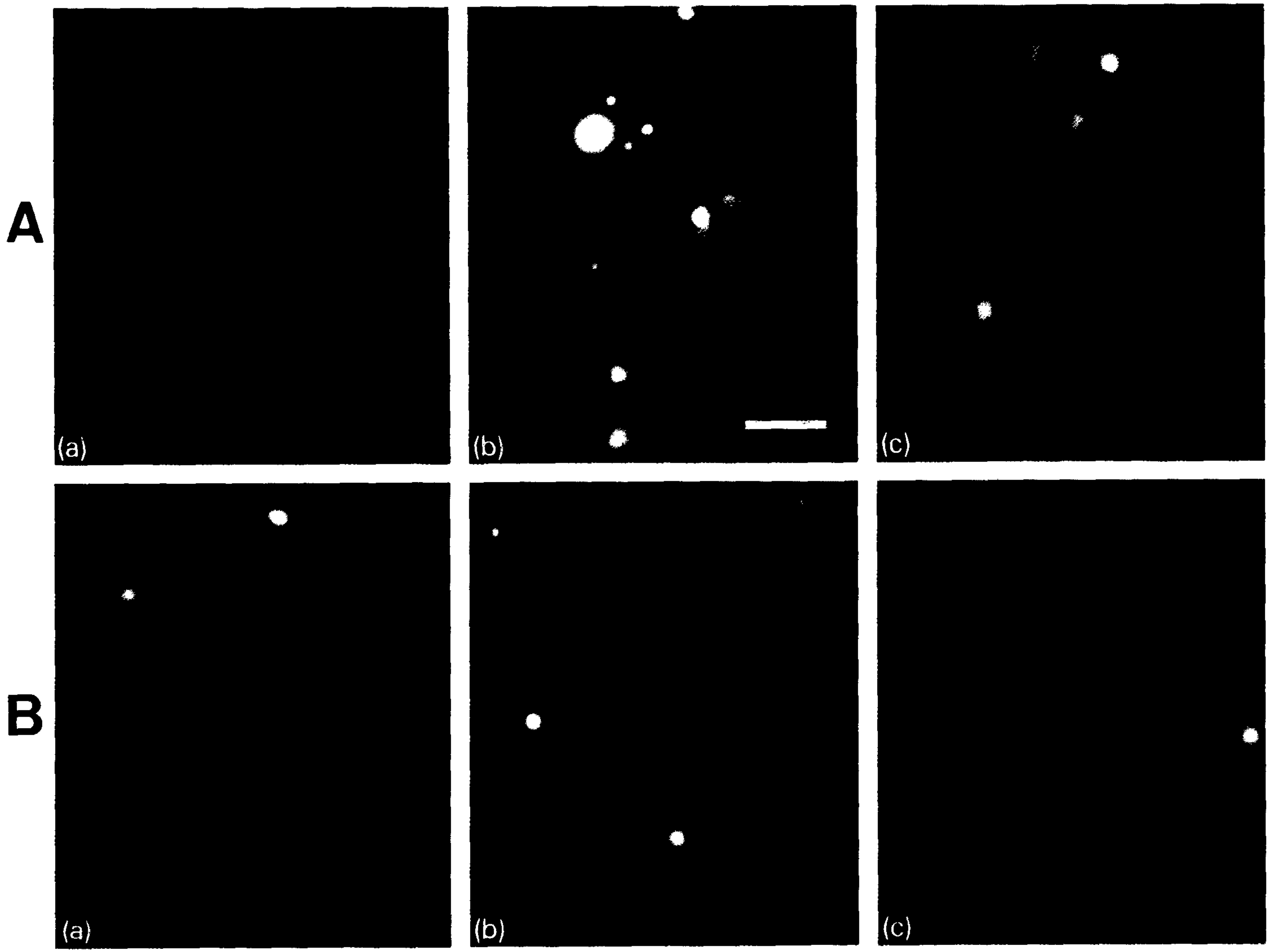

Fig. 4. Fluorescence micrographs of DAPI-stained protoplasts (top row) and sphaeroplasts (bottom row) after enucleation using enucleation medium I (w/o synchronization by nocodazole, but including discontinuous density gradient centrifugation): (a) *S. cerevisiae* 34 *rho*$^+$; (b) *S. cerevisiae* 34 *rho*$^-$; (c) *S. cerevisiae* H_3 *rho*$^+$. Large bright sports visible in some cells are nuclei, but the small spots in *rho*$^+$ strains are mitochondria. In all micrographs, the bar represents 8 μm.

gradient centrifugation (e.g. $20000 \times g$ or $60000 \times g$) did not give better results.

Due to the difficulties with whole cells, protoplasts and sphaeroplasts (Fig. 2a, b, c) were prepared.

Attempts (without synchronization of the yeast culture) to enucleate protoplasts of *Saccharomyces cerevisiae* with enucleation medium I led to the formation, during discontinuous density gradient centrifugation, of much debris, and to aggregation (Fig. 4, top row). This was because enucleated, non-synchronized protoplasts were very fragile. Sphaeroplasts enucleated in medium I showed much less damage (Fig. 4, bottom row, compare with Fig. 2). Some cytoplasts were anchored to cell bodies via long threads and tended to aggregate (Fig. 4c, bottom row). The yield of these cytoplasts was high, and varied with the strain (up to 70% of cytoplasts from all protoplasts, see Fig. 4); but these properties prevented subsequent purification.

Sphaeroplasts of *Saccharomyces cerevisiae* derived from nocodazole-synchronized cultures also gave high yields (on average about 80%, see Fig. 3a, b, c and compare with Fig. 2) of enucleated forms when using enucleation medium II. Unlike the above, these cytoplasts showed neither contraction nor fragility, nor did they aggregate (Fig. 3). The cytoplasts from this procedure could be purified on a Percoll density gradient. Such cytoplasts remained viable (shown by staining with 0.003% methylene blue) for at least 1 day when they were stored in storage medium at 30°C. After this period, extensive aggregation and contraction occurred.

Discussion

It seems that three distinct processes were helpful in successful enucleation:

1) disassembly of the microtubules by use of CB [8,18];

2) disruption of the structural frame-work of the mitotic spindle pole bodies (SPBs) [15];

3) synchronization of the cells in G_1 phase.

The last two processes can both be accomplished with nocodazole [5], and the synchronization is probably the reason for the much better quality of sphaeroplasts (and the resulting higher yield of cytoplasts) after nocodazole.

In the preliminary work using sphaeroplasts or protoplasts in enucleation medium I, there was much damage to the membrane, probably because medium I was damaging to the plasma membrane, leading to loss of the SPBs (probably during centrifugation). This, with the consequent loss of cytoplasm, caused aggregation (Fig. 4, top row). A similar effect was reported for polymorphonuclear leucocytes [9]. It also seems that the depolymerization of the actin cytoskeleton (which plays a central role in yeast morphogenesis) was not sufficiently complete in enucleation medium I, because of the presence of Mg^{2+}, the high level of Ca^{2+}, and the lack of ATP [11]. A low level of ATP in the cell often leads to a loss of cytoplasm [3]. A further problem with enucleation medium I was the pH, which at 7.5 was rather lower than that usually used for protoplasts [2]. A final point is that the culture was not synchronized by nocodazole before treatment with cytochalasin B in medium I and therefore the actin was not depolymerized.

The above considerations led to the formulation of enucleation medium II, which contained 10 μM ATP and much lower concentrations of cations than enucleation medium I (a 10-fold decrease in Ca^{2+}, and no Na^+, K^+ or Mg^{2+}). It is possible that the low concentration of Ca^{2+} (1 mM $CaCl_2$) in enucleation medium II may also have enhanced enucleation (a similar effect is seen in exocytosis [7]). The explanation for the stimulation by Ca^{2+} may be that an increased extracellular concentration of free Ca^{2+} facilitates the depolymerization of spindle microtubules [20]. This correlates with the fact that the behaviour of the cytogel of sphaeroplasts or protoplasts was a function of the Ca^{2+} concentration (see Figs. 3 and 4). It is also known that extracellular Ca^{2+} (about 1 mM) promotes the effect of cytochalasin B, because it binds to the actin filaments which support membrane tension [11,16].

Use of enucleation medium II and sphaeroplasts (instead of the more fragile protoplasts), increased the yield of higher-quality cytoplasts. Although the enucleation yield was similar to that with protoplasts (about 80% on average), aggregation of the cytoplasts was avoided (Fig. 3). This allowed purification of the cytoplasts.

The fact that yeast cells could be enucleated is of interest in itself. In addition, the method described here may be useful for investigations of the nature of nuclear involvement in virus infections in yeast cells (virus–yeast cell interactions) and for production of cybrids (see Introduction).

Further work will be directed towards increasing the life time of the cytoplasts, and to determination of whether regeneration of cytoplast-sphaeroplast fusion products is possible.

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