Fractionation by Differential and Zonal Centrifugation of Spheroplasts prepared from a Glucose-repressed Fission Yeast *Schizosaccharomyces pombe* 972h⁻

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SUMMARY

A method is described for the preparation of spheroplasts in high yield from *Schizosaccharomyces pombe*, by treating cells grown in the presence of glucose and deoxyglucose with snail digestive enzymes. Gentle disruption of such spheroplasts yielded homogenates, from which marker enzymes for nuclei (NAD pyrophosphorylase) and mitochondria (cytochrome *c* oxidase activity and spectroscopically-detectable cytochromes *a* + *a*₃) could be quantitatively sedimented by low-speed centrifugation. In contrast to previous findings with *Saccharomyces carlsbergensis*, cytochrome *c* oxidase and another mitochondrial enzyme, succinate dehydrogenase, were completely sedimentable by zonal centrifugation in sucrose gradients in the presence of either 2 mM-MgCl₂ or 0.4 mM-EDTA. Mitochondria were apparently smaller and of lower buoyant density in gradients containing EDTA. The bulk of the total units of malate dehydrogenase and NADH: cytochrome *c* oxidoreductase sedimented with mitochondria, whereas NADPH: cytochrome *c* oxidoreductase was located in fractions containing no mitochondria. The distributions of mitochondrial enzymes were heterogeneous in populations of mitochondria separated on the basis of size or density. The possible origins of mitochondrial heterogeneity in extracts of *S. pombe* are discussed with special reference to changes in the enzyme activities of cells during the cell cycle.

INTRODUCTION

Difficulties in making spheroplasts in yeasts of the genus *Schizosaccharomyces* (Poole & Lloyd, 1973*a*) have necessitated the use of mechanical methods of cell disruption to isolate sub-cellular organelles (e.g. Duffus, 1965; Rock & Johnson, 1970; Bandlow *et al.*, 1974).

We describe here a rapid method for the preparation and disruption of spheroplasts from concentrated suspensions of organisms grown in the presence of glucose and low concentrations of deoxyglucose, an inhibitor of cell wall synthesis in this organism (Johnson, Lu & Brandwein, 1974). The distributions of marker enzyme activities after fractionation of homogenates indicate that mitochondria are relatively 'intact' but are heterogeneous with respect to size, density and enzyme activities after fractionation in sucrose gradients. Possible sources of this heterogeneity are discussed.

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METHODS

Organism and growth conditions. *Schizosaccharomyces pombe* strain 972h⁻ (kindly supplied by Dr U. Leupold, Institute of General Microbiology, University of Bern, Switzerland) was maintained and grown as described by Poole, Lloyd & Kemp (1973) on a defined medium containing 1 % (w/v) glucose as carbon source. Unless otherwise indicated, deoxyglucose was dissolved in the concentrated vitamins solution before filter sterilization and then added to the sterilized cooled medium to a final concentration of 60 µg ml⁻¹. A stationary-phase starter culture (200 ml), inoculated 24 h previously, was used for inoculation of 10 l cultures, to give an initial population of about 10⁶ organisms/ml. After a lag period of about 2 h, growth commenced with a mean generation time of 2.7 h, identical to that in control cultures lacking deoxyglucose (Poole & Lloyd, 1973*a, b*). The culture was harvested by continuous-flow centrifugation (1 l min⁻¹) during the mid-exponential phase of growth, when the cell population was about 2.0 × 10⁷ organisms/ml. Organisms were still glucose-repressed (Poole & Lloyd, 1973*b*), and deoxyglucose-induced cell lysis was not observed (Poole & Lloyd, 1973*a*).

Preparation and disruption of spheroplasts. Snails (*Helix pomatia*; Gerrard & Haig Ltd, East Preston, Sussex) were deshelled while live; the digestive tract and its associated glands were removed and ground in distilled water (1 ml/snail) at 4 °C. All subsequent operations, except incubation of organisms with enzymes, were performed at this temperature. Combined material from 100 snails was centrifuged at 31 000*g* for 30 min and the supernatant dialysed against distilled water in 6.3 mm Visking tubing for 6 h as described by Birnboim (1971). Solid MgSO₄·7H₂O (and a minimal volume of water) was added to the dialysate to a final concentration of 0.9 M (Poole & Lloyd, 1973*a*). The snail enzyme preparation, pH 6.8, containing 25 to 30 mg protein/ml, was stored at -20 °C.

Cells were washed once with water and once with 50 mM-EDTA (brought to pH 7.0 with 1 M-HCl) by acceleration to 5000 rev./min in the 6 × 250 ml rotor of an MSE 18 centrifuge, followed by immediate deceleration. The cell pellet was weighed, and resuspended in, and gently homogenized with, snail enzyme preparation (3 ml/g wet wt cells). Incubation was at 30 °C in a rotary orbital incubator (120 rev./min) for 50 min; this was sufficient for greater than 90 % spheroplast formation. Intact cells and spheroplasts were removed by centrifuging at 9300*g* for 5 min in the 8 × 50 ml rotor of this centrifuge. The pellet was gently washed twice with 0.8 M-sucrose-10 mM-tris-HCl, pH 7.4, containing either 2 mM-MgCl₂·6H₂O or 0.4 mM-EDTA, under the same conditions.

Spheroplasts were disrupted in approximately 3 vol. of buffer (0.25 M-sucrose-10 mM-tris-HCl, pH 7.4) containing 2 mM-MgCl₂·6H₂O or 0.4 mM-EDTA by single passage through a hand-operated Chaikoff Press, clearance 17 µm (Emanuel & Chaikoff, 1957; Microchemical Specialities Co., Berkeley, California U.S.A.). Partially-digested cells, intact spheroplasts and nuclei were removed by centrifuging at 2000*g* for 5 min to yield a pellet P₀. The supernatant (whole homogenate) was then decanted, care being taken to avoid disturbance of the lower layers.

Fractionation of whole homogenates by differential centrifugation. This was performed as described by Cartledge & Lloyd (1972) in buffers containing 2 mM-MgCl₂.

Fractionation by zonal centrifugation. Rate centrifugations were performed essentially as described by Cartledge & Lloyd (1972), but in an MSE HS zonal rotor running in an MSE 18 centrifuge. Linear sucrose gradients (15 to 60 %, w/w; 400 ml) were generated and loaded into the rotor at 1000 rev./min in 40 min using an Isco Dialagrad pump (Shandon Southern Instruments Ltd, Camberley, Surrey). All sucrose solutions and corresponding

disruption buffers contained 10 mM-tris-HCl, pH 7.4, and either 2 mM-MgCl₂ or 0.4 mM-EDTA as specified. Centrifugation was at 10000 rev./min (5300g; radius at centre of sample zone 4.5 cm) for 20 to 30 min. Unloading was at 1000 rev./min and 30 ml min⁻¹. Fractions (10 ml) were collected at 5 °C; sucrose concentrations were measured with a refractometer and densities were calculated from the data of de Duve, Berthet & Beaufay (1959). In all rate density gradient centrifugations, the duration of sample loading, acceleration to maximum speed and deceleration were recorded and used to calculate the integrated centrifugal field-time at the centre of the sample zone. High-speed zonal centrifugation was performed essentially as described by Cartledge & Lloyd (1972) but with the modifications described above.

Enzyme assays. Details of enzyme assay procedures (Poole & Lloyd, 1973*b*) and their application to the measurement of activities in fractionation studies (Cartledge & Lloyd, 1972) have been described. The method of assay of NAD pyrophosphorylase (EC. 2.7.7.1) was that of Solau & Shall (1971).

Cytochromes. Total cytochrome contents were calculated by measurements of the α absorption bands in difference spectra of dithionite-reduced minus H₂O₂-oxidized preparations at 77 K (Poole & Lloyd, 1974). Wavelength pairs, absorption coefficients, low temperature intensification factors of cytochrome absorption bands and their calculations have been described by Poole, Lloyd & Chance (1974).

Other determinations. Assay methods were: protein in fractions (Lowry *et al.*, 1951), total protein of cells and spheroplasts (Herbert, Phipps & Strange, 1971) and electron microscopy (Cartledge & Lloyd, 1972). Calculations of $s_{20,w}$ (Halsall & Schumaker, 1969) and subsequently of the radius (de Duve *et al.*, 1959) of sedimenting particles were made as described by Poole *et al.* (1971).

Reagents. Alcohol dehydrogenase (yeast), ATP (disodium salt), cytochrome *c* (type III, horse heart), NAD⁺, NADH (type III, yeast), NADPH (type I), *p*-nitrophenyl phosphate, NMN and *cis*-oxaloacetic acid (grade 1) were from Sigma and bovine serum albumin was from Armour Pharmaceutical Co., Eastbourne Sussex. The sucrose used was mineral water sugar from Tate and Lyle Ltd, Cardiff.

RESULTS

Preparation of spheroplasts by the action of snail digestive enzymes on organisms grown in the presence of deoxyglucose

Omission of pretreatment of cells with thiol compounds, and the use of MgSO₄, in preference to equimolar concentrations of sorbitol or sucrose, as an osmotic stabilizer (Foury & Goffeau, 1973) increased the efficiency of spheroplast formation (Poole & Lloyd, 1973*a*). Under the conditions described in Methods, spheroplasts were formed rapidly (within 20 min) in suspensions containing 2×10^9 to 3×10^9 organisms/ml. Protein release from these spheroplasts (protein in whole homogenate expressed as a percentage of total protein in a suspension of unbroken spheroplasts and intact cells) was 76 %. When deoxyglucose (final concentration 70 μ g ml⁻¹) was added to an exponentially-growing culture and growth allowed to continue for a further 30 min (Foury & Goffeau, 1973) subsequent protein release was only 18 %.

Fractionation by differential centrifugation

Of the total units of NAD pyrophosphorylase in the unfractionated suspension of disrupted spheroplasts, 95 % were recovered in the pellet P₀. Since this enzyme has been identified as a nuclear marker enzyme in this organism (Duffus, 1965), this observation

suggests that nuclei remain relatively intact during disruption of spheroplasts and are sedimentable by low-speed centrifugation. Centrifugation at 10^5 g-min of a whole homogenate in Mg^{2+} buffer yielded a pellet (P_1) which contained 94 % of the recovered cytochrome *c* oxidase activity, all the recovered cytochromes *a* + *a*₃, and 50 % of the recovered protein. This fraction also contained 67 % of the recovered cytochrome *c*, the remainder being heterogeneously distributed. Cytochromes *b*₅₅₈ and *b*₅₆₁ showed similar but heterogeneous distributions, maximum amounts (48 and 46 % of the respective recovered amounts) being found in the supernatant obtained following high-speed centrifugation.

Fractionation by zonal centrifugation in gradients containing 2 mM-MgCl₂

After centrifugation in the MSE HS zonal rotor for 20 min at 10000 rev./min ($= 1.38 \times 10^5$ g-min at the sample zone; $\int_0^t \omega^2 dt = 1.58 \times 10^9 \text{ rad}^2 \text{ s}^{-1}$), cytochrome *c* oxidase was heterogeneously distributed in the region of the gradient $\rho = 1.18$ to 1.24 g ml^{-1} (Fig. 1*b*). All of the recovered succinate dehydrogenase (Fig. 1*d*), 90 % of the total NADH: cytochrome *c* oxidoreductase (Fig. 1*c*) and 28 % of the total malate dehydrogenase (Fig. 1*d*) had sedimented to produce complex patterns of distribution between $\rho = 1.15$ and 1.25 g ml^{-1} ; maximum amounts of these enzymes were found to coincide with that of cytochrome *c* oxidase, although considerable heterogeneity in their distributions was evident. The starting zone still contained 82 % of the recovered protein (Fig. 1*a*) and 90 % of the total NADPH: cytochrome *c* oxidoreductase (Fig. 1*c*). Although 30 % of the total amounts of catalase and acid *p*-nitrophenylphosphatase (Fig. 1*b*) were sedimentable, no distinct peaks were observed.

After high-speed centrifugation of a similar homogenate in the B XIV rotor ($> 6 \times 10^6$ g-min at the sample zone), complex profiles of extinction at 520 nm and 260 nm indicated extensive sedimentation of particles from the starting zone (results not shown). A major peak of extinction at 260 nm was attributed to the presence of ribosome monomers which had sedimented to $\rho = 1.12 \text{ g ml}^{-1}$ under these conditions. Cytochrome *c* oxidase was detectable only in that region of the gradient $\rho = 1.20$ to 1.25 g ml^{-1} , together with small amounts of catalase and a major sedimentable zone of acid *p*-nitrophenylphosphatase. Electron microscopy showed that fractions in the region $\rho = 1.23$ to 1.25 contained relatively homogeneous populations of mitochondria (mean diameter $0.4 \mu\text{m}$) but in different 'conformational states' (Hackenbrock, 1967). The outer faces of mitochondria at $\rho = 1.23 \text{ g ml}^{-1}$ bore large numbers of small electron-dense particles, tentatively identified as ribosomes.

Fig. 1. Fractionation of a whole homogenate of glucose-repressed *S. pombe*, by rate-zonal centrifugation in a gradient containing 2 mM-MgCl₂. Cell population at harvesting was 2.00×10^7 organisms/ml. Whole homogenate (19.3 ml), containing 254 mg protein, was loaded on the gradient. The volumes of homogenate (diluted 1 in 10) and of fractions taken for assay were as follows: NADPH: cytochrome *c* oxidoreductase and catalase, 0.1 ml; protein, cytochrome *c* oxidase, acid *p*-nitrophenylphosphatase, NADH: cytochrome *c* oxidoreductase, malate dehydrogenase and succinate dehydrogenase, all 0.2 ml. Centrifugation was at 10000 rev./min for 20 min (1.38×10^5 g-min; $\int_0^t \omega^2 dt = 1.58 \times 10^9 \text{ rad}^2 \text{ s}^{-1}$). Enzyme activities were measured in units/ml. (a) Sucrose density gradient (○), light scattering at 520 nm (—) and 260 nm (---), and protein (●). (b) Cytochrome *c* oxidase (●—●), catalase (○), and acid *p*-nitrophenylphosphatase (●—●). (c) NADH: cytochrome *c* oxidoreductase (■), and NADPH: cytochrome *c* oxidoreductase (▲). (d) Malate dehydrogenase (●), and succinate dehydrogenase (▽). Specific activities of enzymes in the whole homogenate were as follows (recoveries in parentheses): protein (113 %); cytochrome *c* oxidase, 2.62 (20.6 %); catalase, 17.1 (80.6 %); acid *p*-nitrophenylphosphatase, 23.8 (127 %); NADH: cytochrome *c* oxidoreductase, 3.38 (56 %); NADPH: cytochrome *c* oxidoreductase 15.6 (56 %); malate dehydrogenase, 52.0 (64 %); succinate dehydrogenase, 13.3 (124 %).

Fig. 1. For legend see facing page.

Fig. 2. For legend see facing page.

Fractionation by zonal centrifugation in gradients containing 0.4 mM-EDTA

After centrifugation for 30 min at 10000 rev./min in the MSE HS rotor ($= 1.71 \times 10^5 \text{ g-min}$ at the same zone; $\int_0^t \omega^2 dt = 2.27 \times 10^9 \text{ rad}^2 \text{ s}^{-1}$), the major peak of extinction at 520 nm was in the starting zone, but a complex profile throughout the gradient indicated sedimentation of particulate material (Fig. 2*a*). The starting zone still contained approximately 95 % of the total protein (Fig. 2*a*), most of the catalase (Fig. 2*b*), and 44, 74 and 39 % respectively of the total amounts of acid *p*-nitrophenylphosphatase (Fig. 2*b*), NADPH: cytochrome *c* oxidoreductase (Fig. 2*c*) and malate dehydrogenase (Fig. 2*d*). A shoulder of acid *p*-nitrophenylphosphatase activity in fraction 10 was just separating from the sample zone (Fig. 2*b*). Cytochrome *c* oxidase (Fig. 2*b*). NADH: cytochrome *c* oxidoreductase (Fig. 2*c*) and succinate dehydrogenase (Fig. 2*d*), together with 50 % of the total malate dehydrogenase activity, had sedimented away from the starting zone and coincided with the broad zone of extinction at 520 nm (Fig. 2*a*) in fractions 13 to 35. Maximum activities of these enzymes were not coincident in any one fraction.

The observation that replacement of Mg^{2+} with EDTA in disruption buffer and gradients results in an apparent decrease in the rate of sedimentation of cytochrome *c* oxidase was verified by fractionation of a homogenate in the absence of both MgCl_2 and EDTA (not shown). After centrifugation in the HS zonal rotor (corresponding to $0.76 \times 10^5 \text{ g-min}$ at the sample zone), the maximum amount of cytochrome *c* oxidase was present at $\rho = 1.21 \text{ g ml}^{-1}$, but only 35 % of the recovered enzyme had sedimented beyond $\rho = 1.20 \text{ g ml}^{-1}$, whilst under similar centrifugal conditions in the presence of 2 mM- MgCl_2 this proportion was approximately 90 %.

After high-speed centrifugation in gradients containing EDTA (results not shown), a major peak of extinction at 260 nm was observed at $\rho = 1.09 \text{ g ml}^{-1}$, presumably largely due to ribosome monomers, but was broader and occurred at lower ρ than in Mg^{2+} -containing gradients. Acid *p*-nitrophenylphosphatase was heterogeneously distributed; major sedimentable zones were found at a lower density than when fractionation was performed in the presence of MgCl_2 . The total recovered amounts of cytochrome *c* oxidase and succinate dehydrogenase and most of the malate dehydrogenase and NADH: cytochrome *c* oxidoreductase had equilibrated in the density region $\rho = 1.10$ to 1.20 g ml^{-1} . Maximum amounts of cytochrome *c* oxidase and succinate dehydrogenase were coincident at $\rho = 1.13 \text{ g ml}^{-1}$; the density zone $\rho = 1.10$ to 1.15 g ml^{-1} also contained most of the sediment-

Fig. 2. Fractionation of a whole homogenate of glucose-repressed *S. pombe* by rate-zonal centrifugation in a gradient containing 0.4 mM-EDTA. Cell population at harvesting was 2.11×10^7 organisms/ml. Whole homogenate (16.0 ml) containing 146 mg protein was loaded on the gradient. Volumes of homogenate (diluted 1 in 10) and of fractions taken for assay were as follows: catalase, NADH: cytochrome *c* oxidoreductase, NADPH: cytochrome *c* oxidoreductase, malate dehydrogenase and succinate dehydrogenase, all 0.1 ml; protein, cytochrome *c* oxidase and acid *p*-nitrophenylphosphatase, all 0.2 ml. Centrifugation was at 10000 rev./min for 30 min in the MSE HS rotor ($= 1.71 \times 10^5 \text{ g-min}$ at the sample zone; $\int_0^t \omega^2 dt = 2.27 \times 10^9 \text{ rad}^2 \text{ s}^{-1}$). Enzyme activities were measured in units/ml. (a) Sucrose density gradient (○), light scattering at 520 nm (—) and 260 nm (---), and protein (●). (b) Cytochrome *c* oxidase (●—●), catalase (○), and acid *p*-nitrophenylphosphatase (●---●). (c) NADH: cytochrome *c* oxidoreductase (■), and NADPH: cytochrome *c* oxidoreductase (▲). (d) Succinate dehydrogenase (▽), and malate dehydrogenase (●). Specific activities of enzymes in the whole homogenate were as follows (recoveries in parentheses): Protein (140 %); cytochrome *c* oxidase, 9.0 (107 %); catalase, 20.4 (93.8 %); acid *p*-nitrophenylphosphatase, 70.0 (119 %); NADH: cytochrome *c* oxidoreductase, 12.8 (137 %); NADPH: cytochrome *c* oxidoreductase, 11.4 (101 %); malate dehydrogenase, 123 (71 %); succinate dehydrogenase, 21.6 (45 %).

able malate dehydrogenase and 20 % of the recovered NADH: cytochrome *c* oxidoreductase. A second zone of cytochrome *c* oxidase had equilibrated at $\rho = 1.17 \text{ g ml}^{-1}$ and was coincident with the zone containing the maximum amount of NADH: cytochrome *c* oxidoreductase, together with succinate and malate dehydrogenases. Neither of two latter dehydrogenases was detectable in regions of the gradient $\rho < 1.20 \text{ g ml}^{-1}$.

DISCUSSION

The procedure described here for the preparation of spheroplasts of *S. pombe* differs in detail from earlier methods which employ the action of snail enzymes on deoxyglucose-grown cells (Birnboim, 1971; Poole & Lloyd, 1973*a*; Foury & Goffeau, 1973) and yields amounts of homogenate protein sufficient for large-scale subcellular fractionation studies. In the presence of Mg^{2+} , particles with associated activities of mitochondrial marker enzymes, and of NADH: cytochrome *c* oxidoreductase and malate dehydrogenase but lacking catalase and acid *p*-nitrophenylphosphatase activities, sediment rapidly to density regions of the gradient $\rho = 1.20 \text{ g ml}^{-1}$ (Fig. 1) close to their isopycnic density ($\rho = 1.21 \text{ g ml}^{-1}$). Calculated sedimentation coefficients ($s_{20, w}$) of these particles exceed 1.65×10^5 , corresponding to a particle diameter $> 1.20 \mu\text{m}$. The $s_{20, w}$ values for mitochondria, separated in gradients containing EDTA (Fig. 2), range from 5×10^3 (fraction 13) to 3.6×10^4 (fraction 25) and, assuming their median buoyant density to be 1.17 g ml^{-1} , correspond to a range of diameters from 0.23 to $0.90 \mu\text{m}$. The present study suggests that conditions optimal for maintaining integrity of mitochondria from *Saccharomyces carlsbergensis* (presence of Mg^{2+} , absence of EDTA; Cartledge, Cooper & Lloyd, 1971; Cartledge & Lloyd, 1972) may cause aggregation in homogenates from *S. pombe*. Replacement of Mg^{2+} by low concentrations of EDTA also resulted in a significant decrease in median buoyant density of mitochondria from 1.230 to 1.135 (measured in those fractions containing maxima of cytochrome *c* oxidase) but, in contrast to the findings of Cartledge *et al.* (1971), not in the sedimentability of cytochrome *c* oxidase. Kellems, Allison & Butow (1974) have shown that the reversible shift in the density of mitochondria from *Saccharomyces cerevisiae*, induced by starvation of the yeast or by washing mitochondria with 2 mM -EDTA, is due to the depletion of cytoplasmic-type 80S ribosomes from the cytoplasmic face of the outer mitochondrial membrane. In the present study, mitochondria isolated in Mg^{2+} bear ribosome-like particles at their periphery; those isolated in the presence of EDTA exhibited a decreased ratio of extinction at 260 nm (1 in 30 dilution) to units of cytochrome *c* oxidase. These ratios are 0.0047 and 0.027 for mitochondria centrifuged to isopycnic density in gradients containing EDTA or Mg^{2+} , respectively. Furthermore, high-speed centrifugation of homogenates in the presence of EDTA was characterized by an increase in the amount of 260 nm -absorbing material at $\rho = 1.09$ to 1.12 compared with that in the sample zone, consistent with the hypothesis that EDTA depletes mitochondria of associated ribosomes.

Heterogeneity of mitochondria with respect to size and/or density was observed, irrespective of the gradient composition or the time of centrifugation. The relative specific activities of cytochrome *c* oxidase and succinate dehydrogenase (presumably unequivocal marker enzymes of the inner mitochondrial membrane) in fractions 17 and 21 of the experiment shown in Fig. 2 suggest that these fractions contain quite distinct mitochondrial populations and that the observed heterogeneity is not the result of mitochondrial fragmentation. Possible causes of the apparent heterogeneity of mitochondrial populations isolated from various cell types have been discussed by Lloyd (1974). One possibility is that mitochondrial heterogeneity results from heterogeneity at the level of the cell population in organisms

harvested from exponentially-growing cultures. It has been shown that the cellular contents of mitochondrial marker enzymes, cytochrome *c* oxidase and succinate dehydrogenase (Poole & Lloyd, 1973*b*) and of a number of cytochrome components of mitochondria (Poole *et al.*, 1974) fluctuate during the cell cycle of *S. pombe*. Mitochondria isolated from cells from an exponentially-growing culture will therefore be heterogeneous in their complements of these and other components which are synthesized, assembled or integrated discontinuously throughout the cell cycle. They may also be heterogeneous with respect to size and/or density as a result of mitochondrial biogenesis by a process of growth and division. Knowledge of the mode of mitochondrial biogenesis and its temporal relationship with the cell cycle is, at present, too imprecise to define the observed heterogeneity, but the possibility that temporal heterogeneity of mitochondrial structure and function is reflected when cells from an asynchronous cell population are studied is an attractive hypothesis which merits further investigation.

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