Zonal Centrifuge Analysis of a Transformation in 
Ustilago Controlled by Zinc1, 2

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Zonal centrifuge rotors are relatively large, hollow rotors containing density gradients and offer the possibility of quantitative separation of qualitatively pure particulate components of biological systems (1, 2). The possibility is a consequence of the principles of centrifugation in density gradients but to achieve it, we shall have to fashion improved techniques of zonal centrifugation on the anvils of specific biochemical and physiological problems.

A physiological transformation in Ustilago sphacrogena possesses features that led us to examine it with the zonal centrifuge. Grimm and Allen (9) reported that the yeast-like sporidia of this smut fungus can be made to accumulate enormous amounts of cytochrome c (up to 1% dry wt) if one provides high levels of zinc in the growth medium. The concentrations of other cytochromes were also reported to be increased by zinc.

Since the cytochromes of heterotrophic, eucaryotic cells appear to be associated principally with mitochondria (10), we thought that the changes controlled by zinc in Ustilago might well involve the mitochondria of this organism. Consistent with this notion was the report (15) that high-zinc cells were richer in phospholipid than low-zinc cells.

The purpose of this investigation was therefore two-fold: to explore the idea that a zinc-controlled process in Ustilago involved mitochondria, and to test zonal centrifugation on a problem with a yeast-like organism.

Materials and Methods

Organism, Growth, and Harvesting. The organism, Ustilago sphacrogena Burill (7) was obtained from Dr. Paul J. Allen. Culture conditions and harvesting were as described elsewhere (5 and Brown, Cappellini, and Price, in preparation).

The harvested cells at a concentration of approximately 50 mg dry weight per ml were suspended in a mixture of 8% (w/w) sucrose, 0.01 M potassium phosphate pH 7, and 0.03 M magnesium sulfate. All subsequent operations were conducted between 0 and 2°C. The cells were washed in a French Press at 12,000 psig. The brei was clarified by a low speed centrifugation (2000 rpm for 5 min in a Servall SS-1 rotor) to remove clumped material.

Zonal Centrifugation. The general principles and methods of zonal centrifugation are described by Anderson (1). Both A-IX (International Equipment Company, Needham Heights, Massachusetts) and B-IV (Spinco Division, Belmont, California) zonal rotors were employed (2). The A-IX was operated in a PR-2 centrifuge and the B-IV in a modified Spinco L-1, designated ZU-1. The fluid line systems are shown in figure 1.

The starting solution was 0.01 M potassium phosphate pH 7, 0.03 M magnesium sulfate, and the limiting solution was 30% (w/w) or 55% (w/w) sucrose in the A-IX and B-IV, respectively, with the same concentration of salts. Cushions of 55% (w/w) sucrose with salts were employed. The gradient generator was a Spinco model 131. Program cans on the gradient generator were cut to give a 300-ml gradient overlay of 0 to 6% (w/w) sucrose with salts followed by a 1000-ml or 1400-ml linear gradient for the A-IX and B-IV rotors respectively.

The overlay was pumped into a cold-jacketed standpipe (fig 1) and stored until needed. The remainder of the gradient was then pumped into the rotors. Parking speeds were 1500 rpm for the A-IX and 2000 rpm for the B-IV, respectively, except that the A-IX was decelerated to 500 rpm for backing on the sample and overlay. The samples were 20 ml volumes (of Ustilago clarified breis. We often included a few mg of blue dextran (Pharmacia) to mark the location of the original sample zone.

The arct values for the various runs are shown individually. In general the A-IX runs were about
1 \times 10^6 \text{ rev}^2 \cdot \text{min}^{-1} \text{ and the B-IV runs were about } 200 \times 10^6 \text{ rev}^2 \cdot \text{min}^{-1}.

Monitoring and Analysis. The A-IX rotor has plexiglas endplates so that the movement of the zones can be monitored visually. Measurement of the rates of sedimentation was facilitated by scribing arc lines in the endplates at intervals of 1 cm of radius. The lengths of the arcs were coded in sets of 5, so that they appeared in the spinning rotor as lines of periodically increasing brightness.

Both the A-IX and B-IV rotor contents were analyzed at the end of each run by displacing the gradient with 55% (w/w) sucrose pumped to the edge of the rotors with a peristaltic pump (Type 1200 VDC, Harvard Instruments, Harvard, Massachusetts) at a rate of 30 ml per minute. The effluent from the core was passed through a MILAN flow refractometer (Waters Associates, Framingham, Massachusetts) and a 2-mm Oak Ridge-type flow cell (Quaracell, New York) in a DB-spectrophotometer (Beckman Instruments) set at 280 m\text{\AA}. The signals of the 2 instruments, representing refractive index and \(\lambda\) (2 mm), respectively, were recorded simultaneously on a 3-pen potentiometric recorder (Texas Instruments, Dallas, Texas).

We found it necessary to stabilize the voltage on the monitoring circuit with a harmonic-neutralized constant voltage transformer (Sola Electric, Elk Grove Village, Illinois).

The effluent from the rotor was finally collected in 50-ml volumes.

Cytochrome c Assay. Cytochrome c was analyzed in whole cells by a modification of Neilands' preparative procedure (13). Packed cells containing 125 to 300 mg dry weight were frozen in 12-ml polypropylene centrifuge tubes, resuspended in 3 ml of 0.5 M potassium borate buffer, pH 10.8, and shaken at room temperature for 5 hours. The suspensions were centrifuged and the extracted cytochrome c determined in the supernatant from the difference spectra. Samples were reduced with 20 mg of dithionite per ml or oxidized with 0.02 M ferricyanide and read at 550 m\text{\AA} and 542 m\text{\AA}. The following relation (13) was employed for a light path of 1 cm:

\[
C = \frac{\Delta \lambda_{550} - \Delta \lambda_{542}}{19.8} \text{ \mu mole} \cdot \text{cm}^{-1}
\]

The validation of this method with respect to linearity and recovery will be presented elsewhere (5 and Brown et al., in preparation).

Results

We found first of all that the breis of broken Ustilago (and also Euglena gracilis) contain clumped material despite repeated modifications of the medium and conditions of rupture. (We find with several marine algae that clumping occurs only when the nuclei are broken and may therefore result from the release of nucleic acids.) If the clumped material is not removed by low-speed fractional centrifugation, it

![Fig. 2. High zinc cells, B-IV run. Cells grown for 96 hours with \(2 \times 10^{-5}\) M zinc from the time of inoculation; \(110 \times 10^6\) rev\(^2\) \cdot min\(^{-1}\) in the B-IV; ribosome band is incompletely separated from the starting zone; there is a prominent large-particle zone.](https://academic.oup.com/plphys/article/40/6/1278/6090062)

![Fig. 3. UV spectrum of presumed ribosome fraction. Particles from second peak in B-IV run scanned on DB spectrophotometer; particles show strong 260 m\text{\AA} absorption.](https://academic.oup.com/plphys/article/40/6/1278/6090062)
sediments rapidly through the zonal rotor and contaminates the gradient by releasing adhering particles.

We found that the composition of the gradient is critical. Although sucrose alone is sufficient for separating mitochondria of rat liver (14), Ustilago yielded poorly defined zones of particles in gradients lacking ions and cytochrome c remained in the sample zone. The addition of 0.01 M potassium phosphate, pH 7, and 0.03 M magnesium sulfate improved resolution greatly.

Figure 2 is from a preliminary B-IV run of \( \omega t = 110 \times 10^3 \text{ rev}^2 \cdot \text{min}^{-1} \) on cells grown with \( 2 \times 10^{-5} \) M zinc from the beginning. These cells were very rich in cytochrome c. The first peak contained soluble protein and marked the original sample zone. The second peak probably contained ribosomes as judged from absorption spectra of similar peaks in other runs (fig 3). The third peak was broad and appeared buff or pink in the collection tubes. Although the samples were quite dilute, the spectra of this component had a clear Soret peak and weak but detectable \( \alpha \)-bands of cytochrome c. In this and other runs it was the only particulate fraction with detectable cytochrome. Varying amounts of cytochrome remained in the original sample zone.

For subsequent runs, we grew the sporidia of Ustilago for 20 hours in a medium containing \( 4 \times 10^{-2} \) M zinc. The cells at this stage had only small amounts of cytochrome c and we timed the experiment from this point. When \( 2 \times 10^{-5} \) M zinc was added at this zero time, cytochrome c formed rapidly and linearly over the subsequent 24-hour interval. If no zinc was added, cytochrome c formed much more slowly (fig 4). The rates of formation of protein, RNA, and DNA were unaffected under these conditions (5); so that we had eliminated growth as a variable.

Figures 5 and 6 are from B-IV runs of cells collected before and 24 hours after the addition of high levels of zinc. Both samples were centrifuged for \( 200 \times 10^3 \text{ rev}^2 \cdot \text{min}^{-1} \), which separated the ribosome peaks clear of the sample zone. The presumed ribosome peaks are indistinguishable in low and high zinc cells. The low-zinc cells had no major third peak, whereas 24 hours after the addition of zinc the third peak was almost as large as in the cells grown with high zinc from the beginning. The pattern from low-zinc controls grown 24 hours without additional zinc was the same as low-zinc cells taken at zero time.

We then examined Ustilago in the A-IV rotor. The forces obtained (about \( 2 \times 10^5 \text{ rev}^2 \cdot \text{min}^{-1} \)) were insufficient to move ribosomes free of the sample zone, but we obtained a buff colored component from high-zinc cells banding at about the same density as the third major peak in the B-IV runs (fig 7). In low-zinc cells the size of this component was greatly reduced.

![Fig. 4. Ustilago test system. Sporidia of Ustilago have been grown for 20 hours prior to zero time in a medium containing \( 4 \times 10^{-2} \) M zinc. (Figure also shows cells grown in \( 3 \times 10^{-2} \) M.) At zero time the cells are placed in medium containing either \( 2 \times 10^{-5} \) M zinc or no added zinc. The cytochrome content of the cells increases linearly over the ensuing 24 hours in response to the available zinc.](https://academic.oup.com/plphys/article/40/6/1278/6090062)

![Fig. 5. Zero-time cells, B-IV run. Sample grown in \( 4 \times 10^{-2} \) M zinc (see figure 4) to zero time, and centrifuged at \( 200 \times 10^3 \text{ rev}^2 \cdot \text{min}^{-1} \) in the B-IV; ribosome zone moved free of sample zone; large particle-zone is not prominent.](https://academic.oup.com/plphys/article/40/6/1278/6090062)
There were also a number of minor peaks which we have not identified. Several such components can be seen distributed on the leading edge of the sample zone. Since this is the region of overlay, we inferred that the peaks contain material, presumably lipids, considerably less dense than the suspending solution ($\rho = 1.03$).

By recording the position of a band at various times in the A-IX rotor, we should be able to determine the sedimentation rate for these particles. In the case of Ustilago, however, the heavy particles sedimented as a polydisperse system; that is, the bands did not move as a whole to their final positions but appeared to spread into the gradient, then pile up at about $10^6 \text{rev}^2 \cdot \text{min}^{-1}$.

**Discussion**

We have explored the use of zonal centrifuges in the analysis of sporidia of Ustilago. The yeast-like cells were grown first in low followed by high levels of zinc so that they would undergo a transformation involving the formation of large amounts of cytochrome. The particle profiles obtained from the 2 kinds of cells showed 3 principal peaks, including the original sample zone. One of the peaks appeared to be ribosomes. The size of this peak was the same throughout the transformation, which was probably a consequence of the elimination of growth rate as a variable (12). The peak that moved farthest from the origin contained cytochrome and was much larger in the high-zinc cells. These particles could have been mitochondria but they have not yet been subjected to further analysis.

These are general observations. Let us inquire further into the significance of zonal centrifuge separations and into the additional kinds of information that are required.

Although the principles of zonal centrifugation have been described (1, 2), the performance of zonal centrifuge rotors is only beginning to be assessed. In order to evaluate the particle profiles shown above, we should inquire into the types of separation that can be achieved with these rotors and the resolution attainable. In zonal centrifugation as in any density gradient centrifugation, particles may be separated according to their sedimentation rates (rate-zonal separation) and according to their buoyant densities (isopycnic separation). At low $\omega^2$ integrals of the order of $10^6 \text{rev}^2 \cdot \text{min}^{-1}$ only the largest particles (pollen, whole cells, nuclei) can reach their isopycnic point. The separations we observe in the A-IX rotor, therefore, are for the most part rate-zonal. We observe in fact that the bands continue to move with longer centrifugation. At $200 \times 10^6 \text{rev}^2 \cdot \text{min}^{-1}$, as we employed in the B-IV, particles of mitochondrial size are banded isopycnically, but ribosomes, viruses, etc., are still moving.

It has been difficult to determine the ultimate resolution that can be achieved in zonal centrifuges for the simple reason that tiny particles of uniform size and density are not readily available. Tobacco mosaic virus and other viruses are the best-known test particles for characterizing zonal centrifuges, and these migrate in the B-IV as a sharp band less than 50 ml wide at half height (2). Even sharper isopycnic banding of pollen and chloroplasts has been observed.

In view of the kind of intrinsic resolution obtainable with the instrument, we interpret a broad rate-zonal or isopycnic band as evidence of heterogeneity. Thus, the cytochrome-containing particles of Ustilago appear to be polydisperse with respect to sedimentation rate and buoyant density.
We cannot of course conclude that the behavior of particles in the zonal centrifuge necessarily reflects their state in situ. We may well have produced heterogeneity through damage during cell breakage. Particles from different kinds of cells, as in the present case, may have differing sensitivities to damage. In addition the components of the gradient could work profound changes on particles sensitive to osmotic pressure and ions.

Considerably more information is required before we can conclude that differences observed in the zonal rotors have physiological relevance. If the cytochrome-containing particles are mitochondria, we shall have to determine if their ultrastructure has been altered from their state in situ. We must determine if the particles possess an intact electron transport system.

We note that for *Ustilago* and for dehydrogenase-containing particles in *Encephalosporium*, the composition of the gradient is far more critical in density gradient than in fractional centrifugation. Very likely the reason is that a set of particles moving through a density gradient is continuously exposed to fresh solution. The equivalent in fractional centrifugation would be a large number of washes and recentrifugations, a treatment that is known to inactivate many particulate enzymes. This characteristic at the same time accounts for the superior resolution obtainable in zonal centrifugation and is directly analogous to the notion of theoretical plates in chromatography.

This exploratory study shows clearly that the transformation evoked by zinc in *Ustilago* involves a change in sub-cellular particles. The identity of these particles and the nature of their change must await the acquisition of information of the sort outlined above, but the zonal centrifuge patterns observed are not inconsistent with the idea that zinc brings about the formation of more or different kinds of mitochondria.

The idea of different kinds of mitochondria among cell and tissue populations is not new (6, 8, 11). Also, heterogeneity of mitochondria within individual cells has been supported by cytochemical studies on root epidermis (3) and combined cytchemistry, enzymology, and electron microscopy in yeast mutants (4).

Summary

Zonal centrifugation has been employed to separate intracellular particles in the sporidia of the smut fungus, *Ustilago sphaerogena*. Sporidia can be obtained in 2 states, corresponding to media containing high (2 × 10^{-5} m) and low (4 × 10^{-7} m) zinc. The high-zinc cells produce much more cytochrome c than low-zinc cells. High-zinc cells are distinguished by large amounts of heavy particles, possibly mitochondria, which appear as a separate peak in both rate-zonal (ω''t = 2 × 10^9) and isopycnic (ω''t = 200 × 10^9) separations. Ribosome peaks are present and indistinguishable in breis from the 2 kinds of cells. Problems encountered with the zonal centrifugation of these yeast-like organisms are discussed.

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Literature Cited