The Isolation of Oral Structures From *Tetrahymena pyriformis* by Low-Speed Zonal Centrifugation [1]

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SUMMARY

The oral apparatus from indole-lysed *Tetrahymena* has been isolated in sucrose gradients. In the A-XII rotor, kinetosomes and other material band isopycnically at 12 to 18 percent sucrose and oral structures at 45 to 49 percent sucrose. Macronuclei are found only when sucrose gradients extending to 63 percent and higher are used. The significance of methods for obtaining large quantities of pure fractions of oral structures is discussed.—Nat Cancer Inst Monogr 21: 317–321, 1966.

THE RECENT development of low-speed zonal centrifuge rotors (*1*) has made possible isolation of the larger subcellular components in considerable quantity and with higher resolution than obtained with conventional differential centrifugation. The cortical organelles and macronuclei of *Tetrahymena* are too large and sediment too rapidly for isolation by rate-zonal centrifugation in the higher speed zonal rotors used for polysome and ribosome isolation. The studies reported here were begun with the purpose of developing methods for isolating oral structures in sufficient quantity for direct biochemical analysis.

METHODS

Synchronously dividing *Tetrahymena* were grown axenically in modified 2 percent proteose peptone with 0.1 percent liver extract, under the conditions previously described by Padilla and Cameron (*2*). Cells were removed from these cultures and resuspended in a 40 ml capacity, conical test tube. Ten to 20 ml of packed cells were washed in 0.01 M Tris buffer containing 0.005 M Mg^{++} at *p*H 7.5, repacked, and lysed by addition of an equal amount of saturated indole made up in Tris buffer at *p*H 7.5.

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Linear sucrose gradients were used. They ranged from 10 to 55 percent (w/w) with a 55 percent underlay, 10 to 66 percent with a 66 percent underlay, and 30 to 66 percent with a 66 percent underlay. These gradients were mixed and pumped into the rotor as described elsewhere (*3*).

For specific isolation of oral structures and kinetosomes the following method of cell fractionation was used. *Tetrahymena* were collected and lysed with indole, as mentioned.

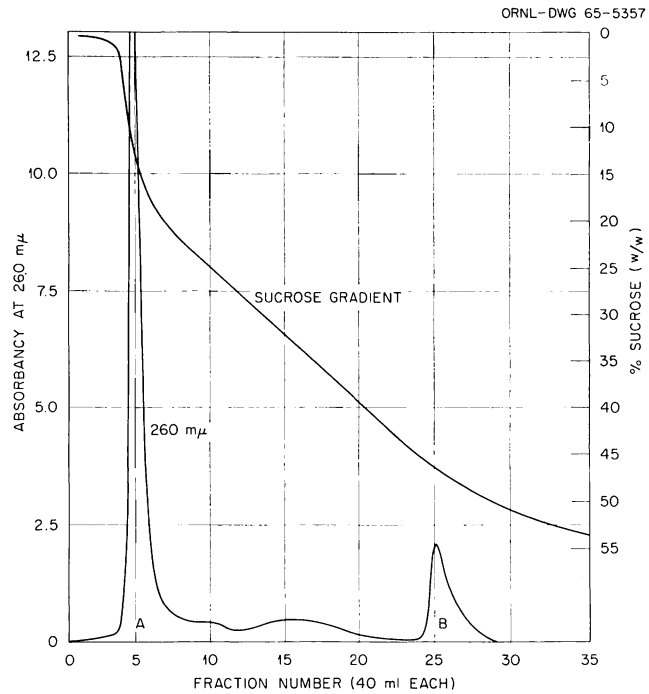
The indole lysate was treated with 4 times its volume of cold 95 percent ethanol (5° C) for 2 to 3 hours and centrifuged; the alcohol-soluble fraction was discarded. The remaining cell lysate was treated with 4 times its volume of cold 1 percent digitonin in 0.4 M KCl as used by Seaman (*4*). After 1 hour, the lysate was spun at 2000 rpm in a refrigerated International preparative centrifuge for 10 minutes and the supernatant was discarded; the pellet was retreated with cold digitonin for 18 to 24 hours to solubilize any remaining unstable cell fractions. Oral structures and kinetosomes were not solubilized by these treatments. Lysates were centrifuged again at 2000 rpm for 10 minutes and the supernatant was discarded; the pellet was resuspended in 10 percent sucrose.

Each 30 ml sample was added to the A-XII rotor, which contained 1300 ml of the sucrose gradient, while the rotor was rotating at a speed of 900 rpm. An overlay of 100 to 130 ml of Tris buffer at *p*H 7.5 was used under pressure with nitrogen to push the sample outward from the core of the rotor. The rotor was brought to a final speed of 3800 rpm to separate and band the various cell fractions in the rotor. The time required for a clear separation of these fractions at this speed, determined visually, varied from 30 minutes to a few hours, depending largely upon the gradient used.

After final separation, the rotor speed was reduced to about 900 rpm and the rotor effluent was monitored through a continuous flow cell with a Beckman DU Spectrophotometer as described by Canning and Anderson (*3*). The percentage of sucrose for each 40 ml sample collected was determined refractometrically, and the contents of each sample were examined with a phase contrast microscope. Cell structures were then related to the absorbance peaks and to their position in the sucrose gradient.

RESULTS

Saturated indole effectively breaks open *Tetrahymena* and allows the separation of intact oral structures and other pellicular elements. A record of optical density showing the position of the kinetosome-containing fraction and oral structures of *Tetrahymena* in the sucrose gradient is shown in text-figure 1. The gradient used in this run was 10 to 55 percent with a 55 percent underlay. Phase contrast observation of the first peak, which reached an absorbance in excess of 2.5, showed that it contained kinetosomes and other digitonin-insoluble material. The second, smaller peak contained the oral structures, as shown in figure 1. The kinetosomal



TEXT-FIGURE 1.—An optical density profile taken from a recording potentiometer showing 260 mμ absorbancy of the separation of kinetosomes (peak A) and oral structures (peak B). *See* text for details.

fraction was found in 12 to 18 percent sucrose and the oral structures were sedimented to the 45 to 49 percent sucrose level.

The phase contrast photomicrograph shows that each of the oral structures, or at least their skeletal frameworks, consists of three compound ciliary membranelles (minus their cilia) and an undulating membrane associated with some of these oral structures. The oral fibers or gullet fibers can be seen extending downward and to one side.

Macronuclei can be obtained when Tris buffer is used and lysed cells are placed immediately into the A-XII rotor after collection of *Tetrahymena* and lysing with indole. Pure fractions of macronuclei have not yet been recovered by banding in this rotor. They have, however, been recovered in apparently good morphological conditions from the rotor wall when a sucrose gradient of 10 to 55 percent was used. When recovered from sucrose gradients, macronuclei and some cellular debris have been found in high-density sucrose levels (63% and higher), depending on their size.

Fractions of mitochondria from *Tetrahymena* have not yet been obtained with these methods. Perhaps these structures became contaminated with microsomal material and other vacuolar fractions. Moreover, in high-density sucrose gradients and at low speeds these particles do not sediment or separate from the soluble or kinetosome-containing fraction.

DISCUSSION

The isolation of oral structures is of interest for several reasons, including the study of the relation of these structures to cell division in synchronized *Tetrahymena* (*5*, *6*). Experimentally, cortical stomatogenesis and cell division can be prevented by the addition of actinomycin D to temperature-synchronized *Tetrahymena* (*7*). Whether this inhibition of cell division is directly correlated with the prevention of oral morphogenesis is not actually known. Williams (*5*), however, has found that oral fibers are absent from isolated oral structures after heat-shock treatment and he has suggested that these fibers may be the "division proteins" in *Tetrahymena*.

Since oral structures in *Tetrahymena* may now be isolated by low-speed zonal centrifugation, it should be possible to study structural protein synthesis and protein changes within these cells during the cell cycle or after experimental treatment.

The role of the kinetosome in ciliate morphogenesis has been discussed at length by Chatton and Lwoff (*8*) and Lwoff (*9*), and biochemical characterization of these structures has been described by Seaman (*4*) and Argetsinger (*10*).

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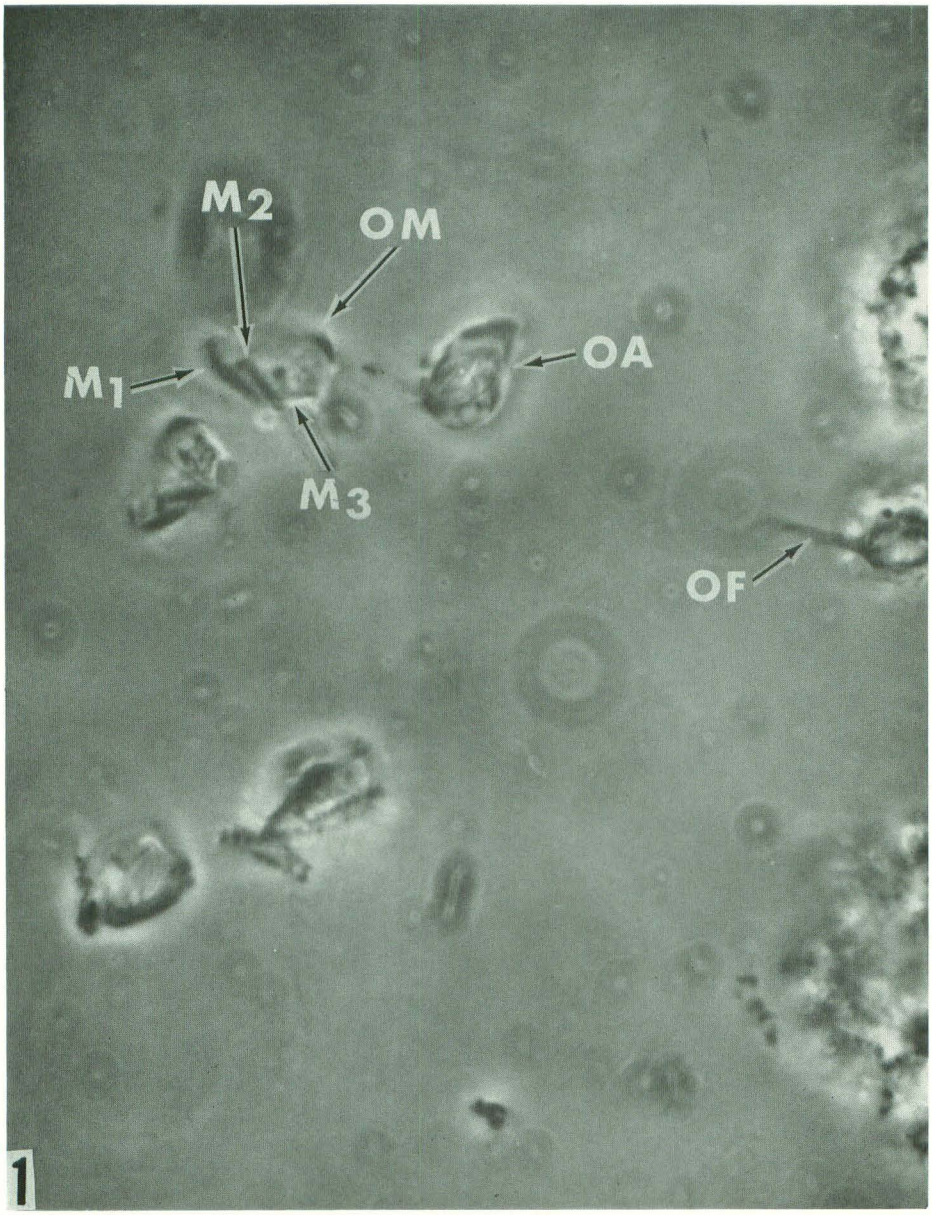

FIGURE 1.—Photomicrograph of the isolated oral apparatus of *Tetrahymena*. Each oral apparatus (OA) consists of an undulating oral membrane (OM), three compound membranelles (M_1, M_2, and M_3), and oral fibers (OF). These structures were isolated from peak B of text-figure 1.