REACTIVATION OF GLYCERINATED CILIA FROM *TETRAHYMENA PYRIFORMIS*

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The ease with which *Tetrahymena* can be grown in relatively large quantity makes it an exceptionally useful source of cilia for biochemical study. Several methods of isolating the cilia have been described (3, 11). Although excellent for many purposes, these procedures all suffer from the limitation that the isolated cilia seem incapable of being reactivated by ATP.

This paper describes a method for isolating cilia from *Tetrahymena* by treatment with 60 per cent glycerol. The preparations of isolated cilia are highly pure and the majority of cilia in them become motile when treated with ATP. The method is based on that used by Hoffmann-Berling (9) and by Brokaw (1) to reactivate sperm tails and protozoan flagella, but certain modifications were necessary in this case.

MATERIALS AND METHODS

One-liter cultures of *Tetrahymena pyriformis*, strain W, were grown in medium containing 1 per cent peptone, 0.1 per cent yeast extract, and phosphate buffer pH 6.5. The cells were harvested by centrifugation, washed at room temperature with a solution containing 0.18 per cent NaCl and 0.2 M sucrose, and resuspended in a sufficient volume of fresh wash solution to give a total volume of 20 ml. A typical culture yielded about 10 ml packed volume of cells after washing. The concentrated cell suspension was cooled to 0°, and then mixed with 100 ml of solution containing 70 per cent (v/v) glycerol, 50 mM KCl, 2.5 mM $MgSO_4$, and 20 mM Tris–thioglycolate buffer, pH 8.3 at 0°. As soon as it was thoroughly mixed, the suspension of cells in glycerol was cooled to −20°, and maintained at this temperature. Vigorous agitation of the suspension on a Vortex mixer for 1 minute caused the majority of the cilia to become detached, but left the cell bodies intact. The bodies were removed by centrifugation at 12,000 *g* for 10 minutes. The supernatant, which consisted of a suspension of pure cilia, was removed and stored at −20°. This stock cilia suspension was used in reactivation experiments simply by diluting it with the appropriate solution containing ATP. The cilia could be recovered from the suspension by centrifuging them at 80,000 *g* for 3 hours, but it was found impractical to maintain the temperature below −10° during this centrifugation and there was some loss of potential for motility.

To obtain pure preparations of isolated cilia with this procedure, it is essential that there be no lysis of cells during the initial harvesting and washing. Any

cells that lyse at this stage pass through the washing steps as fluffy clumps along with the intact cells, and then become dispersed in the glycerol solution to give rise to serious contamination of the cilia with cytoplasmic particles. So long as the cell cultures are not seriously overaged, this lysis is completely prevented by the strongly hypertonic sodium chloride/sucrose solution used for washing. With this precaution the final stock suspension of cilia is highly pure, and no contamination with cytoplasmic particles is apparent by phase-contrast microscopy.

The best reactivation was obtained by taking a small volume of the stock cilia suspension and mixing it with 3 volumes of a solution containing 0.2 mM ATP, 50 mM KCl, 2.5 mM $MgSO_4$, and 20 mM imidazole–HCl buffer, pH 6.8 at 20°. This diluted suspension was immediately transferred to a glass slide, and observed with a phase-contrast microscope at room temperature.

RESULTS AND DISCUSSION

When first observed, the great majority of the cilia showed vigorous bending movements. The detailed pattern of bending varied considerably in different cilia. In the most common type, bending waves were propagated along the length of the cilium from its basal end toward its tip, with a frequency of 2 to 3 waves per second; these cilia usually rotated about their long axis while progressing through the medium, basal end first, at a speed of about 2 μ/second. Other cilia showed only lashing movements of various kinds, and did not progress forward. The percentage of cilia showing some form of movement was usually about 80 per cent initially, about 30 per cent after 5 minutes, and about 1 per cent after 15 minutes. The undulatory type of movement was the most common at the beginning, but it most often degenerated into the lashing type within about 5 minutes. Cessation of movement was frequently followed by fragmentation or by rounding up of the cilia. The duration of motility was not increased by keeping the suspensions cooled to 0°.

Variations of the concentrations of ATP, KCl, and $MgSO_4$ in the reactivation solution affected the frequency of beat in a manner similar to that described for *Polytoma* by Brokaw (1). No movement was observed in the absence of ATP. The pH of the reactivation medium was not critical and some movement could be obtained at all pH's between 6.2 and 8.5; however, the duration of motility was longest at about pH 6.8. Addition of thioglycolate or of polyvinylpyrollidone to the reactivation solution had no clear beneficial effect. The stock cilia suspension in 60 per cent glycerol was usually used for reactivation the same day it was prepared, for the fraction of potentially motile cilia decreased slowly over a period of several days.

As already mentioned, optimal motility was obtained by diluting the stock cilia suspension with reactivation solution in the ratio 1 to 3, and greater dilution caused a decrease in the duration of motility. The occurrence of this optimal dilution ratio suggests that the stock cilia suspension contained some agent with a protective effect on the cilia in the diluted suspension. Whether this protective effect was due to the glycerol or to some substance extracted from the cell bodies is not known.

The effect of the chelating agents ethylene glycol *bis* (β-aminoethylether)-*N*,*N*′-tetraacetate (EGTA) and ethylenediaminetetraacetate (EDTA) on ciliary reactivation was examined in order to determine whether trace amounts of free Ca^{++} are required for motility. It was found that addition of 2 mM EGTA or 1 mM EDTA to the reactivation solution caused only slight inhibition of motility. This small amount of inhibition was not considered significant, for it was about the same at both pH 6.8 and at pH 7.8 in spite of a 100-fold stronger chelating action at the higher pH, and it could not be relieved by further addition of 1 mM $CaCl_2$. The free Ca^{++} concentration in the presence of chelating agent can be estimated by assuming, as an upper limit, that the total adventitious calcium concentration was 10^{-4} M (including that in the reagents and that in the cilia suspension); the amount of EGTA used would then reduce the free Ca^{++} concentration at pH 7.8 to approximately 10^{-9} M (see reference 10 for details of calculation). Therefore these results strongly suggest that reduction of the free Ca^{++} concentration to 10^{-9} M does not inhibit motility of cilia. The lack of effect of EGTA on the motility of cilia is in striking contrast to results obtained with muscle, where chelating agents which reduce the free Ca^{++} concentration below about 5×10^{-7} M completely inhibit the contraction of myofibrils and the syneresis of actomyosin gels (5, 10, 12, 13). Since the Ca^{++} requirement in muscle is involved in control of the contraction-relaxation cycle (13), it is an essential biochemical property of the system. The absence of a Ca^{++} requirement in cilia would mark a fundamental difference from muscle at the biochemical level.

Observation of reactivated cilia which were swimming parallel to the axis of the microscope showed circular patterns, suggesting that the bending waves are helical, rather than planar, in form. This helical wave form, if confirmed by future cinematographic studies, will mark a significant difference between the movements of these cilia and the largely planar bending waves shown by many flagella, including those of *Polytoma* (2) and sea urchin sperm (8).

A fair number of cilia in these preparations become attached by their basal ends to the surface of the microscope slide, and continue to move, swinging around with a circular movement about their point of attachment. It is interesting to note that of approximately 100 cilia observed moving in this manner, all were circling in a counter-clockwise direction. This circling motion of the attached cilia is presumably a modified form of their rotation when swimming freely. The uniform counter-clockwise circling of these isolated cilia recalls the similar circling movements seen when live spermatozoa swim in close proximity to a glass surface (4, 7). The widespread occurrence of these circling movements lends support to the hypothesis advanced earlier (6) that they are related to the enantiomorphic asymmetry of fine structure present in all cilia and flagella.

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REFERENCES

1. Brokaw, C. J., *Exp. Cell Research*, 1961, **22,** 151.
2. Brokaw, C. J., *J. Exp. Biol.*, 1963, **40,** 149.
3. Child, F. M., *Exp. Cell Research*, 1959, **18,** 258.
4. Dewitz, J., *Arch. Ges. Physiol.*, 1886, **38,** 358.
5. Fanburg, B., Finkel, R. M., and Martonosi, A., *J. Biol. Chem.*, 1964, **239,** 2298.
6. Gibbons, I. R., *Nature*, 1961, **190,** 1128.
7. Gibbons, I. R., Proc. 5th Int. Cong. Elec. Micr., paper M2 (1962).
8. Gray, J., *J. Exp. Biol.*, 1955, **32,** 775.
9. Hoffmann-Berling, H., *Biochim. et Biophysica Acta*, 1955, **16,** 146.
10. Portzehl, H., Caldwell, P. C., and Ruegg, J. C., *Biochim. et Biophysica Acta*, 1964, **79,** 581.
11. Watson, M. R., and Hopkins, J. M., *Exp. Cell Research*, 1962, **28,** 280.
12. Weber, A., and Winicur, S., *J. Biol. Chem.*, 1961, **236,** 3198.
13. Weber, A., Herz, R., and Reiss, I., *Proc. Roy. Soc. London, Series B.*, 1964, **160,** 489.