ANALYSIS OF CELL SURFACE CORTEX DURING CILIA REGENERATION

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CONTENTS

CERTIFICATE

The research work embodied in this dissertation
has been carried out in the School of Life Sciences,
Jawaharlal Nehru University, New Delhi. This work is
original and has not been submitted in part or full
for any other degree or diploma of any University.

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(Pratima Ray)

INTRODUCTION

The development, maintenance and evolution of
forms are the features which are common to both the multi-
cellular as well as the unicellular organisms. Some uni-
cellular organisms, such as many protozoans exhibit a wide
diversity of structural and functional features, rarely
encountered in any single cell of multicellular organisms.
The confinement of all the vital and complex components
within the limit of an unicellular form of life provides
us an unique advantage, for the investigation of biological
organizations at the cellular level.

Among the ciliated protozoans *Tetrahymena* has been
extensively used in several biological disciplines like
genetics, cell and molecular biology, developmental biology
etc. Because, it is easier to handle this organism for
its rapid growth, genetic stability and animal characters.

In multicellular organisms, the primary feature which
determines the form and its change is the 'skeletal' system.
This is in form of fibrous matrix in plants and chitinous
and bony matrix in animals. However, in single-celled
organism, this is accomplished by the elaboration of the
cellular architecture which reaches the highest degree of
complexity in ciliate in the form of complex fibrillar

' cortex' . The cortex is a highly differentiated cell
surface comprising of ectoplasmic sculpturing, multiple
pellicular membranes, rows of kinetosomes and other fibri-
llar structures.

The cortical pattern, its chemistry, morophology
and its development in the ciliated protozoan have been
one of the areas of major interest of study. Thus, there
have been several in depth studies of its fine structure
and development.

The cortex and its ciliary components appeared
to be very simple to the early biologists, using the light
microscopes. However, the electron microscopic investigation
has revealed the cortex as a structure of entirely unexpected
complexity (Lwoff, 1950; Corliss, 1952; Metz and Westfall,
1954; Gibbons and Grinstone, 1960).

The ciliate cortex is divided into a somatic cortex
concerned with protection and adhesion and oral cortex
for acquisition and ingestion of nutrients. However, the
cortical architecture is fundamentally similar in both.
It can be interpreted as a fabric composed primarily of
a large number of fundamentally similar and uniformly
spaced ciliary units or kinetides (Pitelka, 1969; Ehret, 1960).
So the kinetide or cilia-kinetosome complex are the funda-
mental components of the ciliate cortex (Grain, 1969;

Pitelka, 1969; Sonneborn, 1970). In addition to a cilium and kinetosome or basal body the kinetid includes a set of membranes and associated fibrills, complexed into an asymmetric structure with a well defined anterior posterior and left right axis (Lynn, 1980). The ciliary units or kinetids are themselve ordered into a characteristic pattern, the most important of which is the linear-array of kinety or the ciliary rows parallel to the anterior posterior axis of the cell (Lwoff, 1950) making the primary meridian. Other association of somewhat modified ciliary units occur in compound organelles with specialized ingestatory functions(Fig. 1)

The ciliate cortex is a highly organized system exhibiting a large number of different other structures beside the ciliary unit, such as mucocyst pores located between the cilia along the primary meridian and also making up the secondary meridian, the parasomal sacs microtubules, contractile vacuole pore. Three sets of membranes, the outer pellicular (outer alveolar), inner pellicular (inner alveolar) and the inner most cytoplasmic membrane also contribute to the cortical pattern of ciliate (Tokuyasu & Scherbaum 1965; Pitelka, 1961). These versatile structures of ciliate cortex obviously contribute to the diverse functional properties such as the mentainence of the typical ciliate shape, impermeability to chemical

substances, ciliate protection, serotype reaction of
ciliates and movement effected by the cilia.

The cortical architecture of _Tetrahymena_, provides
an excellent opportunity for analysis of cortical pattern.
It is fundamentally similar to the ciliate cortex in general.
Oral cortex of _Tetrahymena_ is more specialized exhibiting
four sets of membranelles in characteristic pattern, which
gives tetrahymena its name. Among these the undulating
membrane is most prominent feature (Fig. 1)

The kinetosome or the basal body lying at the
origin of each cilium is the organizing centre. In addition
to the function of providing the origin of the locomotory
unit organell i.e. the cilium, it also provides the site
for nucleation of other microtubular organells ie new
basal body (Allen, 1969; Dippek, 1968). The kinetosomes
are self reproducing bodies (Lwoff, 1950) having genetic
materials within them. New basal bodies are added anterior
to the pre-existing one (Nanney, 1971b, 1975). The basal
bodies in ciliates are homologous to the centrioles of the
multicellular organisms (Pitelka, 1969). They are arranged
in cortical rows which are 17-23 in number in tetrahymena
(Nanney, 1960; Frankel, 1930). The number of cortical
units or kinetids in tetrahymena are fairly constant in

a particular strain but the number of cortical rows or
kineties may vary within certain range (Nanney, 1971a).
This range of variation is also constant for a particular
strain. This cortical stability pattern in strains of
tetrahymena is considered as potentially a useful means
of discrimination of strains (Nanney, 1968).

The cilia are the most prominent structural compo-
nents of the cortex. It is one of the first cell organelle
to be investigated with the electron microscope (Fawcett
and Porter 1954). Among the most complete description of
Tetrahymena cilia is that described by Gibbons (1963) and
Allen (1968b).

The outer limiting membrane and the 9 + 2 array
of microtubules of the axonem predominate in the cross
section of these organells. The limiting membrane is a
continuation of the outer pellicular membrane of the cortex.
The detailed structure includes 9 pairs of peripheral micro-
tubules, each pair consisting of a subfibre-A and subfibre-B
and a pair of central microtubules enclosed by a central
sheath. The other components of the cilium include the
radially oriented spokes extending from central sheath to
each sub-fibre-A. The dense portion at the middle of each
radial spoke often interpreted as the secondary fibre,
oriented longitudinally along the cilium. Two rows of short
projections, the dynein arms having ATPase activity are

(A)

Undulating membrane

Oral membranelle

Primary meridian (Kinety)

Cilia

Secondary meridian

Dynein arm

Inter doublet link

Sub fibre-B

Sub fibre-A

Radial spoke

Secondary fibre

Central sheath

Central microtubule

(B)

Sub fibre-C

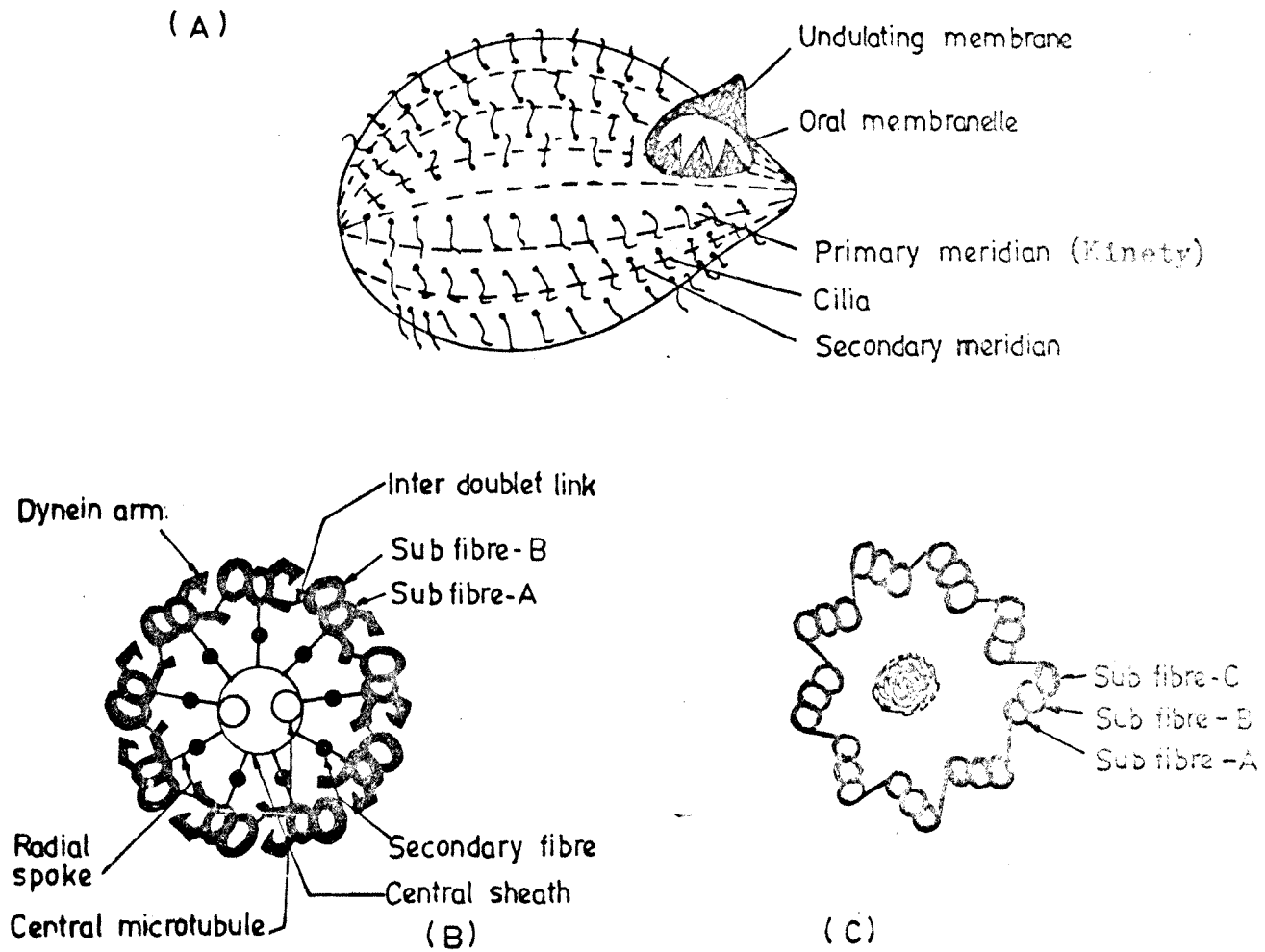Sub fibre-B

Sub fibre-A

(C)

Fig. 1   A - Tetrahymena pyriformis

         B - T.S. of a cilium

         C - T.S. of a basal body

connected to the subfibre-A of each outer doublet.

The essential structure of both the cilium and
the basal body is the microtubule . However, in basal
body, they are present in 9 peripheral triplets of which
in
subfibre-A and subfibre-B are continuous with the doublet
microtubules of cilium. The subfibre-C terminates at the
origin of the cilium and the central pair of microtubule
is absent in the basal body.

At the junction of the cilium and kinetosome,
there are various accessory structures which connect them
with the membrane (Dentler 1977). The central microtubule
cap connects the central microtubules and the dorsal filament
links the outer doublet to the membrane. Besides these,
various other fibres and tubules are associated with the
kinetosome complex which contibute to the cortical pattern
and involved in the ciliary beat (Allen 1969).

The biochemistry of cilia and kinetosomes has been
more elusive than their morphology. Analysis of cilia has
indicated that the bulk of isolated material is protein
(Child, 1959), which accounts for 80% of the weight of the
fraction. The main protein is a globular protein i.e.,
tubulin of molecular weight 55,000. Each microtubule is a
linear assembly of tubulin subunits which is a heterodimer

consisting of two apparently non identical proteins i.e.
α -tubulin and β -tubulin (Bryan & Wilson, 1971). Amino
acid sequencing revealed similarity of tubulin with actin.
It is rich in glutamic and aspartic acid. Tubulin from
various sources has nearly identical properties but recently
the difference of ciliary tubulin and flagellar tubulin
has been found (Stephens 1977 & 1981).

The other protein components of the axonem include
dynein, nexin, calmodulin etc. Dynein having Mg^{2+} - ATPase
activity, sediments at different rates (14S dynein and 30S
dynein) with molecular weight 600,000 (Gibbons and Grimstone
1963 & 1965). Nexin joins the subfibre-A of two adjacent
doublet microtubule (Stephens 1974). Calmodulin has been
found to be associated with the 14S dynein and also has
calcium dependent activity with molecular weight, 15,000
(Gordon at al. 1979).

At present, more attempts have been made to under-
stand the mechanism of tubulin assembly for the formation
of microtubule which is only partially understood. Assembly
of tubulin subunits starts with activated tubulin molecules
containing two tightly bound GTP molecules, one to each of
the two tubulin subunits(α and β). It proceeds through
both lateral and longitudinal association of tubulin molecules.

The sequence of events which link the dimers to form the microtubule is still not clearly known as all the observations were done *in vitro*. But the role of calcium in this process of assembly is no doubt important and is also facilitated by another protein i.e. microtubule associated protein (MAPs).

For the further understanding of the complex mechanism involved in the assembly process of tubulin, various investigators have been trying with different types of known drugs and chemicals. The pioneer work in this aspect was by Borisy and Tayler (1967) who found out the binding of colchicine to the microtubule subunit protein i.e. tubulin. Now, it is clearly known that colchicine can block the polymerization of tubulin and thus inhibit cilia regeneration in *Tetrahymena* (Rosenbaum and Carlson, 1969). Ethylacrylate urea is also known to inhibit the polymerization process and cilia regeneration (Himes and Himes, 1980). The treatment of cilia regenerating cells with actinomycine D, dinitrophenol, puromycin and cyclohexamide have proved that cilia regenerating cells to some extent require protein synthesis and DNA dependent RNA synthesis (Rannested, 1974; Child, 1965). However, most of the ciliary proteins come from the cytoplasmic pool.

Two different hypethesis have been postulated to explain the mechanism of ciliary beat. One explains the co-ordinated, sequential, active contraction of the outer

fibre doublets (Machin, 1958; Brokaw, 1966; Astbury et. al. 1955). The other which is indicated also by high resolution electron microscopy, shows the pairs of tubules instead of undergoing any change in length, slide along each other being attached at the base, suggesting the sliding-filament model which is similar to muscle contraction (Brokaw, 1971 Satir, 1968).

The cilia which are the most important structural as well as functional component of cortex have got the unique property of regeneration. The systems of regenerating cilia in the protozoa have shown to offer many advantages for the investigation of various aspects of the synthesis and assembly of ciliary proteins. These aspects of cilia regeneration have been studied by several investigators (Rosenbaum, Carlsen, 1969; Castro et al. 1973; Rannestad, 1974; Rodriguez and Renau R, 1980; Bird and Zimmerman, 1980; Keenan & Rice, 1980; Guttman & Gorovsky, 1980).

The present investigation has been undertaken not only to find out the cilia regeneration kinetics under our experimental condition but also to correlate different morphological changes of cortical structure those occur during the process of cilia regeneration. This may unravel some aspects of the mechanism and control process of the development of

cellular organells in general. Further understanding may
be obtained from the effect of cytochalasin B which is also
known to interfere various physiological process of cell
including the cell motility. This may provide some additional
information to explain the regulation of cilia regeneration
and the synthesis of various components of the ciliary
apparatus in tetrahymena.

MATERIALS AND METHODS

Tetrahymena pyriformis is a single celled ciliated protozoa. It is a freeliving (a few are parasitic), fresh water organism measuring about 50 µ in length and 30 µ m in width. Normally in log phase of growth, it has a small pear shaped body not distinguishable with unaided eye, but the extreme plasticity of the tetrahymena pellicle permits considerable temporary distortion. The body is uniformly ciliated in longitudinal rows or kineties the number of which varies from 17 - 23 and alternate with secondary meridians of mucocyst pores. The oral area is located close to the anterior pole of the cell. A centrally positioned oval macronucleus is always present. But the micronucleus may be absent in some strains. A contractile vacuole is found in the posterior part of the cell.

Cell Culture:

For the present investigation, the organism used is Tetrahymena pyriformis (W) amicronuclate strain). The organisms are cultured axenically at 24 ± 2°C in the laboratory in 2% proteose peptone supplemented with 0.02% liver extract. For the preparation of culture medium 2 gm of proteose peptone with00.02 gm liver extract is dissolved in 100 ml of double distilled water in a 250 ml conical

flask, filtered, capped with cotton plug and wrapped
with aluminium foil and then the flasks are autoclaved
for 20 minutes in 15 psi. Flasks are inoculated
aseptically with 2 ml. of a 2-days old culture with
the help of a 2 ml. sterilized transfer pipette and is
allowed to grow for 2 days. The generation time of
tetrahymena in our experimental condition has been
found   to be approximately two-hours and forty-five
minutes.

Experimental methods

Selection of the cells:

For our experimental purpose 48 hours log phase
cultures are always selected when the cells are healthy
and actively swimming. The cells are harvested at room
temperature (24 & 2°C) by centrifugation at 200 - 300 rpm
using a Remi centrifuge for 3 - 4 minutes and are concentrated
twice by decantation of the supernatant and resuspended in
their original growth medium. The concentrated cells
are immediately used for the experimental purpose.

Cilia amputation procedure:

The procedure for cilia computation is used as
described by Rosenbaum and Carlson (1969). The whole
operation is carried out at 4°C using an ice bucket.

2 ml. of the concentrated cells are added to 5.0 ml. of medium A (10 mM EDTA + 50 mM CH_3COONa, pH 6.0) in a 15 ml. screw capped centrifuge tube (corning) and thoroughly mixed by gentle shaking. After a minute, 2 ml. of ice cold double distilled water is added and after 2 minutes 0.25 ml. of $0.2 M CaCl_2$ is added. Then the suspension is mixed by inverting the centrifuge tube several times. After 5 minutes the cell suspension is subjected to 3 - 4 shearing with a 10 ml. glass syringe fitted with a 10 gauge needle. Immediately after deciliation procedure, the cell suspension is centrifuged for 2 - 3 minutes and the supernatant is decanted off and resuspended into the original growth medium.

Determination of cilia regeneration timing in normal cells:

The deciliated cells are kept at room temperature (24 ± 2°C) and since deciliated cells are not motile, cilia regeneration is monitered simply by determining the percentage of cells regaining motility with time. At each hour following deciliation an aliquot of cells is observed under the microscope using a haemocytometer and the number of motile and non-motile cells are counted per 0.1 c.u.m.m. of the cell suspension. The percentage of cells moving at a particular time, thus can be calculated at different hours until hundred percent of cells regain their motility. Each experiment is repeated at least for four times.

Cytochalasin B treatment

Dose tolerance:

Before subjecting the cells to cytochalasin B treatment, the extent of viability of the cells at different concentrations of the chemical is taken into account. The dose tolerance test has been done with cytochalasin B, 0.001% to 0.009%. For each concentration of the chemical the deciliated cells are kept in the broth containing the chemical for one hour and the percentage of viable cells is noted using dye exclusion test (0.1% trypan blue). In this test, the living cells first take the dye but immediately after a few minutes the dye will be excluded out, whereas the dead cells will retain the dye permanently. The stock solution of cytochalasin B is prepared in DMSO (10 mg/ml) and stored in the refrigerator. The final solution is prepared in the tetrahymena culture medium at the time of experiment. The cell survival at various concentrations is taken as an index to determine, whether at a particular concentration, the chemical had deleterious effect or not. On the basis of dose tolerance test the concentration of the chemical and the duration of treatment are so selected as not to have any lethal effect on the ciliate.

Duration and time of treatment:

To determine the duration and the time of treatment, four simultaneous experiments are carried out;

1. The cells are pretreated with cytochalasin B for
    2 hour before the cilia amputation procedure
    and then the drug is immediately washed off.

2. The cells are pretreated for two hours before
    deciliation as well as through out the cilia
    regeneration period.

3. The cells are treated for one hour immediately after
    deciliation and then shifted to fresh medium.

4. Cells are only post treated through out the cilia
    regeneration period.

Determination of regeneration kinetics for treated cells

This is determined in similar method as is done in case of control cells. The percentage of motile cells is obtained at different hours after deciliation by counting the treated cells, both motile and non-motile until about hundred percent of cells regain their motility. Four different sets of experiments are carried out to obtain the average timing. For each set of experiment twenty counts are taken.

Observation of cell surface morphology:

Preparation of cell sample for light microscopic observation:

For preliminary observation of cell morphology in relation to their cortical pattern, conventional light microscopic studies are carried out using different staining techniques with the help of Carl Zeiss Amplival microscope (magnification x 160). For this routine observations of cells, (1) normal ciliated tetrahymena (2) cells immediately after deciliation (3) cells with regenerating cilia i.e. 2 hour after deciliation and (4) cells of CCB treated at ½ hour after deciliation are studied.

Giemsa Staining:

For the preparation of slides drop of aliquote is taken on a subbed slide (prepared with 0.5% gelatin) kept in a slanting position for few minutes for partial drying. Fixation is done with 1:3 acetic acid and ethanol for 5 minutes and dehydrated with absolute and 90% ethanol for ten minutes in each. Finally the slides are stained with buffered Giemsa (pH 7.0) and mounted with DPX.

Negative Staining by Nigrosine

The procedure for preparation of negatively stained slides is same as that followed for preparation of Giemsa staining. But here, the staining is done by putting a drop

of nigrosine (10%, prepared in double distilled water).

<u>Silver impregnated slides:</u>

The procedure is followed as described in Chatton-Lwoff silver impregnation technique. Cells are prefixed with champy's fixative (7 parts, 3% $K_2Cr_2O_7$ + 7 parts 1% Cr_2O_3 + 4 parts 2% OsO_4) for 2-3 minutes and then post fixed with Da Fano's fixative (1 gm Cobalt nitrate + 1 gm. NaCl + 10 ml. formalin + 90 ml. double distilled water) for two hours. Samples are prepared using subbed slides kept in 3% silver nitrate solution for 15-20 minutes in cold and dark. Dehydration is done with 70%, 80%, 95% and absolute ethanol, then with Xylene and finally mounted in DPX. Mounted slides are then thoroughly exposed to bright sunlight for the reduction of silver nitrate to metallic silver.

<u>Preparation of specimen for scanning electron microscopic study</u>

For detailed observation of cell surface morphology, the cells observed in scanning electron microscope with the help of a Cambridge stereo scan -S4-10, operated in 30 KV. Cell samples of (1) normal ciliated cells (2) deciliated cells at zero hour (3) cells with regenerating cilia, 2 hour after deciliation and (4) CCB treated cells

at $\frac{1}{2}$ hour after deciliation are prepared. Prefixation
is done with 2.5% glutardialdehyde (in soidum cacodylate
buffer, pH 7.2) in cold for one hour. Cells are theroughly
washed in sodium cacodylate buffer and post fixed in ice
cold 1% osmium tetroxide for one hour. Osmium tetroxide
is completely removed by thorough washing with cacodylate
buffer. Dehydration is carried out at room temperature in
50%, 70%, 90% & absolute ethanol and finally through two
changes of acetone. Thin layer of cells are taken on
a cover glass, lyophilized for 15 - 20 minutes and placed
on metallic stubbs. Cell sample is coated with silver
200 A° thick. Photo-micrographs are taken at different
magnifications.

Source of chemicals:

Gelatin (Bacteriological) $AgNO_3$, ethanol, acetic acid,
xylene, acetone, DPX, EDTA, CH_3COONa, $CaCl_2$, NaH_2PO_4,
KH_2PO_4, $K_2Cr_2O_7$, CrO_3, $Co(NO_3)_2$, NaCl, formalin are all
Analar grade and obtained from M/s BDH, India or M/s
Sarabhai M. Chemicals, India.

DMSO, sodium cacodylate, hog liver extract, OSO_4
are purchased from Sigma Chemical, U.S.A. Proteosepeptone
are obtained from Difco Laboratories, U.S.A. and Giemsa
strain is procured from British Drug House Ltd. England.

Glutardialdehyde is purchased from Riedel, Dehainag seelze-Hannover and Cytochalasin B is procured from Aldrich Chemical Co., U. .A., Trypan blue is obtained from George T. Gurve Ltd., London and nigrosine from Loba Chemie, Bombay.

Abbreviations used:

EDTA	-	Ehtylene diamine tetra acetic acid
NaH_2PO_4	-	Sodium phosphate dibasic
KH_2PO_4		Potasium phosphate di basic
$K_2Cr_2O_7$	-	Potasium dichromate
$CaCl_2$	-	Calcium chloride
C_rO_3	-	Chromium trioxide
$Co(NO_3)_2$	-	Cobalt nitrate
CH_3COONa	-	Sodium acetate
$AgNO_3$	-	Silver nitrate
DMSO		Dimethyl sulfoxide
CCB	-	Cytochalasin B

EXPERIMENTAL RESULTS AND OBSERVATIONS

A.   **Observations on the cell surface cortex of
     Tetrahymena pyriformis**

A series of cytological preparations using various
staining techniques have been made for an analysis of the
general morphology of the cortical structures in tetrahymena
for visualization of the cortex under light microscope.
The observations are further strengthen by a detailed
analysis of the cortical architecture of the ciliate with
the help of scanning electron microscopic observations.
The organism has a spindle shaped cell body, the surface
of which is uniformaly ciliated. The cilia are the most
prominent structural component of the cell surface cortex
and are arranged in parallel rows of kineties as shown
in the photomicrographs of a normal tetrahymena (Figs. 2 &
3). Since the cilia are very minute and submicroscopic
structures, they are not seen in the light microscopic
observation but their positions are represented by the
ciliary groves from where, they originate (Fig. 4). The
kineties are arranged in an anterior posterior direction
making the primary meridian alternating with the secondary
meridian of mucocyst pores seen as rows of pits. The oral
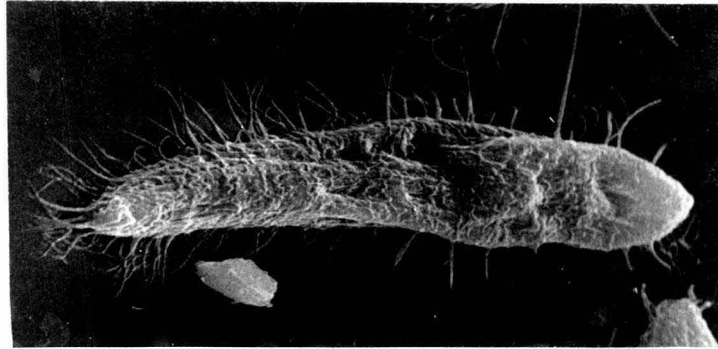area is located close to the anterior pole (Fig. 5).

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Fig. 2. Scanning electron micrograph of tetrahymena pyriformis, showing ventral view. Arrow (⊥) indicates the oral end X4950.
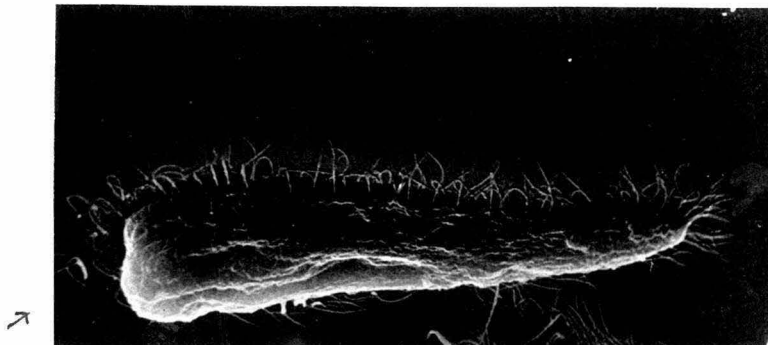

Fig. 3. Scanning electron micrograph of T. pyriformis showing the lateral view. Arrow indicates the oral end X43704.

Fig. 4. Negrosine staining of
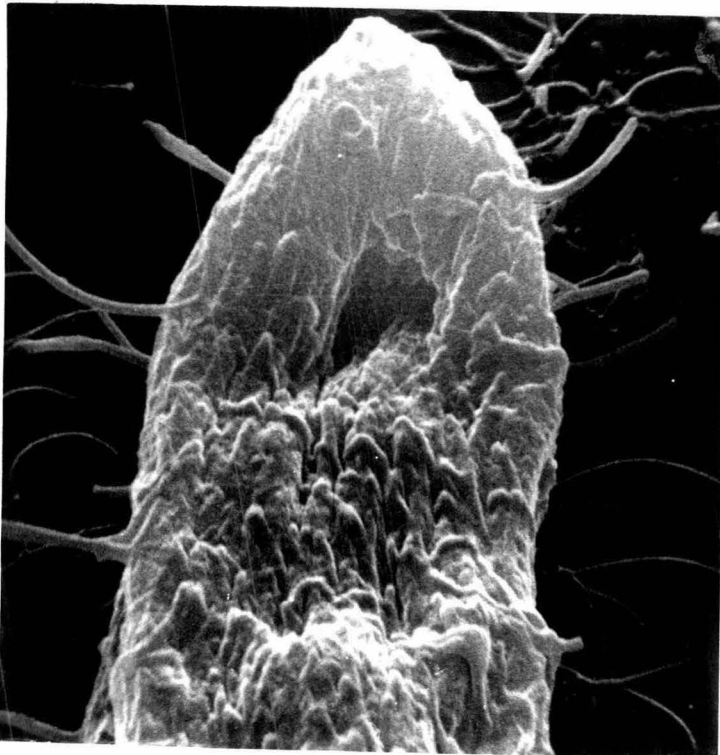T. pyriformis showing the
rows of kineties X 100.

Fig. 5. Scanning electron
micrograph of the interior
region of T. pyriformis
showing the oral grove X 23850.

B.   Kinetics of Cilia Regeneration:

Immediately after deciliation all the cells are
found to be immotile and they settled at the bottom.
However, within very short time after the cilia amputa-
tion a few cells show aberrant type of movement. These
cells slowly rotate on their longitudinal axis and a few
of them display a circular motion moving along a short
circular path. Still a few show non-directed, jerky
movement. However, all these cells are not considered
to be motile cells for our cell motility count. A cell,
for our experimental purpose, is considered to be motile
when it shows the typical ciliary motion as found in a
normal tetrahymena. At one hour after deciliation
ca. 15% of cells have regained their motility while by
second hour the motility index reaches up to ca.50%.
By next hour, about 75% of cells become motile and almost
all cells display the typical ciliary locomotion at the
end of fourth hour. However, our observation at fifth
hour of deciliation shows almost no immotile cells, indicat-
ing a complete ciliogenesis in about 100% of deciliated
tetrahymenae (Figs. 6 & 7).

It has been observed that the deciliation procedure
carried out in cold (ca. 4°C) is most suitable in contrast
to the room temperature (ca. 22°C), where a cell lysis
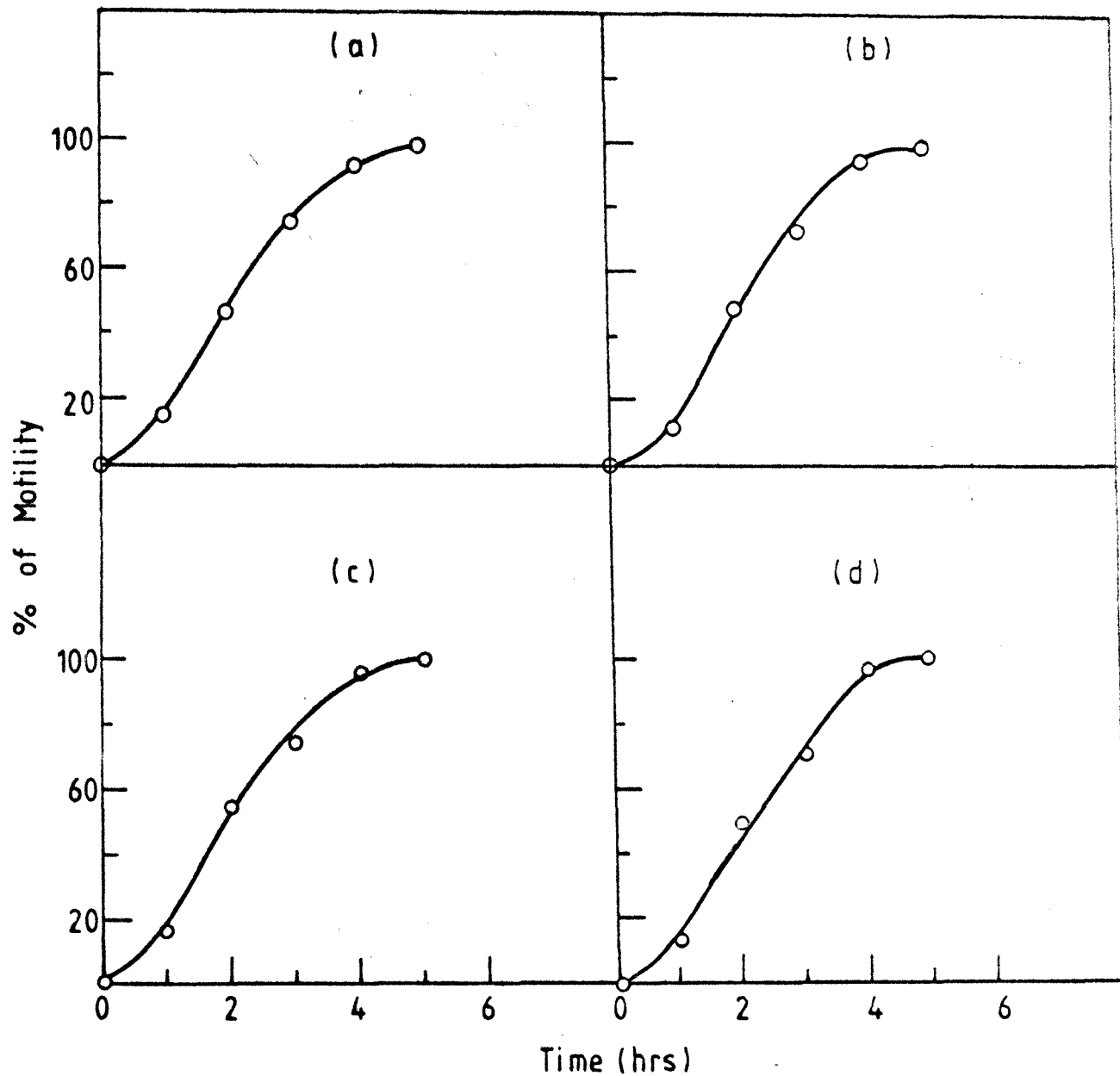between 20 - 25% has been found to occur.

Fig. 6 Timing of cilia regenerations in T. pyriformis, a, b, c & d
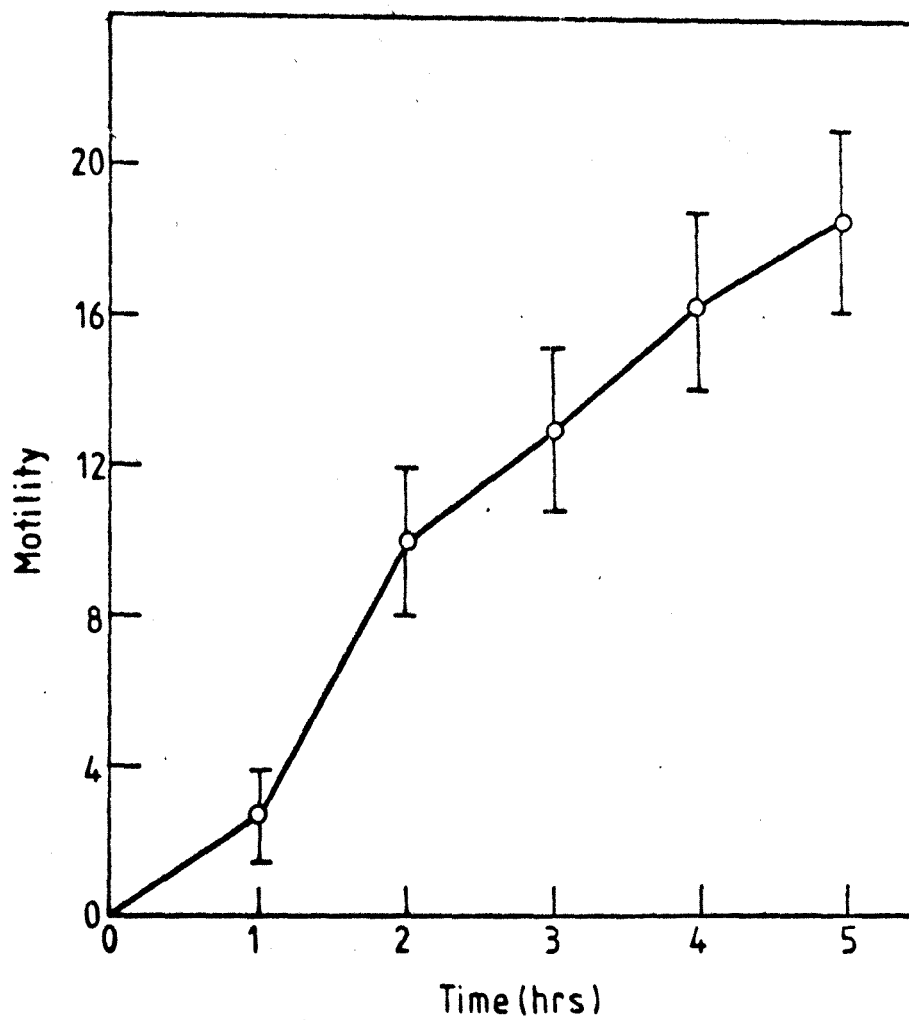   represent four typical experiments showing the regeneration
       kinetics.

Fig. 7   Summary of experiments on cilia regeneration
         in Tetrahymena pyriformis.

C. **General Morphology of the Cells during Cilia Regeneration:**

General morphological pattern of the cortex shows various kinds of alterations after the cilia are removed. Instead of the typical spindle shape, many cells exhibit a spherical form as depicted in Fig. 8 & 9. In the deciliated cells, rows of kineties are indistinct when observed under light microscope. This indicates, the absence of cilia. But, in the scanning electron microscopic observation the ciliary groves look quite prominent and devoid of cilia (Figs. 10 & 11).

In the cilia regenerating cells, at two hour after deciliation, though most of the cells regain their typical cell shape, a few are still seen to be abnormal (Fig. 12). When the cell samples at this hour are observed, it has been found that the cell population contains a mixture of both ciliated and deciliated cells. However, scanning electron microscopic study shows that most of the cells which are observed at this hour do not possess the full complement of ciliary numbers. In fact, the tetrahymenae around this hour bear a few cilia on the cell surface (Fig. 13). This situation might have been possibly due to the fragile nature of the newly formed cilia, which are more sensitive, as compared to the cilia of the normal cells, to the fixation procedures needed for scanning electron microscopic observations.
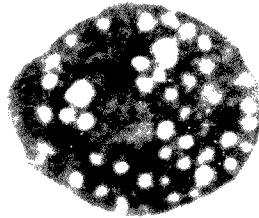
Fig. 8. T. Pyriformis immediately after deciliation
         Note the rounded up and hyper vacuolated
         conditions of the cell. Giemsa stained X 160.
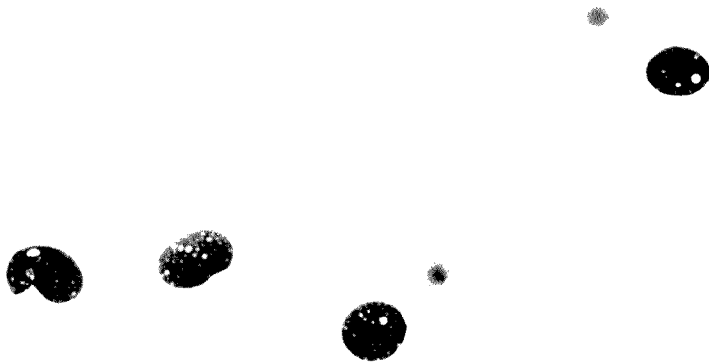

Fig. 9. Showing a group of T. pyriformis immediately
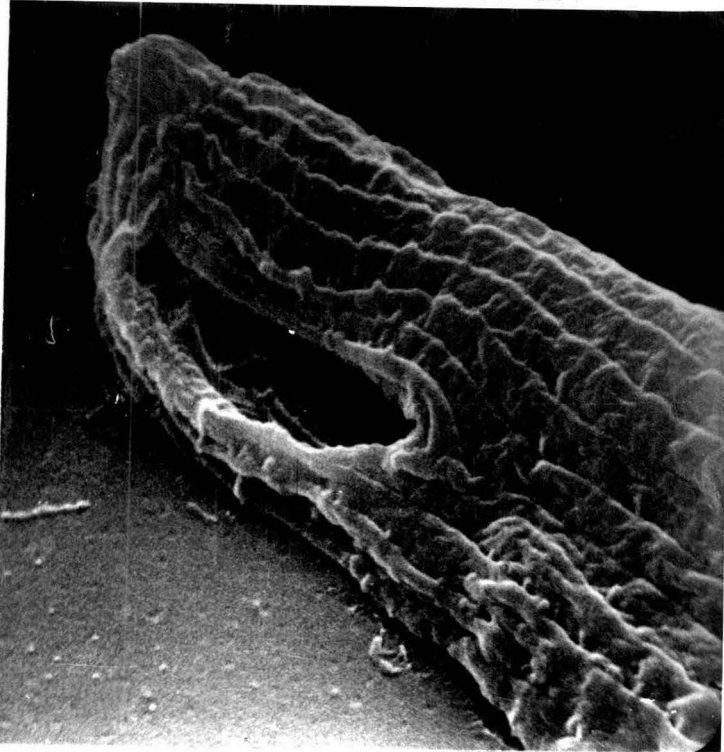   after deciliation. Giemsa stained X63.

Fig. 10 Scanning electron micrograph of anterior end of T. pyriformis immediately after deciliation X 24300

Fig. 11 A deciliated T. pyriformis X8505.

Fig. 12. A group of T. pyriformis two hours after deciliation. Chattow-Lwoff silver imprignation technique X100.
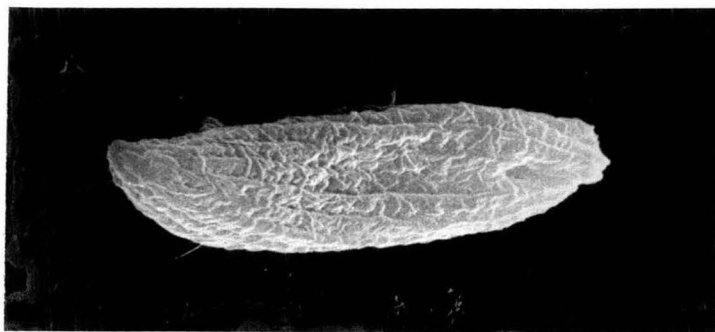

Fig. 13. Scanning electron micrograph of T. pyriformis two hours after deciliation of XB775.

D.   Cytochalasin - B (CCB) Treatment:

The drug CCB, which is well known for producing various kinds of cellular changes has been administered to tetrahymena in order to investigate its effect on cilia regeneration process. It has been found out that the ciliates can tolerate high dose of CCB unlike the mammalian cells for a prolonged period of time. For example a concentration of 25 μg/ml of CCB produces little effect on tetrahymena and causes almost no lethality. On the basis of a dose tolerance experiment, a concentration of 20 μ g /ml. (0.002%) of CCB has been found to be most suitable for our experimental purpose. At this dose, 100% of cell survival has been obtained and no alteration in the cell morphology has been detected, even when the cells are treated with the drug for 24 hours or so.

Four types of experiments have been conducted with CCB on deciliated tetrahymenae:

1.   When the ciliates are pretreated for two hours with CCB prior to their deciliation, the regeneration kinetics of the cilia remain unaffected i.e. around fourth hour after the deciliation, almost total recovery in cell motility has been noticed similar to that of the untreated cells.

2.   When the tetrahymenae are treated with CCB
      immediately after deciliation procedure and kept
      in the drug through out the cilia   regeneration
      period i.e. for five hours, a lag period of one
      hour is noticed before any cell can regain its
      motility. However, at later hours, the cells
      slowly recover somewhat from this initial lag and,
      at about fifth hour after deciliation they show
      about more than 90% motility similar to that of
      the control.

3.   The pre-trea-tment for two hours and post-treatment
      (after deciliation) for five hours show an identical
      result like that of number   2 i.e. only an initial
      lag of one hour in cell motility has been observed.

4.   Post-treatment with CCB for one hour alone, immediately
      after deciliation also shows similar type of result
      as that of number-3. So in effect, the one hour treat-
      ment of CCB immediately after deciliation treatment is
      enough to produce the initial lage of one hour in the
      ciliary motion in tetrahymena. The effect of CCB
      treatment on the process of cilia regeneration has
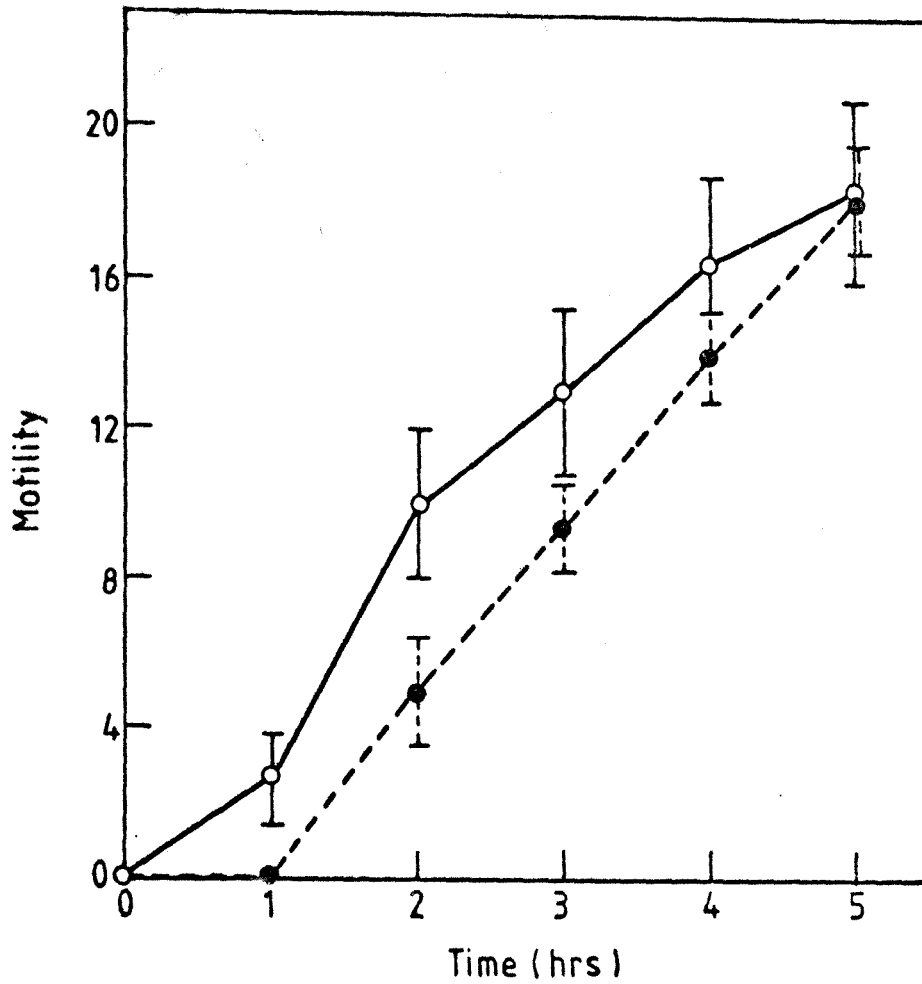   been summerised and depicted in the Fig. 14.

Fig. 14 The effect of cytochalasin B on cilia
regeneration timing. The figure depicts
the summary of four experiments with
cytochalasin B together with the appropriate
controls.

Although CCB does not produce any visible effect
on cell morphology in normal tetrahymena, it produces
a remarkable effect on the deciliated cells. In many
deciliated and CCB treated cells a pronounced alteration
in the cell morphology has been observed. These cells
in many cases demonstrate a change in their cell shape
and these changes are quite visible in light and scanning
electron microscope. In most of the cases, the contour
of the CCB treated deciliated tetrahymenae, appeared
to be twisted and as a result, the arrangement of the
kineties are also found to be quite distorted. In
untreated cells rarely such aberrant morphological feature
has been encountered. The morphological changes in CCB
treated cells together with the changes in the cortical
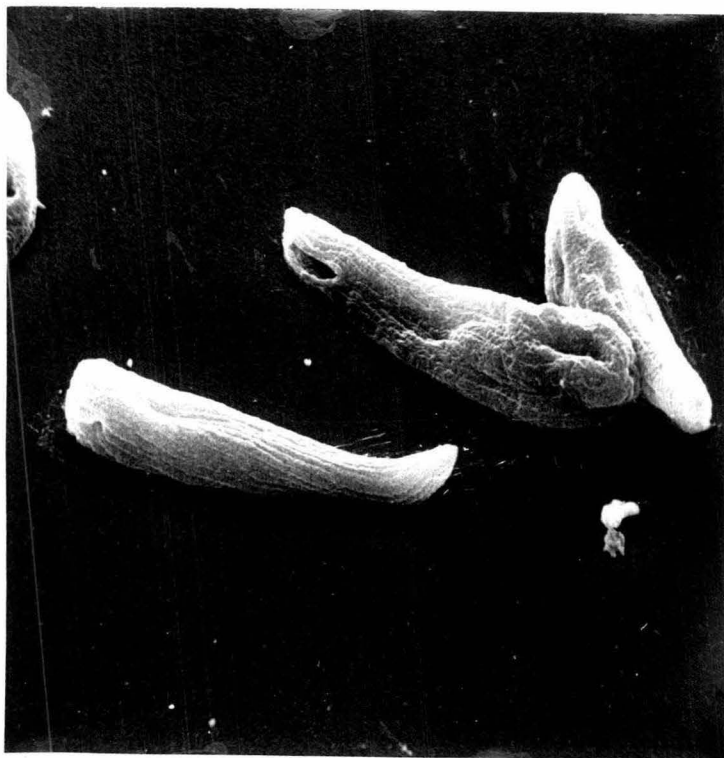architecture are shown in Figs. 15 to 20.
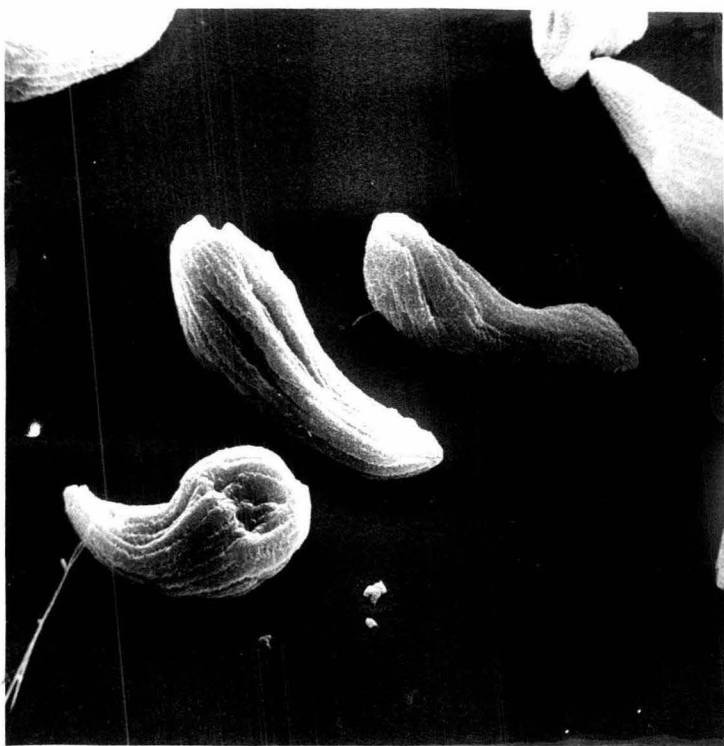
Fig. 15. Deciliated
T. pyriformis X3645

Fig. 16. Deciliated and
cytochalasin B treated
T. pyriformis. Note
the twisted cellular
apparance of the ciliates
X 3150.

Fig. 17. A group of T. pyriformis deciliated and
cytochalasin B treated Chattow-Lwoff silver
imprignation technique X100.

Fig. 18. Scanning electron micrograph of T. pyriformis
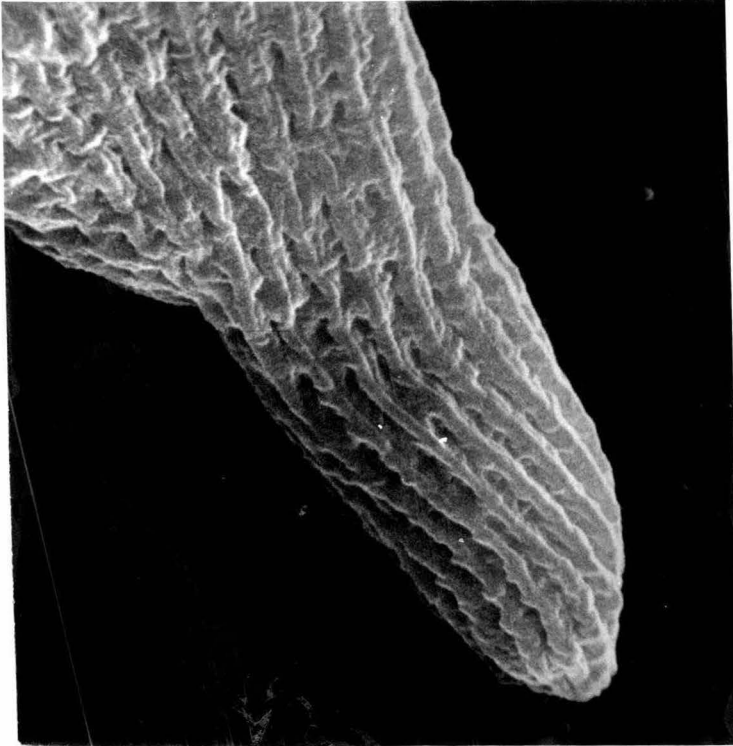deciliated and cytochalasin B treated X8550.

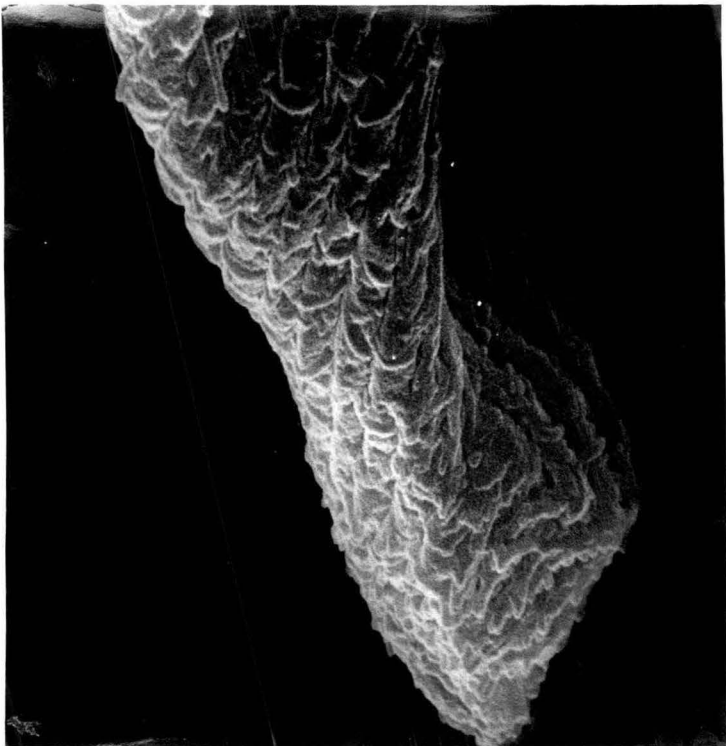Fig. 19. The caudal region of a deciliated T. pyriformis. Scanning electron micrggrph X22525.

Fig. 20. Caudal region of a deciliated and cytochalasin B treated T. pyriformis. Note the distorted caudal region and the rows of kineties. Scanning electron micrograph X20825.

DISCUSSION

There are several studies on the regeneration
process of cilia and flagella which have been tried to
unravel the phenomenon of ciliogenesis both at morpholo-
gical as well as biochemical level (Child, 1965; Auclair,
Siegel, 1966; Rosenbaum, Child, 1967, Rosenbaum and
Carlson, 1969; Castro, Colon et al. 1973; Rannestad, 1974;
Bird and Zimmerman, 1980; Guttman & Gorovsky, 1979; Keenan and
Rice, 1980; Rodriguez and Renaud, 1980). However the
process of assembly of ciliary components, their transfer
and location at specific sites on the ciliate cortex is
still not clearly understood. Moreover, the various
complex molecular interactions, which are involved in the
regulation of cilia regeneration as revealed from the
work of the above mentioned investigators, still remain
largely unresolved.

Our study on the timing of cilia regeneration in
Tetrahymena pyriformis is more or less consistent with
that of Rosenbaum and Carlson(1969) and Guttman and
Gorovsky (1979). Under our experimental conditions, a
total cessation of cell motility has been observed
immediately after the deciliation treatment, indicating

an almost total removal of cilia from the cell surface. This
has been confirmed from the scanning electron microscopic
observations. About an hour after deciliation of the tetra-
hymena, a small population of cells begin to move around.
However, a few cells can be seen moving even within 30 minutes
after deciliation treatment. At later period, there is a rapid
increase in the number of motile cells and at third hour after
deciliation, approximately 75% of cells become fully motile,
reaching upto almost 100% level around fourth hour. Rosenbaum
and Carlson (1969) and Guttman and Gorovsky (1979) have observed
a complete cilia regeneration in tetrahymena within 90 minutes
after deciliation which is fairly a shorter period compared
to our experimental results. This difference in the cilia
regeneration kinetics can probably be attributed to the
difference in experimental conditions viz., growth condition,
temperature, pH, the particular strain of tetrahymena used etc.

We have observed that there exists quite a large
range of difference in the timing of resumption of motility
of the individual cell at any given time. For example,
some cells regenerate their cilia as early as within 30
minutes after deciliation whereas others need as long as
3-4 hours. Other investigators have also observed similar
variations in the timing of cilia regeneration. These
differences can possibly be explained by the asynchronous

state of the experimental cells. It is quite plausible that since the cells remain at various phases of cell cycle, they will have differences also in their physiological states (metabolic conditions), which are reflected in early or delayed regeneration process of the ciliary apparatus.

The aberrant behaviour of cell movement which has been observed in some cells at early phase after the deciliation in our experiments could be due to the short and partial or preferential cilia regeneration at particular area of the cortex which causes the cells to rotate more than to progress. Similar kind of observations have also been reported by Guttman and Gorovsky (1979).

Immediately after deciliation some changes of cell morphology have been noticed in Tetrahymena Pyriformis. The cells become little abnormal in shape and mostly they assume a spherical configuration. Within about two hours, however, most of the cells regain their normal spindle shaped form. This morphological alteration could possibly be due to the action of certain chemical components present in the deciliating agent. As there are several surface acting chemicals e.g. EDTA, Ca^{++} etc. present in this medium, presumably these chemicals might produce some transient effects resulting in the change of the cell shape.

The mode of action of the deciliating agent is not clearly understood. It is believed that EDTA plays the main role in the detachment of cilia from the cortex and addition of Ca^{++} to the deciliating agent causes the destabilization of the cortex, as "the normal stability of the cell surface requires a critical level of Ca^{++}.

The formation of cilium, which requires the synthesis, localisation and assembly of fresh ciliary components, is thought to be stimulated by the deciliation treatment. It has been reported that the synthesis of tubulin (the main protein component of cilia) is highly sensitive to deciliation treatment (Bird and Zimmerman, 1980). For the formation of cilia although most of the protein subunits are available from cytoplasmic pool, a minor portion is synthesised freshly during the regeneration process (Nelson, 1975).

Many workers have used various kinds of inhibitors for a better understanding of ciliogenesis. For the synthesis of ciliary proteins such as tubulin, dynein etc., the transcription of specific mRNA and their translation to the protein subunits are required. These protein subunits

reassemble to form the microtubules and their final arrangement leads to the formation of cilia. The regeneration of cilia, thus, can be blocked at any of these steps. Rosenbaum and Carlson (1969) have shown that the treatment of colchicine can inhibit the cilia regeneration irreversibly. Other investigators in this field have tried with the inhibitors of protein synthesis and also with actinomycin D to explain the regulation of this regeneration process (Child, 1965; Nelson, 1975). Castro at al. (1973) showed that treatment of melatonin, a plant hormone, on the deciliated cells causes a delay in the cilia regeneration timing.

Our investigation with a fungal metabolite, cytochalasin B on the cilia regeneration has shown that this drug affects the cilia regeneration kinetics. In an earlier study Himes and Himes (1980) have used ethyl acetyl acrylate, an analog of cytochalasin-A which inhibits the assembly process of tubulin.

Cytochalasin B has been known to produce a number of diverse cellular effects (Carter, 1967). It causes a marked alteration of cell morphology. Carter, (1967) has reported flattened shape of fibroblast cells caused by CCB at a dose of 1 μg/ml. Sanger and Holtzer (1972) observed a branched cellular configuration and inhibition

of cell movement brought about by CCB in fibroblast and myoblast cells. Recently in 1979, Devoy et al., in their experiment on chick mesoderm cells have found morphological alterations caused by CCB. However, these effects are not observed for all cell types. So it has been suggested that the differences in response to CCB may reflect fundamental differences in cell surfaces (Copeland, 1973).

Cytochalasin B has also been known to affect the cellular adhesion reaction (Sanger and Holtzer, 1972) and to cause nuclear extrusion (Carter, 1967). The inhibition of mucopolysacharide biosynthesis in vitro by CCB has also been observed (Sanger and Holtzer, 1972).

Besides the morphological alterations the drug also brings about a lot of alterations in the physiology of the cells. It causes a depression in phagocytic activity in human blood leucocytes (Malawista, 1971). This drug also has been known to induce some abnormalities in the mitotic process with inhibition of cytokinesis resulting in the formation of multinucleated cells (Carter, 1967; Krishan and Ray Chowdhury, 1969).

Many investigators also have observed that CCB has many adverse effects on the microfilaments, especially studied on the cytoskeletal and contractile elements

(Goldman et. al., 1973; Edds, 1980; Temmink and Spiele,
1981). The drug blocks the polymerization of actin
filament by binding to its end (Spudich & Lin, 1972; Brown
and Spudich, 1979; Fox and Phillips, 1981).

Our results with CCB on the ciliogenesis of
Tetrahymena pyriformis demonstrate that when the drug
is administered prior to the deciliation treatment, it
does not bring about any alteration in the timing of cilia
regeneration. This probably indicates that the cells with
intact cilia, cell membrane and the mucopolysaccharide
coat do not permit the drug to enter inside the cell, or
may be the binding site for CCB is not exposed. This
chemical becomes effective only when the ciliates are
denuded. It is quite obvious from our data that the process
of cilia regeneration remains inhibited for about an hour,
when CCB is applied immediately after deciliation. However,
at later periods they show increased motility albeit at a
lower level compared to the untreated cells. Around fifth
hour after deciliation, the recovery in cell motility
process is much accelerated and becomes almost comparable
to that of the control cells. The pre & post treatment with
CCB does not bring about any additive effect resulting in
any greater inhibition in cilia regeneration. Moreover,

CCB post treatment for longer than one hour also does
not cause any greater suppression in cell motility. So
we can conclude that post treatment of CCB for one hour
immediately after deciliation is most effective in causing
alteration in the timing of cilia regeneration. In this
context, it is interesting to observe that synthesis of
a 80,000 dalton protein (deciliation induced protein, DIP)
begins shortly after the cells are deciliated. The
synthesis of this protein declines around one hour after
deciliation. The synthesis of tubulin (55,000 dalton
protein) can be detected only one hour after deciliation
(Guttman and Gorovsky, 1979). It is a tempting to
speculate that CCB which is only effective during the
first hour after deciliation might interfere with this
DIP proteins thereby causing a lag in the cilia regeneration
process.

A remarkable effect of CCB treatment which is
revealed by our scanning electron microscopic observations,
is the alteration of cell morphology. We have consistently
observed that CCB post treatment produces bizzare cell
shape. Many of the treated cells display a distorted
contour and twisted appearance. High resolution scanning
electron microscopic study reveals that the cell surface
cortex is also disturbed and the orderly rows of kineties

become disarrayed due to the treatment of the drug. This kind of aberrant cell morphology has seldom been encountered in the drug untreated cells. This kind of contorted appearance of CCB treated cells are regulated back to the normal shape at later period. Many kinds of such morphological changes have also been reported by various other investigators. DeVoy et al., (1979) observed a rounding up off cells of chick mesoderm treated with this chemical. CCB causes an immediate distortion in cell morphology in coelomocyte cells (Edds, 1980). Britch and Allen (1981) have observed morphological changes with disruption of microfilament in normal rat liver cells and hepatoma derived cell-line. CCB is also known to cause a change in cell morphology of 3T3 cells (Temmink and Spiele, 1981). This kind of effect can possibly be correlated withthe action of CCB on the cell surface components of tetrahymena. Recently the major cytoskeletal proteins of the cell surface in tetrahymena have been identified and observed under electron microscope. It has also been found that the ' epiplasm' is a continuous layer of fibrous elements found just below the surface membrane. It has also been indicated that the actin protein might also be present in this epiplasmic layer (Williams & Vaudax 1979; and Vaudax-William, 1979). It is already knownthat CCB greatly interferes with

the polymerization and elongation process of the actin
filaments (Spudich and Lin, 1972; Brown and Spudich, 1979;
Fox and Phillips, 1981). So it will not be unreasonable to
conclude that the alterations in the surface architecture
of the ciliate cortex could be due to the action of CCB
on the cytoskeletal proteins including actin. However,
a similar kind of speculation has also been made that
the morphological alterations are to be due to an effect
of CCB on the cytoskeleton which could be a direct disruptive
effect or disruption of microfilament of the cell surface
(Goldman et al., 1973; De voy & England et al., 1979).
We can conclude that the changed cell surface topography
may therefore, affect the precise steps of synthesis,
assembly and transport of the ciliary components by altering
the spatial arrangement of cilia on the cortex. This,
intarn, leads to the change of cilia regeneration kinetics.

SUMMARY

1. Ciliated protozoan, _Tetrahymena pyriformis_ (w)
   are deciliated by EDTA and Ca^{++} pulse method
   and the cilia regeneration kinetics as well as
   the morphology of the cell surface cortex during
   the cilia regeneration are studied with light
   microscope and in more detail by scanning electron
   microscopy.

2. It has been observed that a small population of
   cells slowly start moving at about one hour after
   deciliation and after this, the motility index
   slowly increases, such that, after four hrs of deciliation
   almost all cells regenerate their ciliary apparatus.

3. The observation of cell surface morphology shows that,
   immediately after deciliation all the cells are
   completely devoid of cilia and a few cells show a
   spherical shape.

4. Cytochalasin B which is well known to cause various
   physiological as well as morphological alterations
   of the cells viz., phagocytosis, pinocytosis, cell
   motility and polymerization of actin filament etc.
   has been administered to see the effect of the drug
   on the cilia regeneration of tetrahymena. In our
   study it has been consistently observed that this

drug produces a lag period of one hour in the
cilia regeneration timing. But however, the
effect is temporary and after the lag period
the cells slowly recover to the normal level
at about fifth hour after deciliation.

5. Cytochalasin B also causes a remarkable change
    in the morphology of the cell surface cortex.
    Mainly it causes a twisted configuration of the
    cortical pattern with distorted rows of kineties.

6. It is postulated that these observed effects of
    cytochalasin B on the cilia regeneration timing
    and the morphology of the cortex can be due to two
    reasons. The drug may interfer with the synthesis
    of DIP proteins or the protein components of cell
    surface viz., actin. It may be also possible that
    the drug may affect both these protein components
    simultaneously to bring these observed alterations.

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