The Effect of Colchicine on Sexual Reproduction of Chlamydomonas moewusii

Anne Leslie Menoff

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THE EFFECT OF COLCHICINE ON SEXUAL REPRODUCTION

IN CHLAMYDOMONAS MOEWUSII

A Thesis
Presented to the Faculty of the Department of Biological Sciences
of the State University of New York College at Brockport
in Partial Fulfillment for the Degree of
Master of Science

by
Anne Leslie Menoff
May 1980
THESIS DEFENSE

FOR

Anne L. Menoff
Master's Degree Candidate

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To my parents with much love and many thank yous.
ACKNOWLEDGEMENTS

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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF TABLES</td>
<td>v</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>vi</td>
</tr>
<tr>
<td>LITERATURE REVIEW</td>
<td>1</td>
</tr>
<tr>
<td>The Experimental Organism</td>
<td>1</td>
</tr>
<tr>
<td>Microtubules</td>
<td>2</td>
</tr>
<tr>
<td>Colchicine</td>
<td>15</td>
</tr>
<tr>
<td>Purpose of the Present Investigation</td>
<td>22</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>23</td>
</tr>
<tr>
<td>Culturing Procedures</td>
<td>23</td>
</tr>
<tr>
<td>Cell Harvesting and Gametic Induction</td>
<td>24</td>
</tr>
<tr>
<td>Pairing Percentage</td>
<td>25</td>
</tr>
<tr>
<td>Procedures for Electron Microscopy</td>
<td>25</td>
</tr>
<tr>
<td>Colchicine Treatment</td>
<td>27</td>
</tr>
<tr>
<td>RESULTS</td>
<td>29</td>
</tr>
<tr>
<td>Effect of Increasing Concentrations of Colchicine on ( Chlamydomonas ) Mating Reaction</td>
<td>29</td>
</tr>
<tr>
<td>Further Quantification of the Effects of ( 5 \text{ mM} ) Colchicine on Overall Mating Efficiency</td>
<td>31</td>
</tr>
<tr>
<td>Light Microscope Analysis of Mating</td>
<td>33</td>
</tr>
<tr>
<td>Analysis of the Plasma Papillar Area by Whole Mount Electron Microscopy</td>
<td>36</td>
</tr>
<tr>
<td>Examination of Thin Sectioned Material</td>
<td>37</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>40</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>49</td>
</tr>
<tr>
<td>FIGURES</td>
<td>63</td>
</tr>
</tbody>
</table>

iv
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Colchicine and Principal Derivatives (adapted from Dustin, 1978)</td>
<td>18</td>
</tr>
<tr>
<td>2. The Effect of Colchicine on Motility and Agglutination</td>
<td>30</td>
</tr>
<tr>
<td>3. The Effect of Colchicine on Pairing</td>
<td>32</td>
</tr>
<tr>
<td>4. Electron Microscope Quantification of Papilla Activation in Control and Colchicine-treated Gametes</td>
<td>39</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>1</td>
<td>Whole mount preparation of mixed control gamete</td>
</tr>
<tr>
<td>2</td>
<td>Thin section of mixed control gamete</td>
</tr>
<tr>
<td>3</td>
<td>Whole mount preparation of fused pair</td>
</tr>
<tr>
<td>4</td>
<td>Thin section of cytoplasmic bridge in fused pair</td>
</tr>
<tr>
<td>5</td>
<td>Whole mount preparation of unmixed control gamete</td>
</tr>
<tr>
<td>6</td>
<td>Whole mount preparation of colchicine-treated mixed gamete</td>
</tr>
<tr>
<td>7</td>
<td>Thin section of papilla area of mixed control gamete</td>
</tr>
<tr>
<td>8</td>
<td>Thin section of papilla area of mixed control gamete</td>
</tr>
<tr>
<td>9</td>
<td>Proposed model for the colchicine-labile system responsible for tipping</td>
</tr>
</tbody>
</table>
The effect of colchicine on the mating reaction in *Chlamydomonas moewusii* was investigated. Five mM colchicine was found to cause a 96% inhibition of gamete fusion, without disrupting the preceding agglutination reaction or cell motility. Electron microscope examination of colchicine-treated gametes revealed a 98% inhibition of mating structure activation, suggesting that the drug had rendered the gametes incapable of generating and/or responding to the signal for mating structure activation which normally accompanies sexual agglutination. Light microscope analysis revealed that flagella of adhering drug-treated cells entwined loosely so that treated gametes were unable to establish the flagella tip alignment, and contact between partners' anteriors which facilitates cell fusion in control gametes. The misalignment of colchicine-treated cells is thought to result from a drug induced disruption of the tipping reaction. A model for the colchicine-labile system responsible for tipping is proposed.
LITERATURE REVIEW

The Experimental Organism

Chlamydomonas moewusii is a unicellular bi-flagellated green alga. The two mating types of Chlamydomonas, designated (+) and (-), can be maintained separately in liquid media as motile vegetative cells. The vegetative cells reproduce asexually and have no affinity for each other. Upon transfer to a medium that is low in nitrogen and other salts, the vegetative cells differentiate, without apparent morphological change, into gametes (Trainor, 1959). When (+) and (-) gametes are mixed, they adhere to one another via mating type specific flagellar surface agglutinins. This adhesion of gametes results in agglutination of sexually activated cells into vibrating clusters. Within the clusters, pairs of opposite type gametes move their agglutinins out to their respective flagellar tips. This tipping response brings the anterior cell surfaces into close proximity (Goodenough & Jurivich, 1978). The cells then fuse at the anterior, their flagella disadhere, and the (+) member of the pair resumes flagellar beating. In several hours plasmogamy ensues and a non-motile zygote results (Triemer & Brown, 1975).

Thus, sexual reproduction in Chlamydomonas involves two distinct processes: agglutination and pairing. These have been shown to be genetically separate events (Forest, Goodenough & Goodenough, 1978).
Agglutination, the gametic recognition stage, always preceding pairing, the gametic fusion stage (Weiss, Goodenough & Goodenough, 1977).

Microtubules

Following the introduction of glutaraldehyde as an electron microscopy fixative the ubiquity of microtubules became apparent. In 1963 Slatterback described their occurrence in Hydra and Ledbetter and Porter their distribution in plant cells.

Since that time microtubules have been found in every eucaryote cell in which they were sought and our knowledge of them has increased exponentially.

The Ubiquitous Microtubule

Microtubules are intimately involved in the development and determination of cell shape. They function in elongation of entire cells and in extension of cytoplasmic projections (Tilney, 1963). Microtubules run parallel to the long axis of such cellular extensions as the axons and dendrites of neuronal cells (Wuerker & Kirkpatrick, 1972), dendritic processes of melanophores (Schmitt & Samson, 1968), and the axopodia of various protozoa (Tilney & Porter, 1966). The only known exceptions to the association of microtubules with extensive cell shape changes occur in systems such as the acrosomal filaments of echinoderm sperm (Tinley, 1971) and microvilli of intestinal brush border cells (Tinley & Moosieker, 1971); where the cytoplasmic projections lack microtubules, but rather contain bundles of parallel actin filaments.
The path of moving cytoplasmic components often parallels the course of cytoplasmic microtubules. Disruption of microtubules stops the movement of chromosomes (Inoue & Sato, 1967), mitochondria (Holmes & Choppin, 1968), liposomes, pigment granules (Freed & Lebowitz, 1970) and ribosomes (Rebhun, 1972), for example. Whether microtubules are serving as tracts for transport or are actually supplying motive force is as yet unclear. Centrioles and basal bodies are characterized by their distinctive 9 + 0 arrangement of microtubules. The fibrous framework of the meiotic and mitotic spindle, too, is composed of microtubules (Fulton, 1971).

Microtubules make up the familiar 9 + 2 arrangement of cilia and flagella. Here the protein dynein is found between adjacent outer doublet fibers. Dynein, an ATPase, mediates sliding between adjacent outer doublet fibers, and is responsible for motility (Satir, 1974).

Closely packed longitudinally oriented microtubules are found in connective tissue and muscle insertions. In the extra cellular fibers of vertebrates, microtubules are thought to orient the myofibrils in developing skeletal muscle (Warren, 1968). Microtubules also function as orienting agents in the formation of orthogonal arrays of collagen fibers under embryonic rat epidermus (Branson, 1968), and in cellulose microfibrils in developing plan cell walls (Stein & Stein, 1973).

Microtubules are involved in the mobilization of material for secretion. Colchicine treatment blocks the release of catecholamine (Malawista, 1975), histamine (Poisner & Cooke, 1975), and insulin (Malaisse et al., 1975).
Many types of sensory receptors are modified cilia, and thus microtubules often appear at the site of sensory transduction. For example, when cockroach mechanoreceptors are disrupted with colchicine, action potentials are no longer generated in receptor neurons (Moran et al., 1971). However, the mechanism by which microtubules are involved in sensory transduction remains unclear.

Studies in the area of cell surface organization have revealed some exciting data concerning microtubules. Yahara and Elderman (1975), working with surface changes of lymphocytes, have suggested that an integrated microtubular-microfilament system of surface receptors mediate the responses of the cell to external ligands, including other cells.

It has been established that transformed cells differ in agglutinability by lectins and other multivalent ligands, as well as in surface receptor distribution from their normal counterparts (Puck, 1977). These changes can be induced by colchicine, vinblastine, or colcemid and suggest that microtubules are involved in the expression of surface function perhaps by maintaining or promoting the appropriate distribution of membrane components (Puck, 1972). Microcinematographic experiments reveal that microtubules play a role in mechanical stabilization of cell surfaces (Vasiliev et al., 1970).

The exact nature of microtubule involvement in cell surface organization and the mechanism for coordination of membrane alterations with nuclear events is not yet clear. However, this is an area of research rich in potential for enhancing our understanding of membrane function, and germane to current issues in cancer research.
The above survey suggests that the variety of functions associated with microtubules includes:

1. Cell shape determination
2. Motility
3. Cytoplasmic transport
4. Orientation of cellular structures
5. Sensory transduction
6. Secretion
7. Chromosome movements in cell division
8. Cell surface organization

It would seem appropriate now to turn our attention to the structural characteristics of these versatile organelles in an effort to understand the structure which underlies the microtubule's functional diversity.

**Microtubule Structure**

Microtubules observed with the electron microscope generally appear to be hollow tubular structures about 24 nm in diameter (Amos, 1975). Their walls are made of discrete globular subunits arranged in longitudinal rows or protofilaments.

Under conditions of negative staining incomplete microtubule walls appear as flat sheets of 13 protofilaments. In the assembled microtubule these sheets roll up to form a cylinder with the protofilaments oriented parallel to the long axis of the tubule forming its wall (Erickson, 1974).

The globular subunits of the protofilaments are composed of a tubulin dimer. The constituent molecules of the dimer are distinguished as alpha and beta tubulin. They are of identical molecular weight (approximately 54,000 daltons), but differ slightly in amino acid composition (Eipper, 1974). The tubulin dimer is characterized by a
sedimentation coefficient of $S_{20}^{0}$, $W = 6S$ (Bryan, 1972; Marcum & Borisy, 1974). The dimer is approximately 8 nm long. The substituent protein monomers are each 4 nm long, and split by a longitudinal cleft into two identical lobes (Epper, 1974). In assembled microtubules, they occur as a dumb-bell shaped heterodimer (Amos & Klug, 1974).

Integrating information available from electron microscopy with that from optical and computer processing techniques for image reconstruction, the surface lattice of the prototypical microtubule can be described:

The intact microtubule consists of 13 protofilaments 5 nm apart, rolled up to form a cylinder. The 4 nm monomeric components of the 6S globular subunit, alternate and project in and outwards along radii from the tubule axis forming a 3 start left handed helix composed of the 4 x 5 nm unit cells. (Erickson, 1974; Amos, 1975; Amos & Klug, 1974)

The angle formed by the oblique line between subunits in adjacent protofilaments is 10°. Thus, following the circuit of 13 protofilaments around the microtubule the oblique line would meet the third monomeric unit up. While a 3 start helix can be construed through the individual monomers the staggering of adjacent protofilaments results in an arrangement of alpha-beta dimers which can be viewed as a 5 start right handed helix or an 8 start left handed helix (Erickson, 1974). Owing to the staggering of dimers, the lateral bonds in the tubule are all between alpha and beta units (Marcum & Borisy, 1974; Chasey, 1974).

Under physiological conditions, microtubules generally dissociate into dimers. This indicates that the intradimer bond is stronger than that between adjacent dimers. Under conditions of dissociation,
such as vinblastine for neurotubules and sonication for flagellar tubules, microtubules split into protofilaments. This split supports the suggestion that the intradimer bond occurs within a protofilament (Warfield & Bouck, 1974; Kirschner et al, 1974).

Much of the information concerning microtubule structure comes from studies of cilia and flagella outer doublet microtubules, as they are more stable than cytoplasmic microtubules. However, the surface lattice of single cytoplasmic microtubules and neurotubules are reportedly quite similar to that of the flagellar outer doublet (Erickson, 1974). The outer doublet studies seem to have arrived at a good general model of the microtubule.

**Variability Among Microtubules**

Electron microscopic observations coupled with studies on differential stability among microtubules suggest that, on some level, classes of tubules do exist. In early characterization work, it was noted that ciliary and flagellar tubules were preserved by osmium tetroxide, while cytoplasmic microtubules were not (Tilney & Gibbons, 1968).

Phillips (1966) noted, in spermatids of the fungus gnat Sciara, that axial tubules were initially identical in fixation characteristics to cytoplasmic microtubules, and over the course of spermatogenesis, gradually acquired the fixation characteristics of flagellar tubules. This finding suggests that alterations in microtubules, apart from assembly and disassembly, can be affected in response to changing cell needs. This observation also supports the notion that
differential stability is due to physical and/or chemical differences between microtubules, and is not simply the result of their intracellular locations.

Behnke and Förer (1968) proposed that four classes of microtubules exist. These classes of tubules, based on fixation characteristics and resistance to antimicrotubular agents, in order of increasing stability are:

1. Cytoplasmic microtubules
2. Accessory and central tubules (of the 9 + 2 complex)
3. B tubules
4. A tubules

Comparative morphology also reveals differences between microtubules. For example, a shared common wall appears in outer doublets (Ringo, 1967) and centrioles (Fulton, 1971); dynein arms and spokes are seen attached to axonemal microtubules; filaments and arms are also observed on neurotubules (Daniels, 1972); intermicrotubular linkages are seen as in cilia and flagella (Warner & Satir, 1973); a helical, rather than a straight cylindrical formation has been found in developing spermatocyte microtubules (McIntosh & Porter, 1967).

Most of the morphological variability observed arises from proteins and other materials associated with the microtubule or intermicrotubular linkages, rather than from gross alterations in the microtubule basic structure or surface lattice. Studies on the chemistry of microtubules indicate that the basic subunit is essentially the same for microtubules isolated from a variety of sources (Olmsted, 1976). The differences in stability between microtubules may arise from:
1. subtle subunit modification,
2. association of protein and other material with tubules,
3. intermicrotubular links,
4. local cytoplasmic influences.

**Microtubule Assembly**

Microtubules are a self-assembling protein polymer. The most widely accepted theory of tubule assembly holds that microtubules exist in a state of dynamic equilibrium between the polymeric or intact tubule state, and a disassembled pool of subunits (Stephens, 1973; Borisy et al., 1974; Inoué, 1964). Microtubule inhibitors act by binding to the tubulin subunits and shifting the equilibrium to the dissociated state. Low temperature and high pressure also drive the equilibrium toward depolymerization, presumably by enhancing the affinity between free subunits and their bound water (Marshland et al., 1971). In contrast D$_2$O has a stabilizing effect on microtubules; the weaker electrostatic fields of the D$_2$O molecules facilitate their dispersal from subunits and favor polymerization (Marshland et al., 1971).

In an effort to characterize the overall equilibrium for microtubule assembly, Johnson and Borisy (1975) undertook experiments to identify the protein species present at equilibrium, and their relative concentrations. Sedimentation velocity analysis of the equilibrium mixture revealed two components: A rapidly sedimenting species (>300S) corresponding to the microtubule polymer and a slowly sedimenting species (6S) corresponding to the tubulin dimer. The 6S tubulin dimer is the structural subunit with respect to polymerization.
An analysis of the relative concentrations of polymer and subunit, as a function of total protein concentration, revealed a critical concentration (0.2 mg/ml) below which no polymerization was observed. Above this concentration the amount of polymer formed was proportional to the total protein concentration. The transition towards polymerization was abrupt (Johnson & Borisy, 1975). This process resembles a phase transition, such as the condensation of a gas. It is referred to in the literature as condensation polymerization, a term coined by Oosawa and Kasai (1962) to describe actin polymerization.

In the condensation reaction the polymerizing unit S adds to the polymer, reducing the concentration of S and increasing the concentration of polymer M. The subunit concentration at equilibrium is independent of polymer concentration. The rate at which equilibrium is approached is a direct function of [S] (Johnson & Borisy, 1975; Oosawa & Kosai, 1962). The reaction can be summarized by the following:

\[ M_n + S \xrightleftharpoons[k_2]{-k_1} M_{n+1} \]

\[ K_e = \frac{1}{[S]} \]

The reaction obeys first-order kinetics, the dimers being added one by one. The rate constants \( k_2 \) and \( -k_1 \) are independent of tube length (Johnson & Borisy, 1975). The equilibrium constant is solely a function of the subunit concentration \( K_e = \frac{1}{[S]} \). Thus, as with a phase transition, the equilibrium concentration of subunits is independent of the mass and arrangement of the material in the condensed phase (Borisy et al, 1976).
The microtubule, then, will grow as long as the subunit concentration is high enough to maintain the probability of a subunit adding to the polymer (forward reaction) above the probability of a subunit leaving the polymer.

Equilibrium occurs when the two reactions are of equal probability, or when the subunit concentration equals the reciprocal of the equilibrium constant for the elongation reaction, \([S] = \frac{1}{K_e}\).

The subunit concentration at equilibrium can be viewed as a measure of the free energy change associated with the addition of \(S\) to the polymer (Warner; 1972). The free energy change for the addition of \(S\) to the polymer exceeds that for the association of subunits into an oligomeric form which could serve as a nucleating center for a new microtubule (Borisy et al, 1976). Thus, elongation of existing microtubules is the process preferred over the formation of new nucleating centers. How then might assembly begin?

**Initiating of Microtubule Assembly**

Microtubule assembly can be initiated in vivo on microtubule containing organelles, such as flagellar basal bodies or on the densely staining amorphous structures called microtubule organizing centers (MTOCs) (Snell et al, 1974). MTOCs are most often associated with other cellular organelles; in the nuclear membrane, they form kinetochores (Byers & Goetsch, 1974), and in centrioles MTOCs appear as the centriolar satellites (Bernhard & de Harven, 1960).

These microtubule containing organizing centers and amorphous MTOCs are thought to be involved in cell division and differentiation.
As methods for the in vitro assembly of microtubules became available, the parameters of this process were the subject of numerous investigations (Witman, 1975; Caskin et al., 1975; Olmstead & Boris, 1975). In vitro microtubule assembly was found to proceed optimally at neutral pH (6.9), moderate ionic strength (0.1 M PIPES), physiological temperature (37°C), high protein concentration, and require GTP (Olmstead & Boris, 1975).

Concentrations of the monovalent cations Na⁺ or K⁺ above 240 mM suppress polymerization; maximal assembly occurs when these cations are present at physiological concentrations (150 mM). Divalent ions, especially Mg²⁺, are important for polymerization; although sub-stoichiometric (with respect to tubulin) amounts of either Mg²⁺ or Ca²⁺ will facilitate assembly. As with the monovalent cations, high concentrations of divalent cations inhibit microtubule assembly (Olmstead & Boris, 1975). Maximal polymerization is obtained when the concentration of GTP is at least equal to that of the 6S dimer, and the same as the concentration of Mg²⁺. Olmstead and Boris (1975) suggest that one molecule of Mg²⁺ is tightly bound to the 6S dimer, and that a second Mg²⁺ may occur as a bound, but readily exchangeable, species. It may be, however, that a Mg²⁺-GTP complex is formed, which is tightly bound to the tubulin at some sites, and freely exchangeable at others (Olmstead & Boris, 1975). One study suggests the GTP binds to the 6S dimer and induces a structural change, which renders the subunit capable of assembly (Caskin, 1975).
The in vitro polymerization process involves two distinct phases: nucleation and elongation. The elongation phase is characterized by the addition (or loss, as the equilibrium may dictate) of the 6S tubulin dimer to the incipient microtubule (Borisy et al., 1976). The events and structures involved in nucleation are less well understood.

Under depolymerizing conditions, two forms of tubulin oligomers of sedimentation coefficients $S_{20,w}^0 = 18.6S$ and $30.6S$ respectively, appear in addition to the 6S dimer (Borisy et al., 1976). The relative proportions of 18S and 30S species obtained varies with the pH and ionic strength; an increase in either favors the 18S species. Electron microscope studies have identified the 30S oligomer as a 34nm ring. The 18S component is thought to be of equal diameter, suggesting that they occur as single (18S) and double (30S) ring structures (Rosenbaum et al., 1975; Olmstead et al., 1974).

Sedimentation of these rings from depolymerized microtubules prevents repolymerization. The dependence of microtubule assembly on the presence of these rings led to the proposal that they act as nucleation centers for the polymerization of the tubulin dimer (Murphy & Borisy, 1975). The exact role of these rings in nucleation is not known. What is known is that: (1) these rings exist, along with the smaller amounts of short helical stacks of open rings, in depolymerized microtubule samples (Erickson, 1974), (2) they occur in association with high molecular weight non-tubulin proteins, (3) after the formation of a nucleus or short microtubule segment, these rings
rapidly disappear, leaving the microtubules to elongate through the addition of 6S subunits (Olmstead et al., 1974).

The oligomers cannot serve as direct templates for the microtubules, as the reported diameter for both the rings and the short helical stacks exceeds that of the microtubule. Any one-to-one correspondence between assembled microtubules and ring forms is ruled out for two reasons: (1) the rings "disappear" (break down) before the final number of microtubules appear, and (2) the number of rings is much smaller than the number of microtubules formed (Borisy et al., 1975).

The assembly scheme most consistent with the available data proposes that the role of the oligomer is to uncoil, and in so doing, provide a number of tubulin strands, which associate to form a short microtubule segment upon which the 6S dimers condense (Borisy et al., 1975).

It would seem that the initiation complex need not have the same configuration as the intact microtubule. Perhaps the disassembly of the rings provides some minimum number of subunits necessary for seeding the addition of the 6S dimers, which polymerize and ultimately assume the form of the intact microtubule.

Electron microscope studies by Erickson (1974) have revealed flattened sheets with 13 protofilaments, as well as incomplete walls with attached rings which seem continuous with the protofilaments. As Erickson (1974) suggests, either the flattened sheets or the incomplete walls could be intermediate forms in microtubule assembly.
Finally, since elongation studies indicate that microtubules elongate by consecutive additions of 6S subunits to the tubule end (Borisy et al., 1978), it would seem that the microtubule segments, regardless of their origin, rapidly assume the conformation of the intact tubule, and begin to elongate by the addition of dimers.

Colchicine

Source and Historical Perspective

Colchicine is an alkaloid isolated from the seeds and corms of the autumn crocus Colchicum autumnale and related members of the lily family. Colchicum is a northern hemisphere plant found primarily in the Mediterranean region (Eigsti & Dustin, 1955). Its history extends back to ancient Greece, India, and Egypt. Colchicum is most likely one of the saffron plants mentioned in the Ebers Papyrus, our oldest medical text. The papyrus, written in 1550 BC, instructed doctors to administer the seeds from saffron plants to relieve aches and pains (Eigsti & Dustin, 1955). Extracts of Colchicum were also used in the 17th and 18th centuries to allay joint pains. Today colchicine is considered to be one of the most specific drugs for alleviating the pain of acute gout (Rodnan & Benédek, 1970).

Modern work on colchicine began in the 1920s in the Laboratory of Pathology of Brussels. Albert Dustin, investigating the regulation of mitosis, gave mice small doses of colchicine and noted considerable increase in mitotic figures in all germinative regions; although Dustin did note spindle abnormalities, he thought his work demonstrated that colchicine stimulated mitotic activity. In 1937 it was demonstrated
that colchicine could be used to produce polyploid plants (Blakeslee & Avery, 1937). To this day colchicine remains a major tool for producing the polyploid plants used in agriculture for crop improvement (Dustin, 1978).

In 1955 Bigsti and Dustin published a classic monograph on colchicine. At this time the action of colchicine on mitosis was thought to be its most (if not only) significant effect. Little attention was paid to the possible effects of colchicine on intermitotic cells. The few effects of the drug—not directly related to mitotic arrest mentioned in the monograph included: changes in plant cell walls, central nervous system toxicity, and the therapeutic action in gout. The cause of these changes was, in 1955, still a mystery.

Radioactive Colchicine and the Discovery of Tubulin

In the 1960s Malawista (Malawista, 1965; Malawista et al., 1968) suggested that the various actions of colchicine might be due to a decrease in cytoplasmic viscosity, possibly caused by the destruction of an organized, labile, fibrillar system concerned with cell structure and movements. With the introduction of tritium labelled colchicine (Taylor, 1965; Wilson & Friedkin, 1966), the components of this labile fibrillar system were isolated and characterized. Borisy and Taylor (1967) went on to show that after homogenization the macromolecule which bound colchicine appeared in the soluble fraction of the cell and had a sedimentation constant of 6S. They found colchicine binding activity in a variety of cells and suggested that the binding was correlated with the presence of microtubules. The first direct
evidence that microtubules contained the colchicine receptor was obtained in 1967 when Shelanski and Taylor noted the disappearance of the central part of sperm tail microtubules and the concomitant appearance of tubulin in soluble form.

In 1970 Kirkpatrick et al. revealed that the protein subunits of solubilized microtubules were similar to those of the colchicine receptor. Shortly thereafter Wilson and Meza (1972) demonstrated that the colchicine receptor could be recovered from sperm tail outer doublet microtubules. Previous attempts to demonstrate colchicine binding by outer doublet subunits had been unsuccessful. Wilson and Meza were able to retain colchicine binding because, in contrast to their predecessors, they were able to solubilize the outer doublets without inactivating the protein subunits. Thus, by 1972 it was well established that the protein tubulin, present in the axenome the cytoplasm of interphase cells and in the mitotic apparatus, was the colchicine receptor.

Colchicine Structure Chemistry and Derivatives

Colchicine is a three-ringed tropolone derivative. Ring A is an aromatic ring with 3 methoxy groups. Ring B is a twisted 7 carbon ring bearing a substituted amino group. Ring C is a 7 carbon tropolone ring with one oxygen and one methoxy group (Dustin, 1978). The principal derivatives of colchicine of interest as microtubule poisons are listed in Table 1.
Table 1
Colchicine and Principal Derivatives
(adapted from Dustin, 1978)

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<th>$R^2$</th>
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<td>S - CH$_3$</td>
<td>thiocolchicine</td>
</tr>
<tr>
<td>CO - CH$_2$ - F</td>
<td>S - CH$_3$</td>
<td>fluorthiocolchicine</td>
</tr>
<tr>
<td>CO - CH$_2$ - Cl</td>
<td>S - CH$_3$</td>
<td>chlorothiocolchicine</td>
</tr>
<tr>
<td>CO - CH$_2$ - F</td>
<td>0 - CH$_3$</td>
<td>N-fluoro-desacetylcolchicine</td>
</tr>
<tr>
<td>CO - CH$_3$</td>
<td>OH</td>
<td>colchicine</td>
</tr>
<tr>
<td>CH$_2$-CHCl-CN</td>
<td>0 - CH$_3$</td>
<td>chlorocyanoethyl derivative of N-desacetyl-colchicine</td>
</tr>
<tr>
<td>H</td>
<td>0 - CH$_3$</td>
<td>trimethylcolchicinic acid</td>
</tr>
<tr>
<td>CO-CH$_2$-Br</td>
<td>0 - CH$_3$</td>
<td>bromocolchicine</td>
</tr>
</tbody>
</table>
In a review of antimitotic agents, Dustin (1963) concluded that the following structures were required for activity:

- at least one methoxy group on Ring A
- a 7-membered ring C.

Desacetyl-N-methyl-colchicine (colcemid), a microtubule poison used chemotherapeutically, acts in essentially the same manner as colchicine. X-ray analyses of colchicine, colcemid, and other active colchicine derivatives (Margulins, 1974, 1975), confirm the planarity of the A ring and twisted conformation of ring B, suggesting that the specificity of these compounds' action on tubulin might be explained sterically. Margulins (1975) proposed that the methoxy groups on ring A provide specificity in colchicine and its derivatives by regulating the accessibility of this ring. Accessibility of the benzenoid ring could be regulated by the methoxy groups either blocking the A ring until it reached its binding site and then turning to allow binding, or the methoxy groups could remain rigid and allow access to the ring in some (active) compounds and not in others (in active compounds) (Margulis, 1975).

The substituent groups on ring C are also important determinants of compound activity. Colchicine lacks a methoxy group on ring C and is inactive. In iso-colchicine the positions of the oxy and methoxy groups on ring C are inverted and the compound's antimitotic action is 100 times less than that of colchicine (Eigsti & Dustin, 1955).
The Colchicine Binding Reaction

The binding reaction between colchicine and tubulin has the properties of a bimolecular reaction (Wilson, 1970).

$$\text{colchicine} + \text{tubulin} \rightarrow \text{colchicine - tubulin complex}$$

The colchicine - tubulin bond is noncovalent; chemically unaltered colchicine can be recovered from the complex by extraction (Wilson & Friedkin, 1967). Colchicine binds stoichiometrically to tubulin. Each dimer of tubulin has a single colchicine binding site (Bryan, 1972; Wilson & Mezo, 1973).

The rate of the in vitro colchicine binding reaction is strongly temperature dependent. A 0°C the binding of colchicine to tubulin proceeds so slowly that no binding can be detected with most tubulins (Borisy & Taylor, 1967). Binding activity is detectable at 10°C and proceeds optimally between 20-40°C; the exact optimum temperature varies with the source and method used to isolate the tubulin (Wilson, 1974).

Colchicine forms a very tight complex with tubulin. Wilson (1970) found that after six hours at 37°C only 12% of unlabelled colchicine had been exchanged from the complex for labelled colchicine.

The colchicine binding reaction is not affected by pH between pH 5.5 and 8.5, or by variations in ionic strength between 100-500 mM. This indicates that the interaction between tubulin and colchicine is not electrostatic. The thermodynamics of the binding reaction between colchicine and tubulin have been determined. The reaction is characterized by a positive enthalpy, a positive entropy, and relatively
large free energy change (Bryan, 1972). Considering the data above, it seems likely that colchicine binds to tubulin in a hydrophobic or nonpolar pocket (Wilson & Bryan, 1974).

The Colchicine Binding Site in Assembled Microtubules

In the foregoing discussion of microtubule structure and assembly and the colchicine binding reaction we have considered that:

1. Colchicine prevents microtubule assembly by binding to soluble tubulin (Borisy et al., 1974).

2. With respect to colchicine disruption, at least four classes of microtubules exist; cytoplasmic microtubules, the most labile, represent one end of the spectrum, and the colchicine resistant microtubules of the axoneme, the other (Behnke & Forer, 1968).

3. Axoneme tubulin has essentially the same colchicine binding characteristics as tubulin isolated from labile microtubules. Wilson and Meza (1973) have shown that there are no colchicine binding sites on the surface of intact microtubules.

Considered as a whole, these findings suggest that the colchicine binding site is one of the protein interaction sites of tubulin; once formed the colchicine - tubulin complex is incapable of associating with a second tubule molecule (Wilson & Meza, 1973). This model explains colchicine sensitivity in terms of microtubule turnover rate. In the case of the mitotic apparatus, or cytoplasmic microtubules, which are rapidly assembled and disassembled, (existing in a state of dynamic equilibrium which can be represented as:

\[ S + mt \xrightarrow{N} Mt_N + 1 \] colchicine would bind to the subunits and
shift the equilibrium towards depolymerization. In the case of the axoneme, where microtubules do not exist in a state of equilibrium, the binding of colchicine to tubulin subunits in the cytoplasm, would not disassemble the axoneme. Overall, the action of colchicine in vivo has complex dependency on the rate of colchicine binding and the rate of depolymerization of existing microtubules.

**Purpose of the Present Investigation**

The present investigation examines the effect of colchicine on sexual reproduction in *Chlamydomonas moewusii*. The experiments described focus on two central and related questions. Specifically:

1. Can colchicine inhibit agglutination, or pairing, or both?

2. Does the action of colchicine suggest the participation of microtubules, or other tubulin containing molecules, in the mating reaction of *C moewusii*?
MATERIALS AND METHODS

Culturing Procedures

*Chlamydomonas moewusii* (Syngen I, 96 and 97 UTEX) was grown axenically under continuous illumination of 200-300 ft-c. for two weeks in Bristols Medium (Bold, 1949). The composition of Bristols Medium was as follows:

- Distilled H$_2$O: 1000 ml
- NaNO$_3$: 0.25 g
- CaCl$_2$: 0.025 g
- MgSO$_2$ 7H$_2$O: 0.075 g
- K$_2$HPO$_4$: 0.075 g
- KH$_2$PO$_4$: 0.175 g
- NaCl: 0.025 g
- 1% FeCl$_3$: 0.001 g
- Trace elements: 1.0 ml

The composition of the trace element solution (Anonymous, 1971) was as follows:

- Distilled H$_2$O: 1000 ml
- H$_3$BO$_3$: 0.618 g
- MnCl$_2$: 0.880 g
- ZnCl$_2$: 0.109 g
- CoCl$_2$ · 6H$_2$O: 0.044 g
- Na$_2$MoO$_4$ · 6H$_2$O: 0.024 g
- CuCl$_2$: 0.03 mg
- NaEDTA · 2H$_2$O: 7.44 g
Cells were then transferred to agar plates containing Bristols Medium solidified with 1.3% bacteriologic grade agar. Agar plates were grown under 400 ft.c light intensity on a 16/8 hr. light/dark cycle for 5-9 days. Cultures were maintained by transferring 5 ml of the two-week old liquid cultures to 150 ml of fresh Bristols Medium. All media were sterile and transfers were made using aseptic technique.

Cell Harvesting and Gametic Induction

The 5-9 day old plates were flooded with 50% soil extract (SE) in distilled water. A sterile, glass microscope slide was used to scrape cells from plates into a sterile glass petri dish. Cells were left for 15-18 hours in the dark, followed by 30 minutes in light of 200 ft.c. Mating ability was checked by mixing a drop of each mating type and observing agglutination under a dissecting microscope.

The soil extract was prepared as follows:

1. 1/2 inch of soil was placed in a 3000 ml Erlenmyer Flash and 2 liters of distilled water was added.

2. The mixture was autoclaved and allowed to settle for three days.

3. The mixture was filtered twice using Whatman No. 1 qualitative filter paper.

4. The filtrate was placed in 250 ml flasks and re autoclaved for storage.

The soil was obtained from the edge of a cornfield on East Avenue in Clarkson, New York.
Pairing Percentage

To assess pair formation, pairs and single cells were counted with a hemocytometer. The pairing percentage was calculated according to the following formula:

\[
\% \text{ pairs} = \frac{\# \text{ of pairs} \times 2}{\text{total cell count}} \times 100\%
\]

Procedures for Electron Microscopy

Preparation of Sectioned Material

Cells to be sectioned were prepared as follows:

1. Cells were pelleted by centrifugation at 710 rpm for five minutes in an I.E.C. International clinical centrifuge (model Cl, head #221).

2. The supernatant fraction was aspirated and the pellet embedded in 1.5% agar in induction medium (IM).

Induction Medium was composed of the following:

- distilled H₂O: 100 ml
- sodium citrate: 0.05 g
- CaCl₂ \cdot 2H₂O: 0.005 g
- MgSO₄ \cdot 7H₂O: 0.03 g
- K₂HPO₄: 0.72 g
- KH₂PO₄: 0.36 g
- FeCl₃: 0.001 g
- trace elements: 0.1 ml

pH adjusted to 7.4 ± 0.1

3. The agar block containing cells was cut into cubes 1 mm on a side.
(4) Cells were fixed at room temperature in 2% glutaraldehyde for two hours.

(5) Cells were rinsed in IM four times during the next 30 minutes.

(6) Cells were post-fixed in 2% OsO₄ with 1.8% K₄Fe(CN)₆.

(7) Cells were rinsed in IM four times during the next 30 minutes.

(8) Cells were dehydrated in an ethanol series, as follows:

- 35% ETOH .......................... 15 minutes
- 50% ETOH .......................... 15 minutes
- 70% ETOH .......................... overnight
- 95% ETOH .......................... 15 minutes
- 100% ETOH .......................... 15 minutes (2X)
- Acetone ............................ 15 minutes (2X)

(9) Cells were embedded in firm to hard Spurr low viscosity embedding medium (Spurr, 1969) according to the following infiltration series:

- Spurr: Acetone ............... 1:3 for 1 hour
- Spurr: Acetone ............... 3:1 for 1 hour
- 100% Spurr ..................... 1 hour

(10) Cells were placed in labelled BEEM capsules (Pelco Electron Microscope Supplies) and embedding plastic added.

(11) Capsules were left at room temperature overnight to complete the infiltration process.

(12) Embedding plastic was cured at 62°C for 18 hours.
Sectioning and Staining

Silver sections were cut on an MT-2B Porter-Blum ultramicrotome using a diamond knife. Sections (approximately 50nm thick) were collected on 300 mesh copper grids and post-stained with a saturated solution of uranyl acetate, (9.2 g/100 ml distilled deionized H$_2$O) for thirty minutes in the dark. Grids were then rinsed with distilled water and stained for 15 minutes in lead citrate. Sections were examined under a Hitachi HS-8 electron microscope.

Preparation of Whole Mounts

Cells were fixed for two hours in 2% gluteraldehyde in IM. After two hours cells were rinsed by centrifugation for four minutes at 420 rpm. The supernatant was aspirated and the cells resuspended in SE. This process was repeated for a total of three rinses. Cells were then stained by pelleting and resuspending them in a saturated solution of uranyl acetate. Cells remained in the uranyl acetate solution in the dark, for fifteen minutes. Cells were rinsed twice in SE and then dehydrated in an alcohol series. Following the last alcohol rinse cells were resuspended in amyl acetate. A drop of the cell suspension was placed on a formvar coated grid and examined under the electron microscope.

Colchicine Treatment

Preliminary Treatment

Initial experiments were designed to investigate the effects of colchicine at a variety of concentrations. Stock solutions of colchicine (Sigma Chemical Company) in distilled deionized water were
made in concentrations ranging from 0.2 mM to 20.0 mM. Colchicine was added to an equal volume of cells; the resultant drug concentration was half that of the stock solution.

**Experimental Phase**

In all experiments directly concerned with the effect of colchicine on pairing, the final concentration of the drug was 5.0 mM. Stock solutions of colchicine were made up in 50% soil extract in distilled water. The soil extract-distilled water solution was used because this combination proved the most reliable in inducing gametogenesis; preparation of the drug in the gametic induction medium avoided changes in either tonicity or nutrients available to the cells during drug incubation.

At the outset of all experiments cell concentration was obtained with the aid of a hemocytometer and adjusted to 1 x 10^6 cells/ml. An aliquot of 0.01M colchicine (experimental group) or 50% soil extract in distilled water (control group) was then added to an equal volume of cells, and incubated for three hours. Thus, the final cell concentration was 5 x 10^6 cells/ml and the colchicine concentration 5.0 mM. After three hours equal volumes of opposite mating type cells were mixed. Samples of cells were removed twenty minutes and two hours after gametes had been mixed. Each sample was divided into three portions: one was utilized immediately for light microscopic study, one was fixed and embedded for ultrastructural study, and one was fixed with 1% aqueous iodine for pair counts.
RESULTS

Effect of Increasing Concentrations of Colchicine on Chlamydomonas Mating Reaction

Sexual reproduction in Chlamydomonas depends upon successful recognition of opposite type gametes in the agglutination stage of the mating reaction, and their subsequent fusion in the pairing stage. Initial experiments were designed to determine the effect of increasing concentrations of colchicine on overall mating efficiency, which is represented by the pairing percentage, and to identify the stages of the mating reaction most sensitive to the drug. Gametes were incubated for three hours in one of five solutions ranging from 0.1 mM to 10 mM colchicine. After incubation gametes were observed with a dissecting microscope and scored for motility. Opposite type gametes were then mixed, and mating was allowed to proceed for five minutes. At this time the strength of the agglutination reaction was scored with the aid of a dissecting microscope. The results of these experiments shown in Table 2 reveal the concentrations of colchicine ranging from 0.1 mM to 5 mM had no overt effect on agglutination or motility. Groups incubated at these concentrations were identical to each other and to controls on both measures. These gametes swam vigorously and agglutinated upon mixing. Cells incubated in 10 mM colchicine, however, showed reduced motility and concomitant inhibition of agglutination.
Table 2
The Effect of Colchicine on Motility and Agglutination

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Motility</th>
<th>Agglutination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>0.1 mM</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>0.5 mM</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>1.0 mM</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>5.0 mM</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>10.0 mM</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

Cells were observed with a dissecting microscope at 30x. After scoring for motility, vigor, equal volumes of opposite type cells (5 x 10^6 cells/ml) were mixed, allowed to react for 5 min., and scored for degree of agglutination.

**Scoring**

+ weak
++ moderate
+++ strong
To determine the effect of the drug on overall mating efficiency, opposite type gametes were mixed and allowed to react for two hours. Cells were then fixed and pairing percentages determined. Table 3 shows the sensitivity of the cell fusion stage, of the Chlamydomonas mating reaction, to increasing concentrations of colchicine. At concentrations below 5 mM the drug did not significantly inhibit the establishment of vis-à-vis pairs; pairing percentages in control and the first three drug-tested groups are equivalent. Increasing the drug concentration to 5 mM produced a sharp and nearly complete inhibition of pair formation. This concentration falls within the range for anti-tubulin effects with Chlamydomonas (Flavín & Slaughter, 1974). It is also the concentration which was selected as most efficacious within the shortest period, and least lethal to Chlamydomonas maintained in colchicine for several weeks (Walne, 1966).

A comparison between Table 2 and Table 3 reveals that the adhesion and fusion stages of the mating reaction differ in sensitivity to colchicine. Specifically, 5 mM colchicine produced a 98% inhibition of pair formation, and had no visually detectable effect on the strength of the agglutination reaction.

Further Quantification of the Effects of 5 mM Colchicine on Overall Mating Efficiency

Having established that 5 mM colchicine inhibits cell fusion without effecting cell motility or the strength of the agglutination reaction, it became important to further quantify the drug’s inhibitory effect. A comparison of pairing percentages in controls and cells treated with
Table 3
The Effect of Colchicine on Pairing

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Mean Pairing (Percent of Controls)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 mM</td>
<td>99.3% (+ 15.4)</td>
</tr>
<tr>
<td>0.5 mM</td>
<td>89.5% (+ 10.8)</td>
</tr>
<tr>
<td>1.0 mM</td>
<td>75.3% (+ 20.7)</td>
</tr>
<tr>
<td>5.0 mM</td>
<td>1.7% (+ 2.0)</td>
</tr>
<tr>
<td>10.0 mM</td>
<td>0</td>
</tr>
</tbody>
</table>

Gametes were incubated in colchicine for 3 hours, then mixed, allowed to react for 2 hours, and fixed for determination of pairing percentage. Pairing percentages in Table are expressed as percent of control, and represent the mean of four experiments.

\[
\% \text{ pairs} = \frac{\# \text{ pairs} \times 2}{\text{Total Cell Count}} \times 100\%
\]

Mean pairing expressed as % of controls = \[\frac{\# \text{ pairs, drug group}}{\# \text{ pairs, control}} \times 100\%\]
5 mM colchicine, over the course of 15 experiments, revealed that 5 mM colchicine causes a 96% (± 3.8%) inhibition of cell fusion.

**Light Microscope Analysis of Mating**

A complex sequence of events occurs between agglutination and cell fusion:

Opposite mating type gametes initially adhere at random sites along the flagellar surfaces.

Initial adhesion is followed by the movement of agglutinins to the flagellar tips (tipping), and concomitant spiralization of the paired flagella, bringing the cells anterior surfaces into close proximity.

The plasma papilla, located at the anterior of each cell, begins to grow and ultimately protrudes through the cell wall.

The papilla of the mating partners meet and fuse to establish a vis-à-vis pair connected by a plasma bridge.

The flagella of the vis-à-vis pair cease to be adhesive, and the pair is released from the sexual cluster with their flagella unattached.

Extensive light microscopic examination of the mating process was undertaken to determine the differences in mating behavior, underlying the 96% difference in mating efficiency, between colchicine-treated and control groups.

**Mating Reaction in Control Group**

When opposite mating type control gametes are mixed they adhere to each other instantly, forming vibrating clusters. These clusters increase in size and density and within 2-3 minutes the agglutination reaction reaches its peak. At that time cells are aggregated in large, actively vibrating clusters. Three to five minutes after mixing, control cells begin to partner. In a flurry of activity, the partners' flagella entwine, bringing their anterior surfaces into close proximity. Partners continue to swim about, bobbing towards and away from each other, as if they
were tethered together by the distal ends of their flagella, and attempting to disengage. The flagella are still adhesive, and a third cell may adhere to the flagella of one partner and be pulled along by the pair. The third cell may also be exchanged for one of the partners.

At 8-20 minutes after mixing the agglutination reaction persists and clusters begin to release the first free swimming pairs. Examination of the cells under a phase contrast microscope reveals the primary events marking the transition from partnered cells agglutinated by their flagella to vis-a-vis pairs connected by a plasma bridge. First, the entwined flagella of the partners disadhere and the vigorous uncoordinated movement, typifying cells in the agglutination stage, ceases. Following this momentary quiescence, the pair begins to swim in a coordinated unidirectional manner. The flagella of the (+) partner beat, while the flagella of the (-) partner trail behind. Neither the active flagella of the (-) partner, nor the passive flagella of the (-) partner remain adhesive to other cells; the vis-a-vis pair does not adhere to the flagella of single or paired cells that they encounter.

When the gametes have been mixed for 30 minutes, approximately one-half of the population is found in vibrating clusters and one-half appear as free swimming pairs. At 60-120 minutes the reaction mixture consists almost exclusively of free swimming pairs; free swimming single cells are absent or exceedingly rare, and only a few small vibrating clusters persist.
The Mating Reaction in Cells Treated with 5 mM Colchicine

Like control cells, drug-treated gametes begin to agglutinate upon contact with the opposite mating type. Clustering becomes extensive and generally reaches a maximum in 3-8 minutes. When the agglutination reaction is unambiguously complete, (by 8 minutes after mixing gametes in all cases) clusters of drug-treated cell's appear less dense than those of the controls; clusters of the treated cells do not necessarily contain fewer cells than control clusters, but rather consist of a looser association of cells.

Fifteen to twenty minutes after gametes are mixed, apparent pairs begin to appear in the reaction mixture. These cells are, however, still in the recognition stage of the mating reaction. The flagella of both partners retain motility and adhesiveness. The cell bodies bounce towards and away from each other as the pair moves vigorously about in an uncoordinated manner. The partners' flagella can adhere to those of other paired or single cells, and one partner can be exchanged for another. At this stage in the reaction, colchicine-treated cells differ from controls with respect to the relationship of the partners' flagella. When drug-treated gametes attempt to pair, their flagella loosely entwine leaving gaps between them. In control cells, flagella spiral about each other tightly; they make contact along their entire length, bring cell anterior and flagellar tips into alignment. The alignment of cell anterior allows papilla contact and cell fusion. In colchicine-treated cells, contact between partners' flagella is too loose to establish contact between cell.
anteriors. Cell bodies jiggle and tilt laterally, preventing alignment. The net result is an anomalous persistence of the recognition stage.

Differences in Mating Behavior Between Groups

In summary, the most salient differences in mating behavior between colchicine-treated and control gametes are:

1. Persistence of agglutination (gamete recognition stage) in the treated group vs. establishment of vis-à-vis pairs in controls.

2. Loose entwining of flagella in treated cells vs. flagella spiralization and flagella tip alignment in controls.

3. Misalignment of partnered cells in the treated group vs. contact at the anteriors of control cells permitting cell fusion.

Analysis of the Plasma Papillar Area by Whole Mount Electron Microscopy

Results to this point have shown that gametes treated with 5 mM colchicine are capable of sexual agglutination but not cell fusion. The next question to be answered is where in the sequence of events that follow flagellar adhesion and lead to vis-à-vis pair formation does the drug act: Do colchicine-treated cells produce normal plasma papilla which are prevented from fusing by the misalignment of partnered gametes? Are treated cells producing aberrant papilla? Is colchicine preventing release of the lytic factor which dissolves the anterior cell wall, so that in treated cells, activated papilla remain within the cell oppressed to the undigested cell walls?

An analysis of the plasma papilla area by whole mount electron microscopy was undertaken to begin to answer some of these questions.
Control cells exposed to opposite type gametes and examined as whole mounts, either possessed protuberant papilla, or had fused to establish a vis-à-vis pairs. Figure 1 shows a typical gamete after sexual stimulation and before cell fusion. The outgrown papilla appears as an unambiguous structure between the cell's flagella. Figure 3 shows a pair of cells which have made papilla contact and fused. The cytoplasmic bridge between the partners is shorter and wider than the papilla itself (compare Figures 1 and 3).

Figure 5 shows a gamete which has not been exposed to the opposite mating type. The anterior of this cell is flat as papilla extension does not normally occur in the unmixed gamete. Illustrated in Figure 6 is a colchicine-treated gamete which has been exposed to the opposite mating type. This cell lacks papilla outgrowth and looks like a gamete from unmixed populations.

**Examination of Thin Sectioned Material**

Having established that colchicine-treated cells do not produce protuberant papilla in response to sexual agglutination, it became important to determine whether the drug had also blocked the events preceding papilla outgrowth.

Figures 7 and 8 show drug-treated cells which have been exposed to opposite mating types. Examination of the papilla region reveals no evidence of sexual stimulation. The papilla membrane is of normal density. Fibrillar and granular material lie beneath the papilla membrane and are concentrated above the connecting fiber. A dense granular region exists between the plasma membrane and the cell wall. The wall papilla is intact.
Figure 2 shows a control cell which has been exposed to opposite type gametes. This cell shows obvious papilla activation: There is a concentration of electron dense material on the cytoplasmic side of the papilla membrane. The granular region, which clusters above the central portion of the connecting fiber in the unstimulated state (Figure 9), has spread to fill the papilla. The granular region between the plasma papilla and the cell wall has dissipated. The cell wall above the papilla is beginning to be digested away. The now electron-dense plasma papilla elongates and extends towards the cell wall.

A Quantitative Comparison of Papilla Activation

Examination of thin sectioned material afforded the opportunity for a quantitative comparison of papilla activation. Since the mating structure is only infrequently included in thin section, 300 cells from each group were examined. The results summarized in Table 4 reveal 92% papilla activation in the controls, and 1.2% papilla activation in colchicine-treated cells. Colchicine caused a 98% inhibition of mating structure activation.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of Cells</th>
<th>Plasma Papilla Noted</th>
<th>Plasma Papilla Activated</th>
<th>Percent Activation</th>
<th>Percent Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>300</td>
<td>48</td>
<td>44</td>
<td>92%</td>
<td>--</td>
</tr>
<tr>
<td>5 mM colchicine</td>
<td>300</td>
<td>38</td>
<td>1</td>
<td>1.2%</td>
<td>98.7%</td>
</tr>
</tbody>
</table>

Opposite type gametes were mixed, allowed to react for 20 minutes, fixed, and prepared for thin sectioning.
DISCUSSION

Sexual reproduction in Chlamydomonas involves a highly coordinated, precisely controlled sequence of events. The mating reaction, initiated when (+) and (-) gametes are mixed together, can be subdivided into seven steps (Mesland et al., 1980):

1. Cells adhere to one another at random sites along their flagella.

2. Pairs of adhering cells move adhesion sites out to their respective flagellar tips (tipping) to bring the anterior cell surfaces into contact.

3. One of more signals are sent to the cell bodies.

4. Cells release lytic factors to digest papilla wall.

5. Plasma papilla become activated, increase in density, and grow out.

6. Outgrown papilla meet and fuse to create a narrow cytoplasmic bridge.

7. Flagella of the fused pair lose adhesiveness.

The foregoing experiments reveal that Chlamydomonas gametes treated with 5 mM colchicine are capable of sexual agglutination but not cell fusion. With respect to the seven step mating sequence proposed by Mesland et al. (1980), the drug could be acting to prevent: tipping, transduction of mating stimuli, papilla activation, papilla wall lysis, or papilla fusion. Electron microscope examination reveals
no evidence of a response to sexual agglutination in colchicine-treated gametes. The cells possessed neither the protuberant papilla bridge, nor the increase in papilla density, which normally follows sexual agglutination and precedes cell fusion. The papilla area of mixed colchicine-treated gametes was indistinguishable from that of unmixed controls; i.e., drug group papilla were morphologically normal for cells which had not undergone sexual agglutination. These findings show that colchicine acts as a blocking agent during the early stages of the mating reaction. Treated cells appear to have been rendered incapable of generating and/or responding to the signal for papilla activation that normally accompanies sexual agglutination.

It could be argued that at the concentration used, colchicine disrupts the mating reaction not by its antitubulin activity, but by altering the physiological status of the cells. Evidence from a number of sources suggests that this is not the case. Previous studies (reviewed by Hart and Sabnus, 1976) indicate that most plant cells are highly resistant to colchicine; for example, high concentrations of the drug are necessary to induce the antitubulin activity responsible for mitotic arrest and inhibition of flagella-regeneration.

A recent study on Chlamydomonas reinhardtii by Mesland et al. (1980), lends further support to the notion that colchicine disrupts the mating response by its specific interaction with tubulin. Mesland and co-workers (1980) found that vinblastine and colchicine prevented gamete fusion, while lumicolchicine did not. Lumicolchicine, an analog of colchicine, does not disrupt microtubules, but affects cellular process unrelated to microtubule disruption in the same manner as colchicine.
(Wilson et al., 1974). The effectiveness of two different antitubulin agents, and the ineffectiveness of lumicolchicine, is strong evidence that the effect of colchicine on the mating reaction is a result of its specific antitubulin activity.

Thus, colchicine's inhibition of cell fusion implicates labile microtubules or other tubulin-containing molecules as critical to coupling sexual agglutination with papilla activation. Since the papilla bridge is a microfilamentous structure (and hence not colchicine labile), and the morphology of the papilla area is not disturbed by colchicine treatment, it seems unlikely that the drug's effect is due to a disruption of critical mating structures.

A survey of the literature on sensory transduction offers an alternative explanation. Solter and Gibor (1977) have drawn an analogy between the flagella of Chlamydomonas gametes and the cilia of sensory cells. In ciliated mechanoreceptors and chemoreceptors, microtubules must remain intact for sensory transduction to occur. For example, antitubulin drugs block responses to pheromones in cockroaches (Block & Bell, 1974), and chemotaxis in granulocytes (Bandman et al., 1974), and protozoans (Levandowski et al., 1975). In blowfly taste hairs, colchicine and vinblastine destroy chemosensitivity by disrupting microtubules at the distal end of the receptor cell (Matsumato & Farley, 1978). The prominence of microtubules in sensory transduction suggests that in Chlamydomonas, too, microtubules may function in signal generation or reception.
Agglutination at the flagellar membrane triggers at least two events--papilla activation and papilla wall lysis. In some way the flagella 'acts as a sensory transducer; the stimulus, agglutination, initiates processes at the flagella, resulting in papilla activation at the cell body. The signal for papilla activation might be transmitted by the axoneme microtubules, the flagellar matrix, or by some combination of mechanisms (Mesland et al., 1978). In gametes incapable of generating or responding to such a signal, the mating reaction would be disrupted at a point between agglutination and cell fusion. It is highly unlikely that colchicine-induced inhibition of cell fusion results from the drug's action on the axoneme, as axoneme microtubules are colchicine resistant at this concentration and exposure (Behnke & Porre, 1967). Additional evidence against the involvement of the axoneme in signal transmission comes from work with Chlamydomonas mutants. Paralyzed mutants of Chlamydomonas reinhardii lacking central pair microtubules (Warren et al., 1966), or radial spokes (Witman et al., 1978), are capable of cell fusion.

Apparently, an intact axoneme is not necessary for signal transmission. Flagella length, on the other hand, is a critical factor. Gametes, deflagellated at the time of mixing, are unable to fuse until their flagella reach 50% of their original length (Solter & Gibor, 1977).

One of the consequences of a reduction in flagella length is a reduction in flagella surface area. Several studies suggest that flagella surface events are critical to sexual signaling (Mesland, 1976; Mesland & van den Ende, 1979; Goodenough & Jurvich, 1978).
When flagella of opposite type gametes make contact, interacting agglutinins are transported along the membrane to form clusters at the flagella tips (tipping). These clusters create a strong adhesiveness between mating partners (Goodenough & Jurvich, 1978). In addition to sexual agglutinins, antiflagella antisera (Goodenough & Jurvich, 1979) and other multivalent ligands (Mesland et al., 1980) are bound by normal gametes, to induce isoagglutination and tipping. Ligand and antibody tipping elicits papilla outgrowth in unmixed gametes. In vegetative cells and mutants incapable of tipping, antisera and other ligands do not elicit mating structure activation (Forest et al., 1978). These findings suggest that mating competence is in some way dependent on tipping ability.

Moving agglutinins to flagella tips enables adhering cells to become properly aligned for fusion. The present investigation revealed that colchicine-treated gametes adhere to each other at random points along their flagella, and never become properly aligned at their flagella tips or cell anteriors. The defective alignment in treated cells correlated with a loose and easily disrupted agglutination reaction. These observations suggest that colchicine inhibits the tipping reaction in treated gametes.

It has been proposed that tipping brings signaling components, dispersed in the flagella membrane, together in the flagella tips, and that when a sufficient concentration of these putative signaling components build up at the tips, a papilla-activating signal is generated (Goodenough & Jurvich, 1978; Forest et al., 1978).
The _C. reinhardtii_ mutant, gam-1 is conditionally defective in signal-generation and in antibody and sexual tipping (Forest et al., 1978). Like colchicine-treated gametes, gam-1 mutants are capable of sexual agglutination, and although they possess morphologically normal mating structures, are unable to fuse. It seems that colchicine-treated cells and gam-1 mutants are defective in sexual signaling because they are incapable of tipping: i.e., sexual adhesion does not result in signal-generation and mating structure activation when tipping of agglutinins does not occur.

Two fundamental questions remain to be answered. First, what is the mechanism underlying the clustering and tip oriented movement of agglutinins and second, why does colchicine prevent tipping? In mammalian cells, microtubules interact with the plasma membrane components. For example, colchicine inhibits movement of membrane proteins (Oliver et al., 1974; Ukena & Berlin, 1972) and lipids during phagocytosis, and enhances the movement of concanavalin A-receptor complexes into caps (Oliver, 1976). Colchicine-induced inhibition of tipping in _Chlamydomonas_ suggests that in this system, like in the mammalian one, colchicine-sensitive molecules can direct the distribution of membrane surface receptors.

Bloodgood and co-workers (1977; 1979) have demonstrated the existence of a membrane associated motility system, capable of moving particles along the external surface of the _Chlamydomonas_ flagellum.

This system reportedly involves energy transduction occurring in association with the flagella membrane and a transmembrane
structure(s) (Bloodgood, 1977). Dentler et al. (1980) have identified a dynein-like ATPase, in Tetrahymena and Aequipecten ciliary membranes, which could prove to be the structure underlying Bloodgood's (1977) membrane-associated motility system. This ATPase occurs in association with a membrane tubulin and forms a bridge between the membrane and the outer doublet microtubules (Dentler et al., 1980). Similarly in Chlamydomonas flagella, membrane-microtubule links (Ringo, 1967a), membrane-associated ATPase (Watanabe & Flavin, 1973; Fay et al., 1977, and membrane tubulin (Monk et al., 1980) have been identified.

Based on the above, a model for the colchicine-labile system responsible for tipping in Chlamydomonas can be proposed. As shown in Figure 9, the dynein-like ATPase could provide a membrane-microtubule link and act as a source of energy for moving surface receptors and their bound ligands to the flagella tips. The tubulin molecule could occur as a transmembrane or intergeral protein, occurring in association with a glycosolated species (receptor), at the membrane's external surface. The binding of a multivalent ligand by surface receptors would bring their associated tubulin units into close proximity and induce the association or polymerization of the tubulin (Goodenough, 1980). The association of two or more tubulin units would then permit effective contact between the ATPase and the tubulin-receptor-ligand complex, providing the energy to direct the complexes' tip oriented movement.

To induce tipping and concomitant papilla activation, ligands must be capable of cross linking flagella surface components; monovalent antibodies, for example, are ineffective (Goodenough & Jurvich, 1978).
In light of this finding, a mechanism for colchicine-induced inhibition of tipping can be proposed: The binding of colchicine to the individual tubulin units in the membrane would prevent the association or polymerization of tubulin necessary for the ATPase to function. By disrupting the ATPase "motor," colchicine inhibits tipping and sexual signaling, and thus prevents the mating reaction from progressing beyond initial flagella adhesion.
REFERENCES


Figure 3. Whole mount preparation of gamete pair which have fused and are joined by a cytoplasmic bridge (b). (x 15,200)

Figure 4. Section through cytoplasmic bridge. Cytoplasmic continuity has been established by papilla membrane fusion (arrow). (x 24,000)
Figure 5. Unmixed control gamete. No papilla outgrowth has occurred at the cell anterior (arrowhead). (x 24,000)

Figure 6. Mixed gamete from colchicine-treated group. No papilla extension is noted (arrowhead). The papilla area is indistinguishable from that of the unmixed gamete in Figure 5. (x 24,000)
Figures 7 and 8. Papilla area (P) of mixed gamete from colchicine-treated group. Papilla activation has not occurred. The papilla wall (arrowhead) is intact. A dense granular region exists between the plasma membrane and the papilla wall. Fibrillar and granular material lie beneath the papilla membrane (m) and are concentrated above the connecting fiber. The plasma membrane is at normal density (Figure 7 x 49,000, Figure 8 x 5,500).
Figure 9. Proposed model for the colchicine-labile system responsible for tipping. When a multivalent ligand binds to surface receptors the tubulin units associated with the receptors are brought into close proximitly. The association of tubulin units then permits effective contact between the ATPase and the tubulin-receptor-ligand complex. The energy provided by the ATPase directs the complexes' tip oriented movement. The binding of colchicine to the individual tubulin units in the membrane prevents the association of tubulin units necessary for the ATPase to function. By disrupting the ATPase "motor," colchicine inhibits tipping.
Microtubule

ATPase

Tubulin

ATPase

Tubulin

ATPase

Tubulin