ed hypovirulent strains (H′) about half of the time. When H′ strains were used in paired inoculations with V, there was no significant difference between the mean canker size of the paired H′ and V inoculations and that of the H′ alone (Fig. 2). While H′ alone in this experiment produced a larger canker than H alone in the previous experiment, the growth of V was also greater, an indication that the second experiment was conducted under conditions more favorable for pathogen growth.

Two months after the trees were inoculated, isolations were made from five points equally spaced around the circumference of the cankers, transferred to PDA, and scored for morphology in culture as V or H (H and H′ have the same morphology). Of 40 strains isolated from H plus V inoculations, 18 had virulent morphology, 20 hypovirulent, and 2 sectored; whereas of 43 strains isolated from H plus V inoculations, 5 had virulent morphology and 38 were hypovirulent. We performed heterokaryon tests (*8*), using auxotrophic markers to establish whether or not hypovirulence was cytoplasmically determined. An American virulent methionine auxotroph (*met*-1) (*7*), and a lysine auxotroph (*lys*-1), induced by ultraviolet irradiation and derived from an H′ hypovirulent isolate, were inoculated singly and as pairs in the trunks of two American chestnut seedlings. There were two replications of the inoculations on each tree. After 90 days, isolates were taken from the edges of the resulting cankers. Of the four paired inoculations, canker size was restricted in two and only methionine auxotrophs were isolated from them. The lysine auxotroph could not be reisolated from either single or paired inoculations. Methionine-requiring isolates from restricted cankers were then inoculated into trunks of chestnut seedlings, and the sizes of resulting cankers were compared with those from virulent *met*-1. The cankers formed by recovered methionine-requiring isolates had a mean diameter of 12.5 mm, and those formed by the virulent *met*-1 strain were 38.1 mm in diameter, thus the recovered *met*-1 isolates were hypovirulent. Heterokaryons were forced in culture with the use of a *met*-1 H′ strain and an arginine auxotroph (*arg*-1) derived from an American virulent strain (*7*). Methionine- and arginine-requiring clones were recovered from uninucleate conidia produced by the heterokaryon. Clones of both types had the morphology associated with hypovirulence.

Since hypovirulence appears to be the result of a cytoplasmic determinant transmitted from hypovirulent strains to virulent ones by hyphal anastomosis, the relative abilities of H′ and H strains to limit the size of a canker when coinoculated with a V strain could be due to heterokaryon incompatibility between the French H strain and the American V strain. The H′ was probably derived from American V and therefore heterokaryon incompatibility with the American V was no longer a factor preventing transfer of the cytoplasmic determinant. Furthermore, isolation from the cankers of the paired inoculations of H′ plus V yielded *E. parasitica* with hypovirulent morphology much more frequently than from the cankers of H plus V paired inoculations, which is evidence that hypovirulence is transmitted to American V strains more readily by H′ than by H.

Since in the heterokaryon transfer experiments, the previously virulent strains were reisolated and shown to be hypovirulent, limitation of canker size by hypovirulent strains is not due to elimination of the virulent strains. Induction of a localized host defense response by the H strain as an explanation for hypovirulence has not been ruled out, but, if it is involved, the genes responsible for causing this induction are carried by the cytoplasmic determinant.

Our results in the laboratory, greenhouse, and field, plus the original observation in Europe that hypovirulence can control chestnut blight in nature, suggest that this fungal strain may become a control for the disease in the United States.

N. K. VAN ALFEN*
Department of Plant Pathology and Botany, Connecticut Agricultural Experiment Station, New Haven 06504
R. A. JAYNES
S. L. ANAGNOSTAKIS
P. R. DAY
Department of Genetics, Connecticut Agricultural Experiment Station

References and Notes

1. A. Biraghi, in *Report of the 11th Congress of the International Union of Forest Research Organizations, Rome, Italy, 1953*, p. 643.
2. J. Grente, in *Academie d'Agriculture de France, Extrait du Proces-verbal de la Seance* (10 November 1965), pp. 1033–1037.
3. ——— and S. Sauret, *C.R. Hebd. Acad. Sci. Paris Ser. D Sci. Nat.* **268**, 2347 (1969).
4. J. Grente, *Ann. Phytopathol.* **3**, 409 (1971); *ibid.*, p. 3173.
5. S. L. Anagnostakis and R. A. Jaynes, *Plant Dis. Rep.* **57**, 225 (1973).
6. R. A. Jaynes and N. K. Van Alfen, *Phytopathology* **64**, 1479 (1974).
7. J. E. Puhalla and S. L. Anagnostakis, *ibid.* **61**, 169 (1971).
8. J. L. Jinks, in *Methodology in Basic Genetics*, W. J. Burdette, Ed. (Holden-Day, San Francisco, 1963), pp. 325–354.
9. Financial support from Anne Valk is gratefully acknowledged.
* Present address: Department of Biology, Utah State University, Logan 84321.

14 March 1975

Flagellar Coordination in *Chlamydomonas reinhardtii*: Isolation and Reactivation of the Flagellar Apparatus

Abstract. *The flagellar apparatus (both flagella with basal bodies and accessory structures) of* Chlamydomonas reinhardtii *was isolated from a wall-less mutant and induced to swim in the presence of adenosine triphosphate. The movement of the flagella of the isolated apparatus was largely synchronous and indistinguishable from the movement of flagella on living cells. These observations suggest that the mechanism of flagellar coordination in this biflagellate alga is largely intrinsic to the structure of the flagellar apparatus itself.*

The unicellular, biflagellate alga *Chlamydomonas reinhardtii* offers a simple model system in which to study the mechanism of flagellar coordination. *Chlamydomonas* swims by means of two anteriorly located flagella which generate a ciliary type of beat with two distinct phases: an effective stroke in which the two flagella are held rigid and swept simultaneously back past the cell, bending only near their bases, and a recovery stroke in which a wave propagated from the proximal end of each flagellum passes out to the distal tip (*1*). Electron microscopy of thin sections through the anterior region of *C. reinhardtii* shows that the flagella arise from two basal bodies which lie at approximately a 90° angle to each other (in a "V" configuration) and are connected at their proximal ends by two striated fibers and at their midlateral faces by a single striated fiber (*1*). We refer to the collection of the two flagella and the basal bodies with connecting fibers as the flagellar apparatus.

Demembranated individual flagella from *C. reinhardtii*, detached from their basal bodies, can be reactivated in vitro (*2*). We now report a procedure for the isolation of the entire flagellar apparatus from *C. reinhardtii* under conditions that allow its subsequent reactivation and analysis in vitro.

A wall-less mutant of *C. reinhardtii*, strain CW92 (*3*), was used to facilitate isolation of the flagellar apparatus. Cultures (1.5 liters) of CW92 were grown to approximately 10^6 cells per milliliter in medium 1 of Sager and Granick (*4*) supplemented with 0.2 percent sodium acetate; they were grown at 25°C with continuous aeration and on an illumination cycle of 14 hours light and 10 hours dark. The cells

were harvested by centrifugation at 200*g* (IEC-R centrifuge, rotor 267) for 15 minutes at room temperature and were then suspended at 0°C in 100 ml of a solution containing 10 m*M* HEPES (*N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid), 5 m*M* $MgSO_4$, 1 m*M* DDT (dithiothreitol), 0.5 m*M* EDTA (ethylenediaminetetraacetic acid), 25 m*M* KCl, *p*H 7.0. The cells were then sedimented at 800*g* (IEC-CL centrifuge, rotor 215) for 5 minutes and were resuspended in 5 ml of the same solution. At this point approximately 3 percent of the original population of cells spontaneously released their flagellar apparatus intact; 30 percent released single flagella into solution and the balance of the population remained flagellated. A subsequent 5-minute centrifugation (800*g*, IEC-CL rotor 215) sedimented the cell bodies, but the detached flagellar apparatus and single flagella remained in the supernatant. The flagellar apparatus were reactivated by mixing the suspension with an equal volume of the following solution: 30 m*M* HEPES, 5 m*M* $MgSO_4$, 1 m*M* DTT, 0.5 m*M* EDTA, 25 m*M* KCl, and 2 m*M* ATP (adenosine triphosphate), *p*H 7.0. Both the isolation and reactivation solutions are basically those described by Allen and Borisy (*2*), with the exception that our isolation solution did not contain the detergent Nonidet P40 (Shell Chemical, London, England) and our reactivation solution contained no polyethylene glycol.

The isolated flagellar apparatus had the characteristic "V" configuration described for the flagella in situ. Electron microscopy of samples negatively stained with 1 percent uranyl acetate indicated that most of the structures observed in situ were also present in the isolated apparatus (unpublished results). The flagellar membrane was largely intact and terminated just below the distal end of the basal body, at the point where the flagellar membrane becomes continuous with the cell membrane. The striated fiber connecting the midlateral faces of the two basal bodies was present, although the two smaller proximal fibers were only rarely visualized. Other accessory structures that we observed attached to the isolated flagellar apparatus included the probasal bodies (*5*) and the remnants of the four bundles of four microtubules each which constitute the flagellar root system (*1*).

Upon the addition of ATP to a final concentration of 1 m*M*, more than 90 percent of the isolated flagellar apparatus began swimming with a motion typical of living *C. reinhardtii* cells. The movement of the flagella on living cells and in the reactivated apparatus was examined by phase microscopy with Zeiss optics. The form and synchronization of the flagellar beat

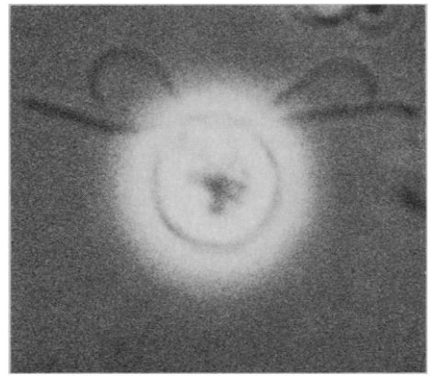

Fig. 1. Living *Chlamydomonas* cell showing the two flagella each in two positions of the beat cycle. Phase optics, × 2240; flash rate, 31 hertz; exposure time, 1/15 second.

were analyzed from photomicrographs in which stopped images of the moving flagella were recorded by means of short pulse illumination with a xenon flash tube (Strobex 136, Chadwick-Helmuth, Monrovia, Calif.). In some instances, illumination flash rate and exposure time were adjusted to obtain multiple images of the flagella in the same photograph (Fig. 1). Each of the flagella was caught in two positions of the beat cycle, illustrating both the effective stroke and recovery stroke; the synchronous nature of the flagellar motion is evident.

The isolated apparatus were observed to swim considerable distances across the microscope field, although more frequently they were seen to tumble in and out of the plane of focus. More convenient for photographic purposes were those flagellar apparatus which became attached by their bases to the microscope slide and remained beating in a fixed position. More than 500 images of reactivated flagellar apparatus stopped in motion have been recorded. Of these, more than 70 percent showed the two flagella beating in synchrony (Fig. 2a), and the remainder showed various forms of asymmetric beating such as that shown in Fig. 2b. It was possible to follow the motion of a particular flagellar apparatus and see it change from synchronous to asynchronous beating and then return to synchronous motion; however, the flagella beat predominantly in the synchronous mode.

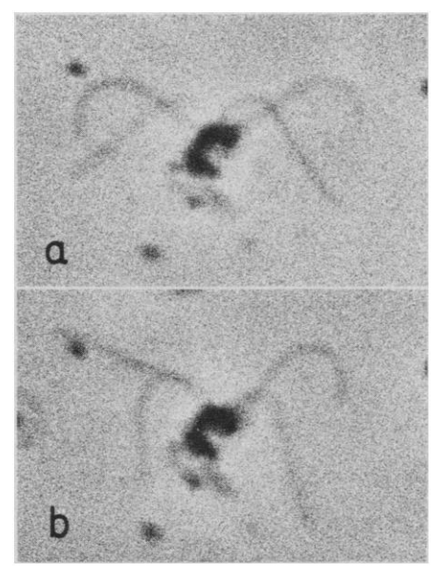

Fig. 2. Isolated, reactivated apparatus, attached to the microscope slide by means of a piece of debris, showing both synchronous (a) and asynchronous (b) beating of the flagella. Phase optics, × 2240; flash rate, 31 hertz; exposure time, 1/15 second.

To determine whether the integrity of the flagellar membrane was required for the synchronous beating, the isolated apparatus were demembranated by addition of the detergent Nonidet P40 to a final concentration of 0.1 percent in the reactivation solution (*2*). Apparatus previously seen to be extremely refractile by phase contrast microscopy became much less refractile upon the addition of detergent, indicating extensive solubilization of the flagellar membranes. However, the form of beating of these demembranated apparatus was indistinguishable from the apparatus unexposed to detergent.

Clearly, the flagella of living cells must have the capability to beat both in and out of synchrony in order to allow the cell to move in straight lines and to turn in response to external stimuli. Our observations suggest that the synchronous movements do not require the cell cytoplasm or the cellular or flagellar membranes but, rather, are inherent in the flagellar apparatus itself. Whether the occasional asynchrony observed in vitro results from differential resistance encountered by the flagella in the vicinity of constraining surfaces (glass slide or cover slip) or is a more fundamental property of the isolated apparatus is not known.

Studies on ciliary coordination dating back over a century have suggested two somewhat opposing theories for the mechanism of coordination (*6*). One theory proposes that coordination is nervelike or "neuroidal" in nature and dependent upon an internal excitation mechanism (*7*), whereas the other theory, the "mechanical" theory, maintains that the action of one cilium mechanically stimulates the next one to action (*8*). Both theories, originally derived from studies of coordination of the swimming plates of ctenophores, have been used to account for the ciliary coordination observed in other multicellular organisms and protozoa.

The neuroid theory as adapted for protozoa envisaged the rootlet fiber system

connecting the basal bodies of adjacent cilia as a neuromotor system, transmitting regulatory impulses from an undetermined control center (*9*). More modern versions have proposed that the rhythm of a group of cilia is dependent upon a pacemaker cilium and, in some instances, that potential changes occur during a ciliary beat which then spread electrotonically to adjoining cilia causing them to beat in turn (*10*).

The mechanical theory has been refined and coordination according to this view is now considered to arise from viscoelastic coupling in the medium between autonomously beating oscillators. Accordingly, synchrony or metachrony results because the energy dissipation in overcoming the viscous drag of the medium is minimized when neighboring cilia beat in phase or with a constant phase relationship (*11*).

Recent studies have shown the neuroidal theory to be implausible, and the mechanical model of coordination is now generally accepted (*12*). Curiously, the ctenophore used in the study responsible for the inception of the neuroidal theory (*8*) seems to represent a special case which provides the only well-documented exception to the mechanical theory. In ctenophores, the existence of both neuroidal and mechanical mechanisms of coordination have been demonstrated, each characteristic of a particular class of ctenophore (*13*). However, since the swimming plates of ctenophores comprise up to thousands of cilia, the motion and coordination of these structures may represent phenomena distinctly different from the motion and coordination of individual cilia.

The synchronous movement of flagella in the isolated apparatus of *Chlamydomonas* may present another and different exception to the mechanical theory. The synchrony is clearly not explainable by neuroidal mechanisms involving elements of the cell cytoplasm and has been shown to be unaffected by solubilization of the flagellar membranes. However, it is also unlikely that the coordination of flagellar beat results from viscoelastic coupling through the medium since the two flagella sweep away from each other during the effective stroke and generate opposing waves during the recovery stroke. Thus one flagellum is not passively following the line of least resistance created by the beat of the other.

Another possibility is that flagellar motion in this organism represents a new category of coordination in which the movement of the individual flagella is coupled by structural elements intrinsic to the apparatus itself. Some such coupling mechanism at the base of the flagella seems required by (i) the structural simplicity of the functional apparatus and (ii) the apparent exclusion of the other theories of coordination. A plausible candidate for the coupling of motion in the flagellar apparatus might be the striated fiber connecting the basal bodies (*1*).

JEREMY S. HYAMS
GARY G. BORISY
Laboratory of Molecular Biology, University of Wisconsin, Madison 53706

References and Notes

1. D. L. Ringo, *J. Cell Biol.* **33**, 543 (1967).
2. C. Allen and G. Borisy, *ibid.* **63**, 5a (1974); *J. Mol. Biol.* **90**, 381 (1974).
3. J. Hyams and D. R. Davies, *Mutat. Res.* **14**, 381 (1972).
4. R. Sager and S. Granick, *J. Gen. Physiol.* **37**, 729 (1954).
5. R. R. Gould, *J. Cell Biol.* **65**, 65 (1975).
6. H. Kinosita and A. Murakami, *Physiol. Rev.* **47**, 53 (1967).
7. M. Verworn, *Pfluegers Arch. Ges. Physiol.* **48**, 149 (1890).
8. G. H. Parker, *J. Exp. Zool.* **2**, 407 (1905).
9. C. Grave and F. O. Schmitt, *J. Morphol. Physiol.* **40**, 749 (1925); L. G. Worley, *J. Cell. Comp. Physiol.* **5**, 53 (1934).
10. M. A. Sleigh, *J. Exp. Biol.* **34**, 106 (1957); *Int. Rev. Cytol.* **25**, 31 (1969).
11. J. Gray, *Proc. R. Soc. Lond. Ser. B* **107**, 313 (1930); K. E. Machin, *ibid.* **158**, 88 (1963); J. R. Blake and M. A. Sleigh, *Biol. Rev.* **49**, 85 (1974).
12. H. Machemer, in *Cilia and Flagella*, M. A. Sleigh, Ed. (Academic Press, London, 1974), p. 199; M. A. Sleigh, in *ibid.*, p. 287.
13. S. L. Tamm, *J. Exp. Biol.* **59**, 231 (1973).

21 January 1975; revised 18 April 1975

Taste Aversions to Sexual Attractants

Abstract. *The vaginal secretion of female hamsters serves as a sexual excitant or attractant for the male even in the absence of previous sexual experience, but attraction to the secretion can be altered with surprising ease by pairing ingestion of the secretion with gastrointestinal illness.*

Many mammalian species rapidly learn to avoid tastes or odors which have been paired with gastrointestinal illness (*1*), and these learned aversions have been demonstrated with a wide variety of taste and odor stimuli and several different methods of producing illness (*2*). Although dozens of different tastes have been used in these studies, there is little evidence that the nature of the taste (for example, sweet, bitter, or salty; palatable or unpalatable) is an important factor in the formation of taste aversions: as long as the taste is readily distinguished from other tastes, the animal can form an aversion to it. While the particular taste employed has little effect on the strength of aversions, past experience with the taste is extremely important. Familiar tastes are less readily associated with illness than are novel tastes (*3*), and tastes which have been paired with recovery from illness are even more resistant to association with illness than are other familiar tastes (*4*). Perhaps experience confers a new meaning on a taste—"This taste is safe" or "This taste always makes me feel better"—and that new meaning can then interfere with later associations between the "meaningful" taste and illness.

The important variable here may not be experience per se, but the "meaning" or information which experience provides. If that is the case, certain tastes or odors which communicate important information to a species even without prior experience might also be difficult to associate with illness. A number of these pheromone-like substances have been implicated in the communication of various mammalian species, and one of the most thoroughly studied examples is the vaginal secretion of the female hamster. This secretion has several effects on the behavior of male hamsters: its odor alone is highly attractive to males and elicits approach, sniffing, and licking behavior (*5*), and when the secretion is applied to a variety of inappropriate hamster partners, such as castrated or anesthetized males, it elicits attempted copulation (*6*). These behavioral responses are not dependent on experience with the vaginal secretion after weaning (*7, 8*). Females deposit the secretion on the substrate with a special scent-marking behavior, the frequency of which varies cyclically with the estrous cycle (*9*); the secretion is also sniffed, licked, and consumed by males when females extrude it as part of normal mating sequences (*6–8*). Thus the variety of male responses to the vaginal secretion and the various conditions under which females extrude it suggest that the secretion serves at least two communication functions: the attraction of males to females and scent-marked areas and the elicitation of male sexual behavior. If the strong sexual "meaning" of this secretion interferes with the formation of new associations to its taste and odor, it should be difficult to produce aversions to the secretion by pairing it with illness; on the other hand, if it is possible to produce strong aversions to this biologically important substance, then responses to this pheromone-containing secretion are more easily altered by experience than we have supposed.

Having first established that hamsters (like all other rodents tested) show strong, long-lasting aversions to tastes which have been paired only once with gastrointestinal illness (*10*), we performed a standard taste-aversion experiment using vaginal secretion from estrous females as the taste stim-