prevented suppression of the colliculi.

Four major conclusions are drawn from this work. First, these data confirm Sprague's dramatic report (*2*) that occipitotemporal decorticate cats are capable of extensive visual behavior provided that intercollicular influences via the collicular commissure are prevented. Second, such decorticate cats behaved as if only their nasal retinas maintained functional central connections, and it is noted that most —up to 99 percent (*9*)—of the cat's retinotectal pathway is crossed. Third, cats with lesions largely limited to geniculocortical areas also behaved as if they have no functional temporal retina, and presumably this obtained because the remaining cortex had visual afferentation only from the midbrain via the pulvinar and lateral posterior nucleus complex. Fourth, the cats with large occipitotemporal lesions plus a split of the collicular commissure behaved on all tests precisely as did binocularly deprived cats (*4*), and this strengthens an earlier suggestion (*7*) that such deprived cats develop visually guided behavior only through retinotectal and not geniculocortical pathways.

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5. I have observed such long-term blindness in three other cats with large, bilateral, occipitotemporal lesions. These cats, like C6, are being retained for further study. In the context of Sprague's suggestion that each colliculus receives balancing facilitatory corticotectal and inhibitory collicular commissure inputs, it is interesting that bilaterally decorticate cats remain blind. This implies that, although each colliculus loses a facilitatory input and is "depressed," it still manages to inhibit the other through its commissure.
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22 March 1974

Assembly of Chick Brain Tubulin onto Isolated Basal Bodies of Chlamydomonas reinhardi

Abstract. *Basal bodies isolated from* Chlamydomonas reinhardi *will serve as initiation centers for the assembly of chick brain microtubule protein subunits (tubulin) into microtubules. The rate of microtubule assembly is tubulin-concentration dependent; this assembly occurs onto both distal and proximal ends of the basal body microtubules, with distal assembly greatly favored. In vitro assembly of brain tubulin also occurs onto the mid-lateral aspects of the basal bodies, presumably onto the fiber connecting the two basal bodies.*

Basal bodies are the assembly sites for the microtubules of all eucaryotic cilia and flagella. Because of the difficulties which have been encountered in their isolation, little is known about their biochemistry or how they function in the organization and assembly of microtubules (*1*). By contrast, their ultrastructure (see Fig. 1), development, and movements in the cell are well defined, and there are excellent reviews on these topics (*2*). One approach to the determination of the role of basal bodies in the assembly of microtubules has been the use of flagella-regenerating systems in flagellated cells such as the biflagellate alga *Chlamydomonas*. In these systems the flagella are detached by physical or chemical procedures and new flagella regenerate from the remaining basal bodies. There have been a variety of studies on the synthesis and assembly of flagellar proteins during this regeneration process, including some which demonstrated that the flagellar microtubules elongate by distal assembly of subunits (tip growth) (*3, 4*). In order to learn more about the role of basal bodies as sites for microtubule protein (tubulin) assembly it would be advantageous to be able to study the assembly of tubulin onto basal bodies in vitro. It became possible to carry out such studies with the development of methods for the in vitro assembly of brain tubulin by Weisenberg (*5*). By use of these methods for the in vitro assembly of tubulin along with new procedures described here for the isolation of basal bodies from *Chlamydomonas*, we now show that the isolated algal basal bodies can act as assembly sites for chick brain tubulin. As with the distal directionality of microtubule assembly seen during flagellar regeneration in vivo, the in vitro assembly of tubulin onto isolated basal bodies occurs principally by distal addition of subunits, although there is also some assembly onto the proximal ends of the basal bodies.

Cultures of strains 21 gr (+ mating type) and 6145C (− mating type) of *Chlamydomonas reinhardi* were grown to a cell density of approximately 2×10^6 cells per milliliter in Medium I of Sager and Granick (*6*) supplemented with 3 g of sodium acetate per liter and three times the normal amount of phosphate buffer. Growth was at 25°C with continuous aeration on a cycle of 13 hours of light and 11 hours of dark. Four liters of each strain were harvested, resuspended in 4 liters of M-N medium (Medium I of Sager and Granick without NH_4NO_3, at *p*H 7.6), and placed in continuous light for 18 hours, during which time the cells differentiated into gametes (*7*). The two strains of gametes were harvested and mixed together in 1 liter of M-N medium for 30 minutes to allow mating to occur (*8*). During the mating reaction a cell wall lysin is released into the medium which causes dissociation of the cell walls from approximately 90 percent of the cells (*9*). These wall-less cells were then deflagellated in M-N medium using the *p*H shock method (*4*). The suspension of cells, cell walls, and flagella was centrifuged at 1500*g* (2700 rev/min, IEC PR-6 centrifuge, rotor No. 253) for 3 minutes at 25°C, sedimenting the cell bodies and leaving the cell walls and flagella in the supernatant. The supernatant was removed, the cell bodies were resuspended in 500 ml of TE buffer (10 m*M* tris, 1 m*M* EDTA, *p*H 7.5 at 25°C) at 4°C and centrifuged again as above. All subsequent steps were carried out at 4°C. The wall-less, flagella-less cells were resuspended in 50 ml of TE to which was then added 50 ml of 1 percent Nonidet P-40 (Shell) in TE. The suspension was stirred vigorously for 10 minutes, and homogenized by hand in 20-ml aliquots in a 25-ml glass-Teflon homogenizer (Arthur H. Thomas Co.) with five up-and down strokes; 20-millimeter aliquots of this suspension were layered over 20 ml of 25 percent sucrose-TE in 50-ml, conical, polycarbonate centrifuge tubes.

This preparation was centrifuged at 1500*g* (IEC PR-6 centrifuge, as above) for 10 minutes to sediment intact cells, whitish starch-like granules, and other dense material. The green supernatant above the 25 percent sucrose-TE layer was removed, relayered over 25 percent sucrose-TE, and centrifuged again as above. Thirty-milliliter aliquots of this second supernatant were layered over a discontinuous sucrose-TE gradient (10 ml of 40 percent over 10 ml of 50 percent sucrose-TE) in 50-ml round-bottomed centrifuge tubes and centrifuged at 14,000*g* (9,250 rev/min, Sorvall RC-2 centrifuge, rotor No. HB-4) for 1 hour. After centrifugation the greenish band of material at the 40 to 50 percent sucrose-TE interface was removed, diluted 1 : 1 with TE, and centrifuged at 35,000*g* (17,000 rev/min, Sorvall RC-2 centrifuge, rotor No. SS-34) for 30 minutes to sediment the basal bodies. The basal bodies were resuspended in 3 ml of 0.5 percent Nonidet P-40 in TE, rehomogenized as above, layered over a discontinuous sucrose-TE gradient (3 ml each of 50, 55, and 60 percent sucrose-TE) in a 12-ml round-bottomed centrifuge tube, and centrifuged at 14,000*g* (Sorvall, No. HB-4 rotor) for 45 minutes. The whitish band at the 55 to 60 percent sucrose-TE interface contained basal bodies relatively free of contaminants (Fig. 2).

As shown in Figs. 2 to 4, the isolated basal bodies are structurally intact and have ultrastructural characteristics identical to *Chlamydomonas* basal bodies in situ (*10, 11*) (see legend of Fig. 3 for description). Basal bodies have four potential sites for tubulin assembly: the proximal and distal ends of the microtubules which compose the basal bodies, the basal cup, and the distal connecting fiber between the two basal bodies (see Fig. 3). The distal connecting fiber apparently acts in vivo as a microtubule organizing center (*12*) for the cortical microtubules that encircle the cell just underneath the cell membrane (*10, 11*).

Isolated basal bodies were incubated with tubulin subunits to determine if the basal bodies would act as sites for the assembly of chick brain tubulin into microtubules in a manner similar to the assembly of chick brain tubulin onto isolated flagellar axonemes of *Chlamydomonas* and sea urchin sperm, as has been described (*13, 14*). Brain tubulin was used in these studies because it has not yet been possible to dissociate and reassemble *Chlamydomonas* flagellar microtubules in vitro.

Tubulin from chick brain was isolated by in vitro assembly methods essentially as described by Weisenberg (*5*). Chick brains were homogenized at 0°C in a volume of PM buffer (1 m*M* $MgSO_4$, 2 m*M* EGTA, 1 m*M* GTP, 100 m*M* PIPES, *p*H 6.9) equal to 1.5 times the weight of the brains and centrifuged at 35,000*g* (17,000 rev/min, Sorvall RC-2 centrifuge, rotor No. SS-34) for 1 hour at 4°C. The supernatant (S-1) was removed and used as a crude preparation of tubulin subunits (*15*). To obtain more purified tubulin preparations, a portion of the S-1 supernatant was incubated at 37°C for 30 minutes to assemble the tubulin into microtubules. The microtubules were harvested by centrifugation at 27,000*g* (15,000 rev/min, Sorvall SS-1 centrifuge) for 15 minutes at 25°C, resuspended in PM, and incubated at 0°C for 30 minutes to depolymerize the microtubules. This suspension was centrifuged at 105,000*g* (40,000 rev/min, Beckman L2-65B centrifuge, rotor No. 50Ti) for 1 hour at 4°C to remove any large aggregates of tubulin in the supernatant which had not been dissociated by the cold treatment. The supernatant (S-2) from this centrifugation was also used as a source of tubulin subunits. Quantitative polyacrylamide gel electrophoresis showed the crude, S-1 supernatant to be composed of 25 to 30 percent tubulin and the S-2 supernatant to be composed of 65 to 70 percent tubulin.

One volume of isolated basal bodies in TE was mixed with 3 volumes of the purified tubulin preparation (S-2) in PM and incubated at 37°C for 30 minutes. The final concentration of tubulin in the incubation mixture was

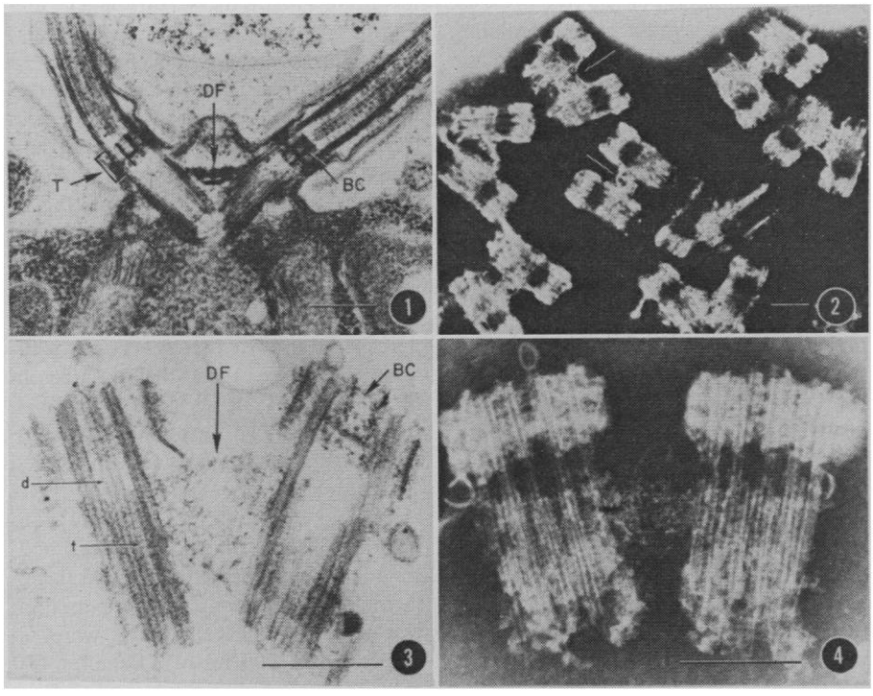

Fig. 1. Longitudinal section through the basal bodies and flagella of *Chlamydomonas* [from Ringo (*10*)]. The basal bodies are connected by the distal connecting fiber (*DF*) which acts as a microtubule organizing center for the submembrane microtubules (*16*). The transition region (*T*) between basal bodies and flagella is characterized by a dense basal cup (*BC*), from which the two flagellar central microtubules arise, and by a dense, rigid membrane surrounding the axoneme. This membrane contains the "flagellar necklace" (*19*). Fig. 2. Low magnification micrograph showing an isolated basal body preparation. Basal bodies are usually found in pairs, connected by the distal connecting fiber (arrows) (*16*). Negatively stained with 2 percent phosphotungstic acid (PTA). Fig. 3. Longitudinal section through a pair of isolated basal bodies. The distal connecting fiber (*DF*), basal cup (*BC*), and the membrane surrounding the transition region remain intact throughout the isolation procedure. Clearly visible are the doublet (*d*) and triplet (*t*) microtubules. Fig. 4. Isolated basal bodies negatively stained with 2 percent PTA. The membrane surrounding the transition region appears as a light "cap." Also visible are the distal connecting fiber (*16*) and the doublet and triplet microtubules. The short C tubules are striated and extend for the proximal one-third to one-half of the basal body; this is typical of protozoan basal bodies (*1, 2*). The bars represent 0.3 μm.

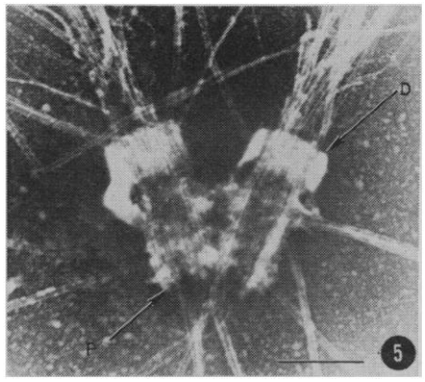

Fig. 5. Isolated basal bodies incubated with chick brain tubulin (S-2, approximately 4 mg/ml) for 10 minutes at 37°C, fixed with glutaraldehyde, and negatively stained with 2 percent PTA. Microtubules arise from the distal end (*D*) and, to a lesser extent, from the proximal end (*P*) of the basal body microtubules. Microtubules also arise from the region of the distal connecting fiber (*16*). Bar represents 0.3 μm.

varied from approximately 1 to 6 mg/ml. Aliquots of the incubation mixtures were fixed at 5-minute intervals during the 30-minute incubation by the addition of 4 volumes of 2 percent glutaraldehyde (in 0.1*M* phosphate buffer, *p*H 7.0), negatively stained with phosphotungstic acid and examined in a Philips 200 electron microscope.

As shown in Figs. 5 to 7, isolated basal bodies can act as sites for the in vitro assembly of brain tubulin. Assembly occurs onto both distal and proximal ends of the basal bodies; in addition, in the majority of cases there is assembly of tubulin onto the midlateral aspects of the basal bodies near the region of the distal connecting fiber (Figs. 5 and 7) (*16*). These same results—tubulin assembly onto all three assembly sites—are also obtained if the *crude S-1 supernatant* is used as a tubulin source. With both tubulin preparations (S-1 or S-2), assembly of tubulin onto the distal end and onto the region of the distal connecting fiber is much more rapid than assembly onto the proximal end of the basal body (Fig. 5). With low concentrations of tubulin (1 mg/ml) distal assembly and assembly from the region of the distal connecting fiber can be detected with short incubation times (5 minutes), whereas proximal assembly can be detected only with long incubation times (30 minutes). With high concentrations of tubulin (4 mg/ml), assembly onto all three sites can be detected within 5 minutes after the incubation has started. These results are similar to those reported previously for tubulin subunit assembly onto isolated axonemes of *Chlamydomonas* flagella and sea urchin sperm where assembly likewise is tubulin-concentration dependent and bidirectional, but favors distal addition (*13*). In the latter studies tubulin assembly occurs onto both of the outer doublet microtubules (A and B) and onto the two central microtubules of the axoneme (*13*). In the present studies it is not yet known if brain tubulin is being assembled onto all three of the basal body microtubules (A, B, and C). Neither is it known if the basal cup from which the two central axonemal microtubules arise in vivo is acting as an assembly site in vitro. Further work is required to determine more precisely these tubulin initiating sites in the basal body.

The fact that isolated basal bodies from a green alga will support assembly of tubulin isolated from chick brain emphasizes the extent to which the assembly sites on the tubulin subunits have been conserved throughout evolution. Other studies, which have shown that chick brain tubulin will assemble onto isolated, microtubule-depleted mitotic apparatuses of marine eggs (*17*) and HeLa cells (*18*) as well as onto isolated axonemes of sea urchin sperm (*13*), also emphasize this conservation of tubulin assembly sites.

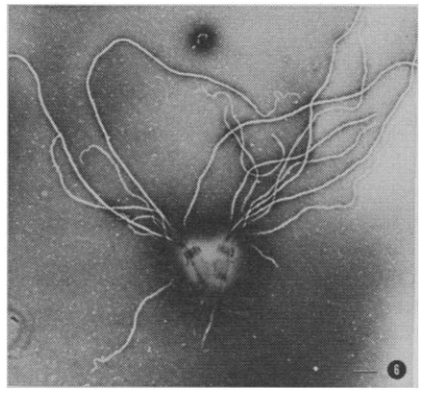

Fig. 6. Isolated basal bodies incubated with chick brain tubulin (approximately 2 mg/ml) for 10 minutes at 37°C, fixed with glutaraldehyde, and negatively stained with 2 percent PTA. It is clear that both microtubule initiation and the rate of assembly are considerably greater on the distal ends of the basal bodies as compared to the proximal ends (*13*). Because the basal bodies absorb much larger amounts of PTA than do the microtubules and appear very dark in the electron microscope, this micrograph has been photographically masked and dodged in order to show more clearly the connections between the in vitro assembled microtubules and the basal bodies. Bar represents 0.3 μm.

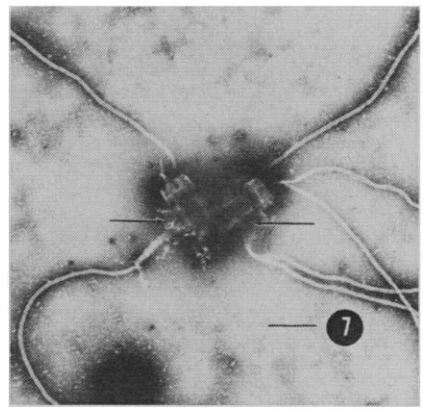

Fig. 7. Isolated basal bodies incubated with chick brain tubulin to show assembly of microtubules onto midlateral (arrows) and distal aspects of the basal bodies. Bar represents 0.3 μm.

The procedures described in this report for the isolation of basal bodies will now permit the comparison of the biochemical composition of basal bodies isolated from a variety of flagellar microtubule structural mutants of *Chlamydomonas* such as those lacking central pair microtubules (9 + 0 mutants), temperature-sensitive paralyzed mutants, and various flagella-less mutants. Perhaps of even more importance, basal bodies from these mutants can now be assayed for their ability to act as assembly sites for tubulin in vitro. The use of isolated basal bodies from these mutants along with in vitro tubulin assembly procedures and the appropriate in vitro complementation studies should now make it possible to define the various steps in the assembly of the eucaryotic flagellar axoneme.

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16. The microtubules which were observed to originate from the midlateral aspect of the isolated basal bodies which had been incubated with brain tubulin in vitro may be assembling onto the distal connecting fiber. This fiber between the two basal bodies has been described as the in situ site of attachment for the submembrane microtubules. However, in order to determine if this fiber is serving as an in vitro assembly site for microtubules, thin sectioning and electron microscopy will be required. Some basal bodies were observed that supported tubulin assembly from both distal and proximal ends, but not from their midlateral aspects. In these cases the presumptive microtubule assembly sites associated with the distal connecting fiber may be missing.
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Immediate Hypersensitivity Responses in Flatfish

Abstract. *Fungal extracts that precipitate with human C-reactive protein caused immediate erythema on subdermal injection into marine flatfish. Only species with calcium-dependent serum precipitins to these fungi showed skin reactions. Immediate hypersensitivity in a nonreactive species could be induced after injection with serum from reactive species. The transferable serum factor (or factors) was heat sensitive.*

In recent years phylogenetic aspects of antibody structure and function in poikilotherms have received a great deal of attention (*1*, *2*), but little attempt has been made to study the phylogeny of immediate and delayed hypersensitivity reactions. There have been a few attempts to demonstrate immediate hypersensitivity reactions in fish. These studies were based on methods that were designed to demonstrate systemic or passive cutaneous anaphylaxis, but results have been inconclusive (*1*, *3*).

Using some marine teleost species belonging to the order Heterosomata (flatfish), we have found that intradermal injections of some fungal extracts produce immediate (type 1) skin reactions (*4*) in the injected fish. Flatfish were selected for examination since, in many respects, they are ideal experimental animals for a study of immediate hypersensitivity in poikilotherms. Plaice, *Pleuronectes platessa* L., and the closely related flounder, *Platichthys flesus* (L.), are readily available throughout the year and are easily maintained in the aquarium. Both species are easy to handle, the accessible caudal vein is suitable for intravenous injection and blood collection, and, most important, skin reactions can be clearly observed on their nonpigmented under surfaces.

Fish used in our study were seinenetted in shallow water off the Aberdeenshire coast and transferred to aerated seawater tanks where they were maintained at 11° to 14.5°C. Experiments were generally carried out on fish that had been in the aquarium for periods ranging from 24 hours to 16 months, but a few fish were examined within 1 hour of capture. Both male and female fish, varying in age from 1 to 10 years, were used. Extracts of the fungi *Aspergillus fumigatus, Candida albicans, Epidermophyton floccosum, Micropolyspora faeni, Trichophyton mentagrophytes,* and *Trichophyton rubrum* and an extract from the house dust mite *Dermatophagoides farinae* were prepared from culture filtrates and whole mites by methods already described (*5*). These preparations are widely used as allergens in human skin tests.

The extracts and a peptido-polysaccharide isolated from *E. floccosum* (*6*) were injected (0.2 ml) into the skin of plaice at concentrations of 10 mg/ml in 0.19*M* NaCl. Extracts of *A. fumigatus, E. floccosum, T. mentagrophytes,* and *T. rubrum* and *E. floccosum* peptido-polysaccharide produced immediate erythema reactions in the skins of 69 of 70 plaice tested (Fig. 1). The one plaice that failed to react did show a slight response to *T. mentagrophytes* extract. In general, skin reactions were most pronounced when *E. floccosum* whole extract and *E. floccosum* peptido-polysaccharide were injected, but no skin reactions appeared after the injection of *E. floccosum* extract from which the peptido-polysaccharide had been removed with concanavalin A (*6*). No skin reactions were observed when plaice were injected with saline or with extracts from

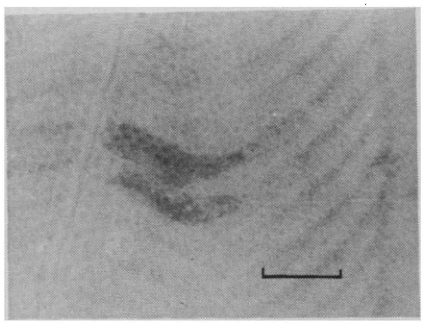

Fig. 1. Erythema reaction observed on the undersurface of plaice (*Pleuronectes platessa*) 5 minutes after subdermal injection of 0.2 ml of *Epidermophyton floccosum* extract (10 mg/ml in saline). The bar is equivalent to 0.75 cm.

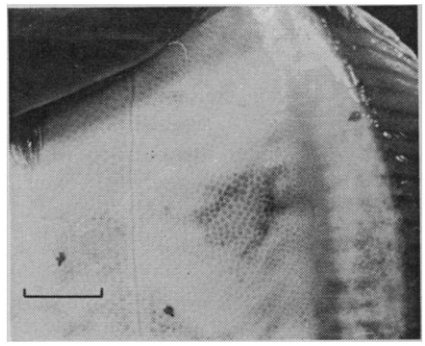

Fig. 2. Erythema reaction observed on the undersurface of flounder (*Platichthys flesus*) 1 hour after subdermal injection of 0.2 ml of *Epidermophyton floccosum* extract (10 mg/ml in saline). Flounder injected intravenously 24 hours earlier with plaice serum (1.5 ml serum per 100 g of the body weight of the flounder). The bar is equivalent to 2 cm.