

Synthesis, Transport, and Utilization of Specific Flagellar Proteins during Flagellar Regeneration in *Chlamydomonas*

STEPHEN P. REMILLARD and GEORGE B. WITMAN

Department of Biology, Princeton University, Princeton, New Jersey 08540. Dr. Remillard's present address is the Department of Biology, Brandeis University, Waltham, Massachusetts 02154. Dr. Witman's present address is Cell Biology Group, Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts 01545.

ABSTRACT We labeled gametes of *Chlamydomonas* with 10-min pulses of $^{35}\text{SO}_4^{-2}$ before and at various times after deflagellation, and isolated whole cells and flagella immediately after the pulse. The labeled proteins were separated by one- or two-dimensional gel electrophoresis, and the amount of isotope incorporated into specific proteins was determined. Individual proteins were identified with particular structures by correlating missing axonemal polypeptides with ultrastructural defects in paralyzed mutants, or by polypeptide analysis of flagellar fractions. Synthesis of most flagellar proteins appeared to be coordinately induced after flagellar amputation. The rate of synthesis for most quantified proteins increased at least 4- to 10-fold after deflagellation. The kinetics of synthesis of proteins contained together within a structure (e.g., the radial spoke proteins [RSP]) were frequently similar; however, the kinetics of synthesis of proteins contained in different structures (e.g., RSP vs. α - and β -tubulins) were different. Most newly synthesized flagellar proteins were rapidly transported into the flagellum with kinetics reflecting the rate of growth of the organelle; exceptions included a central tubule complex protein (CT1) and an actinlike component, both of which appeared to be supplied almost entirely from pre-existing, unlabeled pools.

Isotope dilution experiments showed that, for most quantified axonemal proteins, a minimum of 35–40% of the polypeptide chains used in assembling a new axoneme was synthesized during regeneration; these proteins appeared to have predeflagellation pools of approximately the same size relative to their stoichiometries in the axoneme. In contrast, CT1 and the actinlike protein had comparatively large pools.

When the flagella are amputated from many types of eucaryotic cells, the cells regenerate new flagella.¹ This phenomenon has been frequently studied to learn more about the induction and regulation of synthesis of a defined set of proteins and how these proteins are transported and assembled into a developing cell organelle (12, 37, 39, 43, 44, 49). Several *in vivo* labeling studies have demonstrated that synthesis of the tubulins and other flagellar proteins is stimulated after flagellar loss (6, 15, 23). *In vitro* translation studies of isolated mRNA have shown that during regeneration there is an increase in the levels of translatable mRNAs for the tubulins and other flagellar proteins (24, 47), and studies using cDNA probes complementary to tubulin mRNA have provided evidence that tubulin mRNA

is accumulated after flagellar amputation (30, 41). These findings are in good agreement with an *in vivo* study using an inhibitor of RNA synthesis which suggested that tubulin synthesis during ciliary regeneration requires new mRNA production (15). Formation of a new flagellum therefore appears to involve the transcription of specific mRNAs and their translation into proteins which are subsequently used to assemble the organelle or to replenish protein pools. Flagellar regeneration thus provides a model system for studying the regulation of gene expression as well as organelle morphogenesis and assembly.

Flagellar regeneration in the unicellular green alga *Chlamydomonas* is particularly suited to such studies. The flagella are easily detached from *Chlamydomonas*, and populations of cells will synchronously regenerate new flagella (39). The kinetics of regeneration have been well-characterized, and

¹ The terms flagella and cilia will be used interchangeably throughout this report.

inhibitor studies have clearly shown that normal regeneration is dependent on new protein synthesis (23, 39). The detached flagella can be readily isolated, free of cell body proteins and contaminating proteases (48, 51); this greatly facilitates analysis of the individual flagellar proteins. As a result, a large number of *Chlamydomonas* flagellar proteins have now been identified with either the flagellar membrane (19, 48) or with specific axonemal structures (11, 14, 18, 34–36, 48–50).

Although a considerable amount of information is available on the synthesis of the tubulins after flagellar loss in *Chlamydomonas* (7, 23, 24, 30, 41, 47), relatively little is known about the synthesis of the many proteins which are assembled into the flagellum in fixed ratios relative to the tubulins. In vivo studies have indicated that after deflagellation, the dyneins and several other axonemal proteins are synthesized with kinetics similar to that of the tubulins (23, 24); however, other flagellar proteins are synthesized with different kinetics (23). Analysis of in vitro translation products of mRNAs isolated from regenerating cells have shown that levels of translatable mRNAs for a large number of proteins are elevated in regenerating cells (24); several of these products comigrated with flagellar proteins, but they were not identified with specific flagellar structures.

To understand how the cell controls and coordinates the formation of a complex organelle such as the flagellum it is essential to know more about how the synthesis of the other axonemal components is regulated relative to that of the tubulins during flagellar regeneration. It is also important to have information on the relative pool sizes of the various flagellar proteins and their rates of transport into the growing flagellum. We have investigated these phenomena in *Chlamydomonas*. Our results, presented here, indicate that: (a) synthesis of most flagellar proteins is stimulated at the same time soon after deflagellation; (b) proteins contained within the same structure often appear to be coordinately synthesized, but (c) proteins in different structures are not coordinately synthesized; (d) most newly synthesized flagellar proteins are rapidly transported into the flagellum with kinetics which reflect the rate of growth of the organelle, and do not necessarily coincide with the time course of their synthesis in the cytoplasm; and (e) large pools of at least two flagellar proteins (the actinlike protein associated with the inner dynein arm, and a protein of the central tubule complex) are present in the cell body and are the source for most of the protein of these types used in assembling a new flagellum.

MATERIALS AND METHODS

Materials

$^{35}\text{SO}_4$ (as $\text{H}_2^{35}\text{SO}_4$, carrier free, 43 Ci/mg) was obtained from New England Nuclear (Boston, MA). DNase I and RNase A were obtained from Boehringer-Mannheim Biochemicals (Indianapolis, IN).

Dictyostelium actin was kindly provided by Dr. J. S. Condeelis (Albert Einstein College of Medicine). Gel casting materials for electrophoresis were purchased from Bio-Rad Laboratories (Richmond, CA) unless noted otherwise. Carrier ampholytes (pH ranges 5–7 and 3–10) for isoelectric focusing were obtained from LKB Instruments, Inc. (Rockville, MD). Ultrapure urea for two-dimensional gels was purchased from Schwarz/Mann Div., Becton, Dickinson & Co. (Orangeburg, NY). Other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) unless stated otherwise.

Cells and Cell Cultures

Wild-type (1132D) and mutant (*pf-1*, *pf-14*, *pf-18*, and *pf-19*) strains of *Chlamydomonas reinhardtii* were cultured at 24–27°C in minimal medium 1 (MM) of Sager and Granick (40), with a threefold increase in the potassium

phosphate concentration, as previously described (51). Gametic cells were obtained by transferring vegetative cells ($4\text{--}6 \times 10^6$ cells/ml), ~6 h after the beginning of the light period, into the nitrogen-free medium (NFM) of Sager and Granick (40), modified to contain 0.13% ($2 \mu\text{M}$) of the normal concentration of SO_4^{2-} . The gametes ($\sim 1\text{--}2 \times 10^7$ cells/ml) were harvested the next day by centrifugation and resuspended in their conditioned medium at a density of 2×10^7 cells/ml for use in the regeneration studies.

Flagellar Isolation and Fractionation

Cells were collected, washed, and their flagella were isolated as described by Witman et al. (51), except that cells were deflagellated by addition of dibucaine to a final concentration of 0.615 to 1.25 mM, and the cell bodies were removed by sedimentation through a layer of 25% sucrose in 10 mM HEPES (pH 7.5), 5 mM MgSO_4 , 1 mM DTT. In the regeneration experiments, the flagella were pelleted using a Beckman 75 Ti rotor (Beckman Instruments, Inc., Fullerton, CA) at 40,000 rpm (143,000 g) for 30 min at 4°C to insure that the shortest flagella were pelleted. The isolated flagella were then resuspended in 5 ml of HMDEK (10 mM HEPES, pH 7.5, 5 mM MgSO_4 , 1 mM DTT, 0.5 mM EDTA, 25 mM KCl). After removal of an aliquot for electrophoretic analysis, the remaining flagella were demembrated by the addition of Nonidet P-40 (NP-40) (BDH Chemicals, Ltd., Poole, England) in HMDEK to a final concentration of 1.0%. The axonemes were then sedimented in a Sorval SS-34 rotor (DuPont Instruments–Sorval Biomedical Div., DuPont Co., Newtown, CT) at 16,000 rpm (31,000 g) for 20 min at 4°C, and the pellet was prepared for electrophoretic analysis. The membrane and matrix proteins were recovered from the resulting supernatant fraction by acetone precipitation. The supernate was mixed with 9 vol of acetone prechilled in an EtOH-dry ice bath, the mixture was spun in the SS-34 rotor at 10,000 rpm (12,000 g) for 10 min at 4°C, and the supernate was removed. The residue, containing the membrane and matrix proteins, was prepared for electrophoresis.

Whole Cell Solubilization

Whole cells were prepared for electrophoretic analysis by the following procedure. The cells ($6\text{--}10 \times 10^7$ cells) were pelleted and resuspended in 15 ml of a solution (modified from Howell [17]) containing 50 mM Tris HCl, pH 7.5, 200 mM NaCl, 1 mM EDTA, 2 mM MgCl_2 , 80 mM β -mercaptoethanol, 1 mM PMSF (phenylmethanesulfonyl fluoride), and 1 mg/ml L-lysine. The cells were sedimented again and the pellet was frozen in an EtOH-dry ice bath for storage at -85°C until solubilization. The frozen cells were solubilized by a modification of the sonication procedure of O'Farrell (32). The major changes were the addition of 2 mM PMSF and 1 mg/ml L-lysine to the sonication buffer, the addition of 0.25% NP-40 during the nuclease digestion, and a low-speed centrifugation (IEC 253 rotor [Damon/IEC Division, Needham Heights, Ma], 2,000 rpm [1,100 g], for 10 min at 4°C) to remove any nonsolubilized material.

Flagellar Amputation and Regeneration

Gametes were placed in a Virtis 100-ml fluted glass flask and deflagellated by shearing for 75 s in a Virtis Model 23 homogenizer set at full speed. The cells were then placed on a magnetic stirrer under a fluorescent lamp and allowed to regenerate. For determination of regeneration kinetics, aliquots of cells were removed at 5-min intervals and fixed by mixing 1:1 with a solution of 2.5% glutaraldehyde in isotonic saline diluent (Fischer Scientific Co., Fair Lawn, NJ). Flagellar lengths on a minimum of 25 cells were then measured using an ocular micrometer on a Zeiss Universal microscope outfitted with Nomarski interference contrast optics at $\times 800$.

Isotopic Labeling of *Chlamydomonas*

Vegetative cells were labeled by growing them for 4 d to a density of $3\text{--}4 \times 10^6$ cells/ml in MM modified to contain 4.8×10^{-2} mM SO_4^{2-} and 1.0 mCi $\text{H}_2^{35}\text{SO}_4/125$ ml. Steady-state labeled gametes were obtained by transferring labeled vegetative cells into 125 ml of low SO_4^{2-} -NFM containing 43 μCi $\text{H}_2^{35}\text{SO}_4$, and allowing gametic differentiation to proceed. Under these conditions, the specific activity of $^{35}\text{SO}_4^{2-}$ was the same in both MM and low SO_4^{2-} -NFM. These procedures resulted in a specific activity of between 100,000–200,000 cpm/ μg of TCA-precipitable protein. Gametes were pulse labeled by placing 5-ml aliquots of a gametic cell suspension in 25-ml Erlenmeyer flasks containing $^{35}\text{SO}_4^{2-}$ (0.5 mCi carrier-free $\text{H}_2^{35}\text{SO}_4$, per 5 ml of medium) plus 25 $\mu\text{g}/\text{ml}$ chloramphenicol; after 10 min, the pulse was stopped by addition of unlabeled MgSO_4 to a final concentration of 3.2 mM. Close adherence to our growth and labeling conditions was necessary to achieve uniform rates of uptake of label at the various times throughout regeneration.

Measurement of Incorporation into TCA-Precipitable Protein

The incorporation of isotope into TCA-precipitable protein was measured by a batch filter paper disc method (Rosenbaum et al. [39], as modified from Mans and Novelli [28]). The background was <0.3% of the maximum number of counts among the filters.

Protein Determination

The protein concentration in various samples was determined by the method of Bradford (5), using the microassay described in the Bio-Rad Protein Assay Dye Reagent Kit (Bio-Rad Laboratories), and with bovine gamma globulin as the protein standard.

Gel Electrophoresis

One-dimensional electrophoresis of proteins in SDS polyacrylamide gels was carried out by the method of Laemmli (20, 21), modified to give better resolution of the high molecular weight components of the whole flagella. The plug consisted of 8% acrylamide; the separating gel (32 cm long by 16 cm wide by 1.5 mm) was a 5–15% linear acrylamide gradient, 0–8% linear glycerol gradient; and the stacking gel (2 cm long) was 3% acrylamide. The running buffer contained 0.024 M Tris base, 0.192 M glycine, and 0.13% SDS. Samples were prepared by boiling for 1 min in a sample buffer containing 0.124 M Tris HCl, pH 6.8, 10% glycerol, 2% SDS, 0.001% Bromophenol Blue, and 10% β -mercaptoethanol. The samples were electrophoresed at 60 V until the tracking dye was 1 cm into the separating gel, at which time the voltage was increased to 120 V until the dye front reached the plug (~16 h).

The flagellar components were also analyzed by two-dimensional gel electrophoresis (NEPHGE SDS). The methods used were those of O'Farrell et al. (33), except that the second dimension used the SDS polyacrylamide gel system described above.

All gels were initially fixed in 20% methanol (MeOH)–7.5% acetic acid (HAc). If the gels were to be stained, they were then washed with 35% ethanol–7.5% HAc for 24 h to remove the ampholytes, and subsequently washed with 20% MeOH–7.5% HAc followed by staining for 3 h in 45% MeOH and 9.0% HAc containing 0.25% (wt/vol) Coomassie Brilliant Blue. After destaining in 20% MeOH–7.5% HAc, the gels were photographed using Kodak High Contrast Copy or Panatomic X films. For the quantification procedure described below, the destained gels were washed with 2.5% HAc just before excision of relevant spots.

Fluorography

One- and two-dimensional gels containing ^{35}S -labeled proteins were prepared for fluorography according to the procedure of Bonner and Laskey (4) and Laskey and Mills (22).

Quantification of Two-dimensional Gels

To determine the amount of ^{35}S in specific flagellar components, axoneme, whole flagella, or whole cell samples were electrophoresed with unlabeled axonemal protein (75 $\mu\text{g/gel}$ unless stated otherwise) as a carrier. After staining, spots corresponding to particular proteins were excised, solubilized with NCS Tissue Solubilizer (Amersham/Searle Corp., Arlington Heights, IL) (1), and counted. Control experiments using two-dimensional gels in which various amounts of labeled material were loaded along with the carrier showed that the recovery was linearly proportional to the counts loaded (see Results).

RESULTS

Identification of Structural Proteins in the Flagellum

The *Chlamydomonas* flagellum is composed of a membrane-bounded axoneme containing nine outer doublet microtubules, two central microtubules, and a number of microtubule-associated structures, including the inner and outer dynein arms, the radial spokes and spoke heads, and the projections of the central tubules (Fig. 1). When analyzed by two-dimensional gel electrophoresis, the flagellum is seen to contain α - and β -tubulin and over one hundred other proteins (Fig. 2a). To

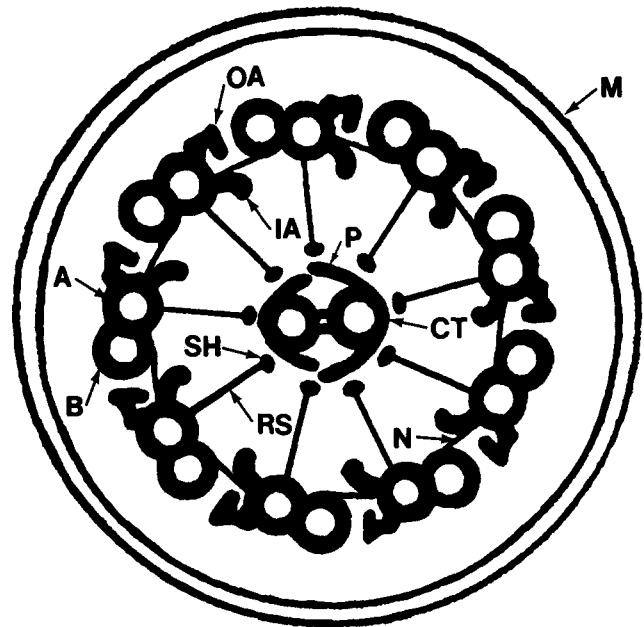


FIGURE 1 Diagram of a cross section of a *Chlamydomonas* flagellum viewed from base to tip. A, A-tubule; B, B-tubule; CT, central tubule; M, flagellar membrane; IA, OA, inner and outer dynein arm, respectively; N, nexin link; RS, radial spoke; SH, spoke head; P, central sheath projection.

determine which of these proteins are associated with particular axonemal structures, we have (a) compared the proteins of isolated axonemes of wild-type with those of paralyzed mutants containing known ultrastructural defects in order to correlate missing proteins with missing structures in the mutants and (b) analyzed the polypeptides of various flagellar fractions isolated using selective solubilization procedures.

Fig. 2b shows a fluorograph of a two-dimensional gel of axonemes obtained by treating isolated flagella with the detergent NP-40 to dissolve the flagellar membrane; note that several proteins are present in greatly reduced amounts relative to whole flagella (Fig. 2a). These proteins (arrows in Fig. 2a) represent components of the flagellar membrane and matrix. Two of these proteins, having molecular weights of 65,000 and ~125,000, (*m* and *m'* in Fig. 2a), have been identified as major membrane components by analysis of membrane vesicles isolated using the procedure of Witman et al. (48) (results not shown; see reference 38).

To identify proteins associated with the central tubules, the central tubuleless mutants *pf-18* and *pf-19* were analyzed. In these mutants, the central tubules are replaced by a core which runs longitudinally down the center of the axoneme (46); this core is frequently lost when the axonemes are isolated (48, 50, 51). Fig. 3 shows fluorographs of two-dimensional gels of axonemes of wild-type and mutant *pf-19*. A number of proteins were found to be reproducibly missing in both the *pf-18* and *pf-19* axonemes; five of these are indicated in Fig. 3. These proteins presumably represent components of the central tubule–central sheath complex. The four high molecular weight (HMW) proteins previously shown by Witman et al. (51) to be associated with the central tubule complex were not resolved in the two-dimensional gels.

Similarly, to identify components of the radial spokes, mutants *pf-14*, missing the entire radial spoke (50, 51), and *pf-1*, missing only the spoke head (34), were analyzed. 14 proteins

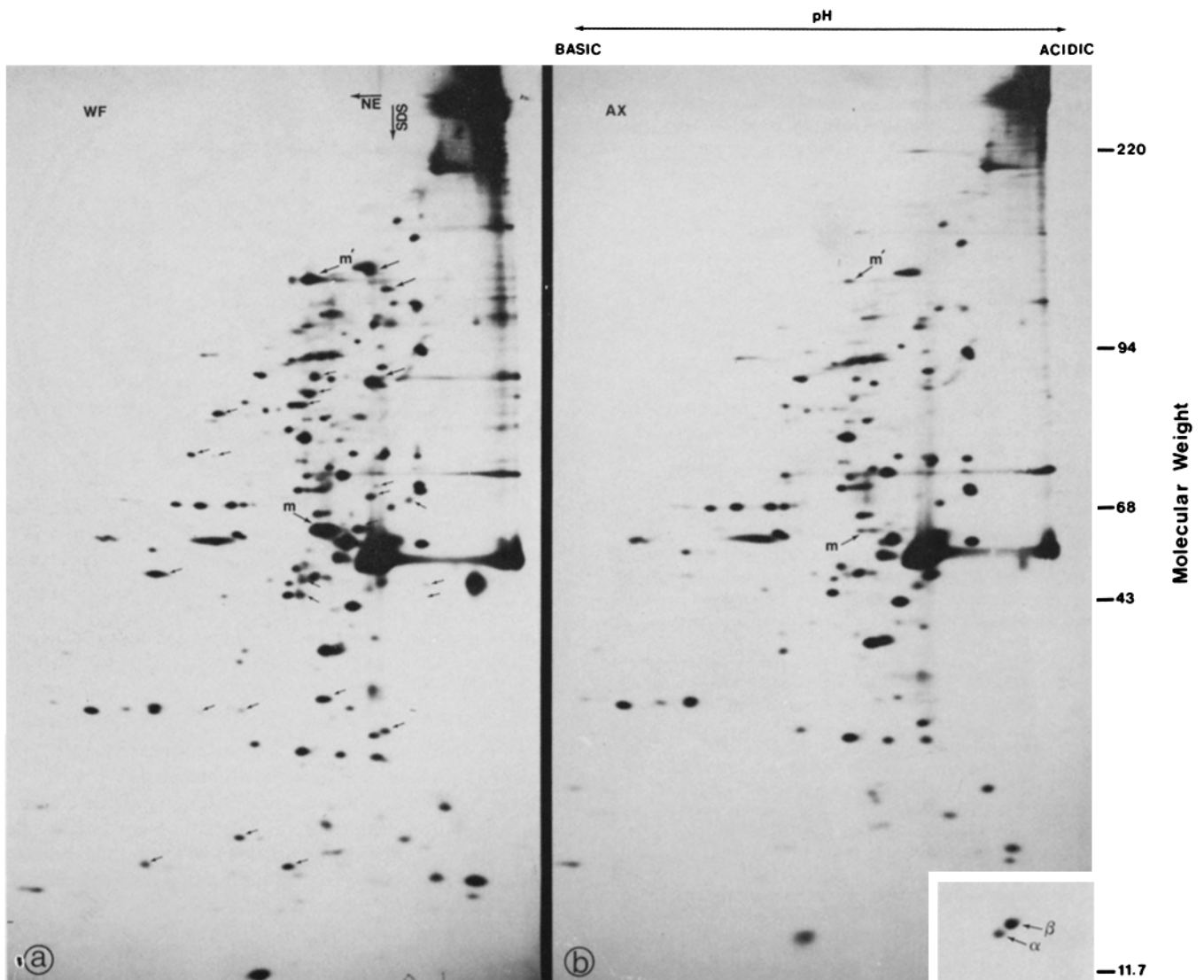


FIGURE 2 Fluorographs of two-dimensional gels of whole flagella (*WF*) (*a*), and axonemes (*AX*) (*b*) labeled to steady state with ^{35}S . *NE*, NEPHGE dimension; *SDS*, SDS PAGE dimension. Very little of the 65,000 and 125,000-mol wt membrane proteins (*a*: *m* and *m'*, respectively), remain in the axoneme preparation *b*. Arrows in *a* indicate minor membrane and matrix proteins. The major high molecular weight membrane protein (48) is not resolved in our two-dimensional gel system. Gels were loaded with $\sim 200,000$ cpm and exposed for 2.5 d. Inset in *b* shows a shorter exposure of the tubulin region. Identification of the upper and lower tubulin spots as β - and α -tubulin, respectively, is based on their isoelectric points and the relative amounts of ^{35}S taken up into their cysteine and methionine under steady state conditions (42, 45, and see text). β -tubulin may run ahead of or behind α -tubulin in SDS acrylamide gels, depending on the source of SDS used (2; and see Fig. 13). The molecular weight standards ($\times 10^3$) used for calibration of the gels were cytochrome C (11,700), ovalbumin (43,000), bovine serum albumin (68,000), phosphorylase A (94,000), and myosin (220,000).

(R1-R14, Fig. 4*a*) were found to be reproducibly missing in *pf-14* axonemes (not shown). These proteins are probably all components of the radial spokes. Six of the 14 proteins missing in *pf-14* (proteins R1, R2, R7, R8, R10, and R11) were also missing in *pf-1* (not shown), indicating that these proteins are contained in the spokehead. These results are in good agreement with previous studies of the protein deficiencies in *pf-14* and *pf-1* axonemes (34, 51).

Proteins associated with a unique group of three protofilaments which form the wall between the lumens of the A and B tubules of the outer doublets (48) were identified by analysis of the isolated partitions on two-dimensional gels (results not shown) (13; C. R. Gattass and G. B. Witman, manuscript in preparation). Four of these proteins relevant to this study are indicated in Fig. 4*a*.

A number of low and medium molecular weight proteins associated with the 12s and 18s dynein ATPases were identified by coelectrophoresing the purified dyneins (11) with isolated axonemes. Four of these components analyzed in the present study are indicated in Fig. 4*a*. One protein (*A*, Fig. 4*a*), possibly associated with the 12s I dynein contained in the inner arm (10), coelectrophoresed with actin isolated from *Dictyostelium* (38). This agrees with reports that an actinlike component can be isolated from axonemes (35), and that a protein with an electrophoretic mobility identical to that of actin is missing in mutant *pf-23*, which lacks the inner arm (18).

Fig. 4 summarizes the proteins of interest to this study which have been identified with particular structures in the axoneme. With the exception of the α - and β -tubulins, each of these proteins comprises a small percentage of the axonemal protein

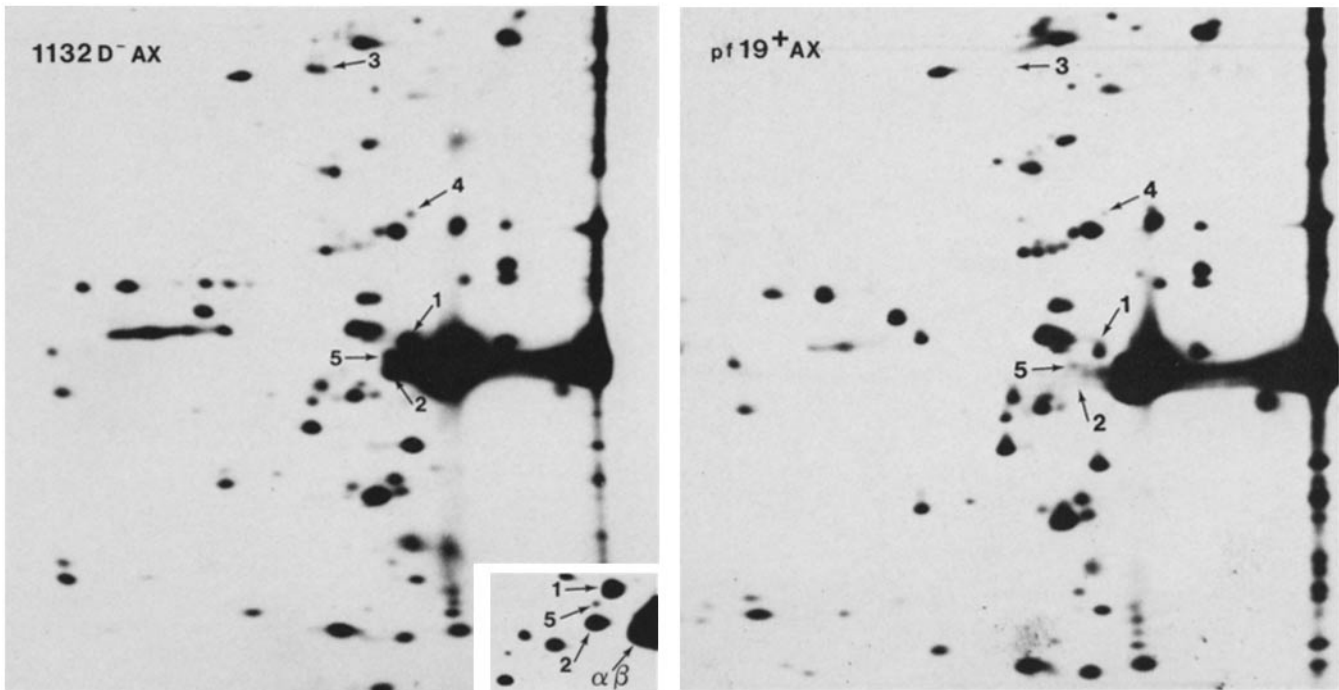


FIGURE 3 Fluorographs of NEPHGE-SDS gels of axonemes of wild-type (1132D) and the central-tubuleless mutant *pf-19*. The numbered arrows in the wild-type gels indicate some of the proteins missing in the mutant; the positions of these deficiencies are indicated by numbered arrows in the mutant fluorograph. ~200,000 cpm were loaded and the gels exposed for 2.5 d. Inset: Portion of a fluorograph of another gel of wild-type axonemes. Polypeptides CT1, CT2, and CT5 are more clearly resolved from the tubulins (α , β) at this lower loading and shorter exposure. 100,000 cpm, 1.5-d exposure.

in either Coomassie Blue-stained gels or fluorographs of ^{35}S -labeled proteins. Fig. 4*b* shows the results for an ^{35}S -labeled preparation run on a two-dimensional gel and quantitated as described below. The identified minor proteins individually contained 0.03% to 0.26% of the TCA-precipitable radioactivity loaded onto the gels. These values do not necessarily represent the relative amounts of these proteins in the axoneme, but reflect (a) the relative amounts of sulfur-containing amino acids in the proteins and (b) the relative amounts of the proteins entering our gel system (some material does not enter the first-dimensional gel).

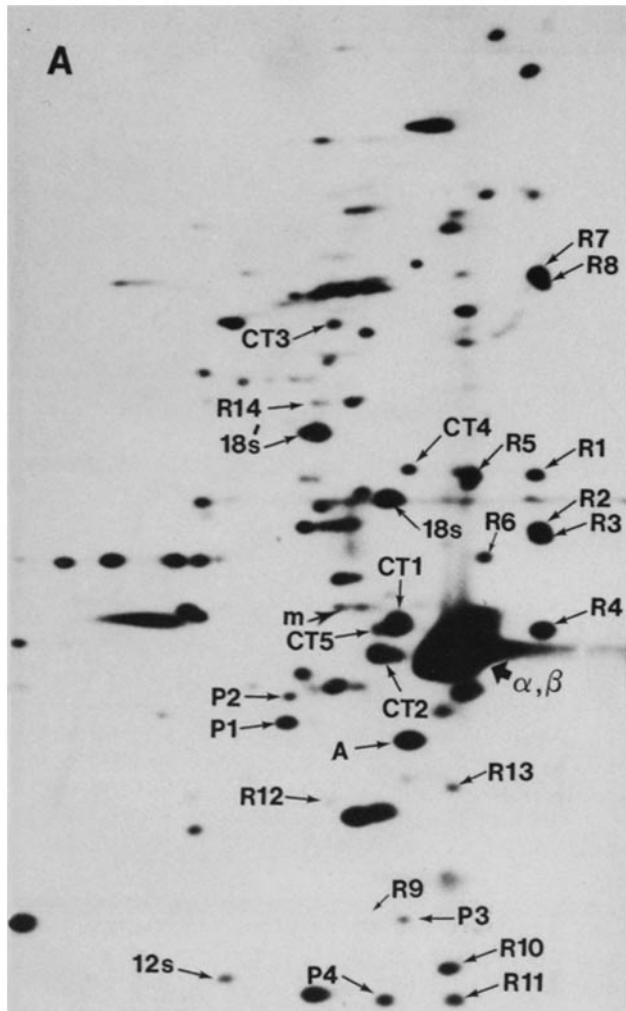
Experimental Design

Gametic cells rather than vegetative cells were used for studies on protein synthesis during flagellar regeneration for several reasons. Gametic cells appear to have smaller pools of unassembled flagellar protein than vegetative cells. Cycloheximide-treated gametes regenerate flagella of only one-fifth to one-tenth the normal length (23), as compared with the one-half normal length flagella of vegetative cells so treated. Gametes also have a 10-fold higher rate of $^{35}\text{SO}_4^{-2}$ incorporation into TCA-precipitable protein (unpublished result). Both factors result in an increase in the specific activity of proteins labeled during regeneration. Moreover, deflagellated gametic cells regenerate flagella more slowly than vegetative cells (23). The time required for regeneration is 140 min for gametes as compared to 90 min for vegetative cells. The final length of the regenerated flagella is approximately the same in both gametes and vegetative cells. This longer regeneration time makes possible increased temporal resolution in pulse label experiments.

The kinetics of flagellar regeneration in a population of gametes is illustrated in Fig. 5. The time periods chosen for pulse labeling are superimposed on the curve. These periods included a time before deflagellation (to determine basal levels of synthesis, transport, and assembly of flagellar proteins), several times during the regeneration process, and a time when the flagellum has nearly regained its normal steady-state length. The rate of incorporation of $^{35}\text{SO}_4^{-2}$ into TCA-precipitable protein before deflagellation and at the different times during regeneration are shown in Fig. 6; the rate varied by no more than $\pm 12\%$ within an experiment. After the pulse, whole cells, whole flagella, or flagellar fractions were isolated and electrophoresed on one- and two-dimensional gels.

For quantification, these labeled samples were run together with unlabeled axonemal carrier protein on two-dimensional gels. Fig. 7 shows a Coomassie-Blue-stained gel of an ^{35}S -labeled whole cell preparation coelectrophoresed with unlabeled axonemal material. It should be pointed out that although some of the heavily labeled spots in the fluorographs were not always well resolved due to β -particle spreading and saturation of the emulsion, the flagellar components appeared as discrete spots in the stained gels (cf. Figs. 7 and 10*a*, 30-min gel). Spots of interest were excised, solubilized, and counted, all as described in the experimental procedures.

To check the accuracy of our quantitation procedures over a range of cpm/spot, we determined the amount of label in the α - and β -tubulins on gels loaded with different amounts of ^{35}S -labeled axonemes together with the standard amount of unlabeled axonemal carrier protein (75 μg). The radioactivity recovered was linearly proportional to the radioactivity loaded for both species of tubulin (Fig. 8). Similar results were obtained for other proteins quantitated. The difference in incorporated counts between the α - and β -tubulins is due to the



B

Protein	³⁵ S cpm in AX
	%
α-Tubulin	6.20
β-Tubulin	9.57
R1	0.05
R2	0.11
R3	0.07
R4	0.26
R5	0.07
R6	0.04
R7	0.08
R8	0.05
A	0.16
18s'	0.11
CT1	0.26
CT2	0.24
P1	0.12
P2	0.03

FIGURE 4 (A) Fluorograph of NEPHGE-SDS gel of wild-type axonemes summarizing proteins relevant to our study which were identified by analysis of mutants and flagellar fractions. (B) Quantification of the label in components of axonemes labeled to steady-state with ³⁵S and coelectrophoresed with 50 μg of unlabeled axonemes. Percentages were calculated relative to the total TCA-precipitable material loaded per gel and do not reflect the relative amounts of each protein in the axoneme (see text). α, β: α- and β-tubulins, respectively; R1-R14: radial-spoke proteins; A: actinlike component; CT1-CT5: central-tubule proteins; P1-P4: partition components; 12s, 18s: proteins associated with the 12s I and 18s dyneins,

difference in cystine and methionine content between the two proteins (42, 45).

One cannot assume that the same proportion of each flagellar protein enters the gel system. However, because the values obtained for individual proteins are directly proportional to the amount of radioactive protein loaded, comparison of the

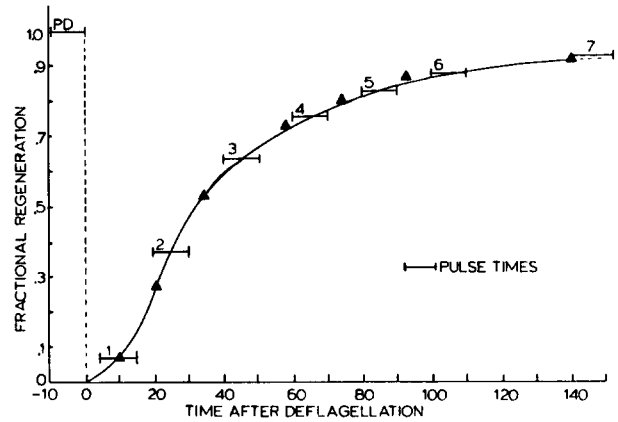


FIGURE 5 Regeneration kinetics for a population of gametes deflagellated at time zero, with the periods (labeled PD and 1-7) of pulse labeling superimposed on the regeneration curve. Initial length of flagella was 12 μm.

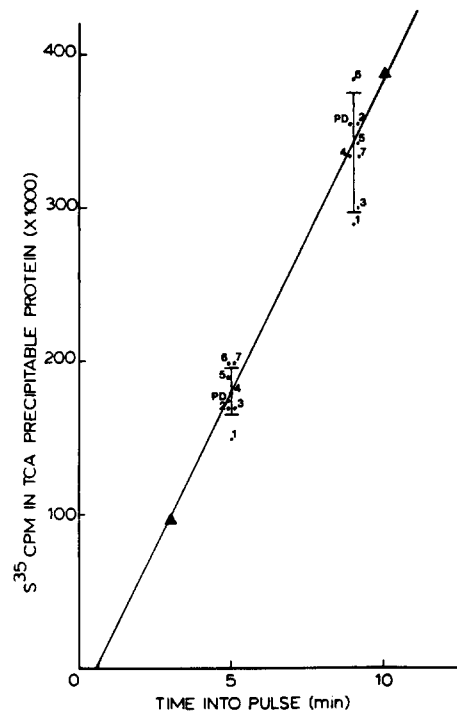


FIGURE 6 Incorporation of ³⁵SO₄⁻² into whole gametes before deflagellation and during regeneration. Counts per minute incorporated into TCA-precipitable protein (per 25-μl aliquot of the cell suspension) is plotted against time into the pulse for each of the pulse periods (1-7 and PD) indicated in Fig. 5. The pulses were done in the presence of chloramphenicol to block chloroplast and mitochondrial protein synthesis. Chloramphenicol had no effect on the kinetics of regeneration, and a comparison of the patterns of synthesis of several proteins during regeneration in the presence and absence of chloramphenicol indicated that the drug did not affect the synthesis of flagellar proteins. The calculated linear regression line (▲) is also shown.

respectively; m: residual amount of the 65,000-mol wt membrane protein.

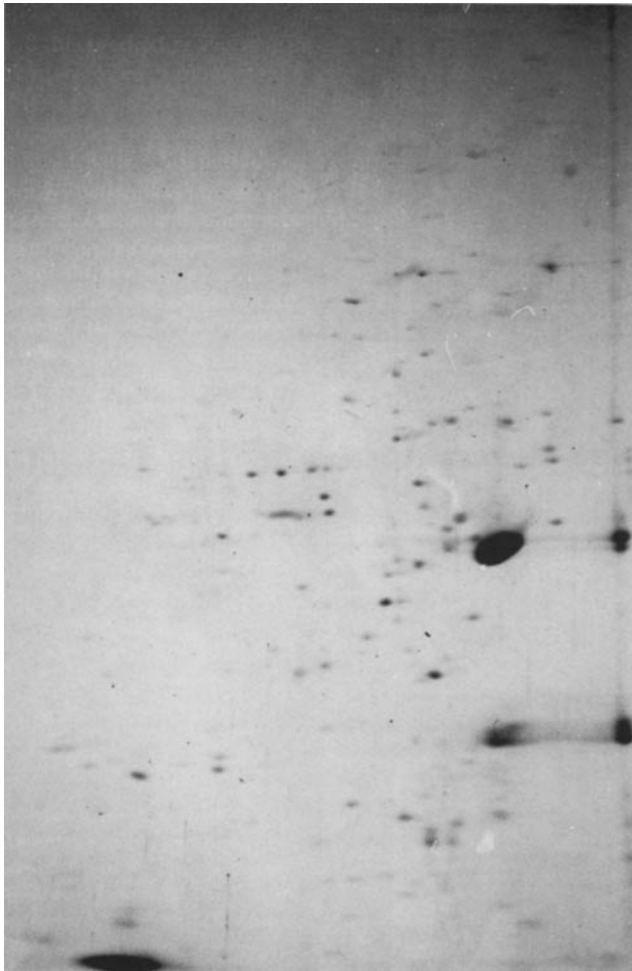


FIGURE 7 Two-dimensional gel stained with Coomassie Brilliant Blue. 50 μg of axonemes were coelectrophoresed with 10 μg of ^{35}S -labeled whole cell protein isolated at 30 min into regeneration. The axonemal proteins are visible as discrete spots; α - and β -tubulin could be resolved in the actual gel.

relative amounts of radioisotope in spots at different times during regeneration will give an accurate indication of the relative timing and amounts of increases or decreases of label within or between specific proteins.

Synthesis of Flagellar Proteins During Regeneration

To study the time of induction of flagellar proteins and their rates of synthesis at various times after deflagellation, regenerating gametes were pulse labeled at the previously indicated times and whole cells isolated and analyzed by gel electrophoresis. Fig. 9 shows a fluorograph of a one-dimensional gel of these samples along with a whole flagellar standard. Uptake of label into the α - and β -tubulins was substantial as early as 15 min after deflagellation and reached a peak 30–50 min into regeneration (see below for quantitation). The 65,000-mol wt membrane protein was labeled most heavily at the earliest time point. Label appeared in the high molecular weight dynein components only during the second and subsequent time points. Some minor proteins (e.g., see arrow in Fig. 9, right hand margin) were maximally labeled near the end of regeneration.

To achieve better resolution of individual proteins, these

samples were analyzed on two-dimensional gels. Portions of the fluorographs are shown in Fig. 10a. As noted earlier, the overall rate of incorporation of $^{35}\text{SO}_4^{-2}$ remained constant during regeneration; the increase in the amount of isotope in the region of the gels shown was offset by a complementary decrease in other portions of the gels (see Fig. 9).

The first panel in Fig. 10a (PD) shows protein synthesis in the nondeflagellated cells. Substantial synthesis occurred for the α - and β -tubulins, the central tubule complex protein 1 (CT1), and the 65,000-mol wt membrane protein. This synthesis was not due to flagellar regeneration in a subpopulation of the cells which might have spontaneously lost their flagella; light microscope examination of >100 cells showed that before deflagellation all cells had fully grown flagella. Nor was the synthesis due to slight flagellar growth in the entire population of cells; gametic flagella undergo virtually no change in length over several hours under our experimental conditions (38).

The synthesis of most flagellar components was coordinately stimulated following flagellar amputation. The gel patterns for the regenerating cells showed that tubulin synthesis was greatly elevated for the first 50 min after deflagellation, and then steadily declined to predeflagellation levels. The central tubule proteins CT1 and CT2 were not well resolved (in the fluorographs) during the early times, but CT2 synthesis clearly peaked between 15 and 50 min, and then declined to background levels by 90 min into regeneration (circle, 90 min). Synthesis of the 65,000-mol wt membrane protein (*m*) and the radial spoke proteins (marked by arrows in 15-, 30-, and 50-min gels) also peaked early during regeneration. A large number of nonflagellar proteins of molecular weight $<65,000$ were induced after deflagellation; these may represent "stress-in-

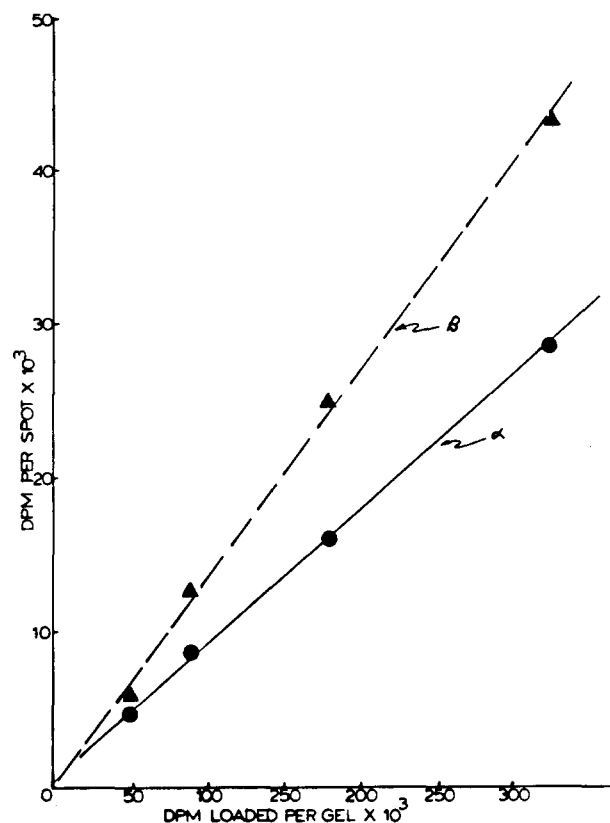


FIGURE 8 Quantification for two-dimensional gels in which various amounts of ^{35}S -labeled axonemes (60,000 dpm/ μg of TCA-precipitable protein) were coelectrophoresed with 75 μg of unlabeled axonemes. Gels were stained and spots corresponding to α - and β -tubulin were cut out, solubilized, and counted.

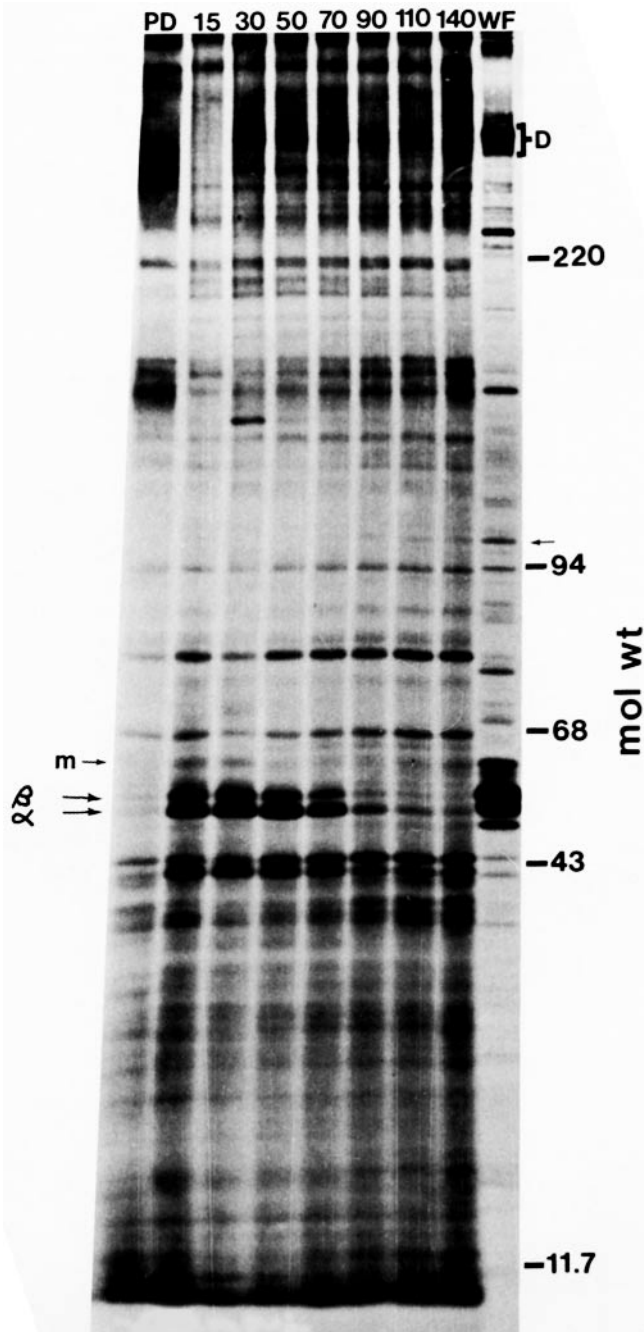


FIGURE 9 Fluorograph of a 5–15% acrylamide gradient gel of whole cell proteins from regenerating cells prepared at the indicated times during regeneration. Cells were pulsed with $^{35}\text{S}\text{O}_4^{-2}$ for 10 min immediately before their isolation. Each channel was loaded with an approximately equal number of cells. PD: predeflagellation sample; WF: steady-state-labeled whole flagella; α , β : α - and β -tubulin, respectively; m: 65,000-mol wt membrane protein; D: high molecular weight dynein components; arrow (right hand margin) indicates a component labeled late in regeneration. 55,000 cpm/channel; 2-d exposure. Molecular weight standards ($\times 10^3$) were the same as for Fig. 2.

duced proteins" similar to those synthesized in starved *Tetrahymena* as a response to deciliation, heat shock, or release from anoxia (16). In *Chlamydomonas*, several proteins are induced by heat shock; the same proteins are a subset of those induced after flagellar amputation (29).

The changes in the uptake of label into certain of the flagellar components were quantitated as described above. Fig. 10 b and c show the results for the α - and β -tubulins, the 65,000-mol wt membrane protein, the radial spoke proteins 1–4, the central tubule complex proteins 1 and 2, and the actinlike component.

Synthesis of the tubulins peaked between 30 and 50 min into regeneration (Fig. 10 b: α , β); the rate of tubulin synthesis at this time was approximately 10-fold higher than the predeflagellation rate. The relatively high level of incorporation of isotope into α -tubulin compared to β -tubulin may reflect preferential synthesis of the α -tubulin (cf. Figs. 4 and 8).

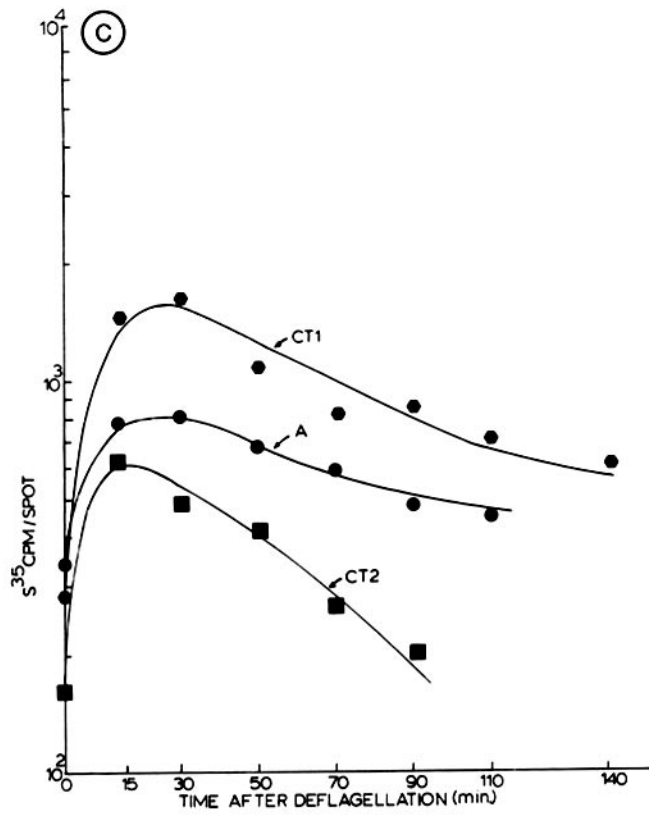
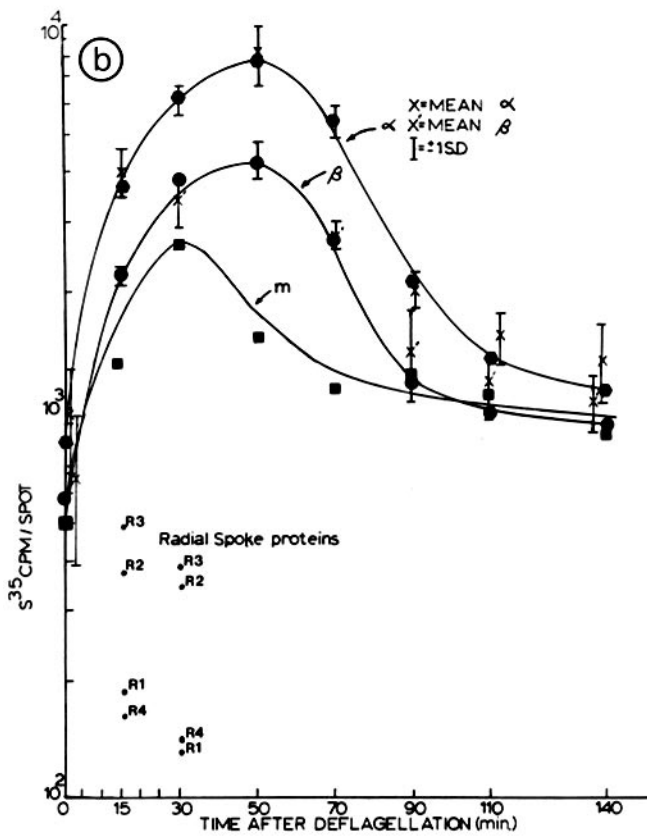
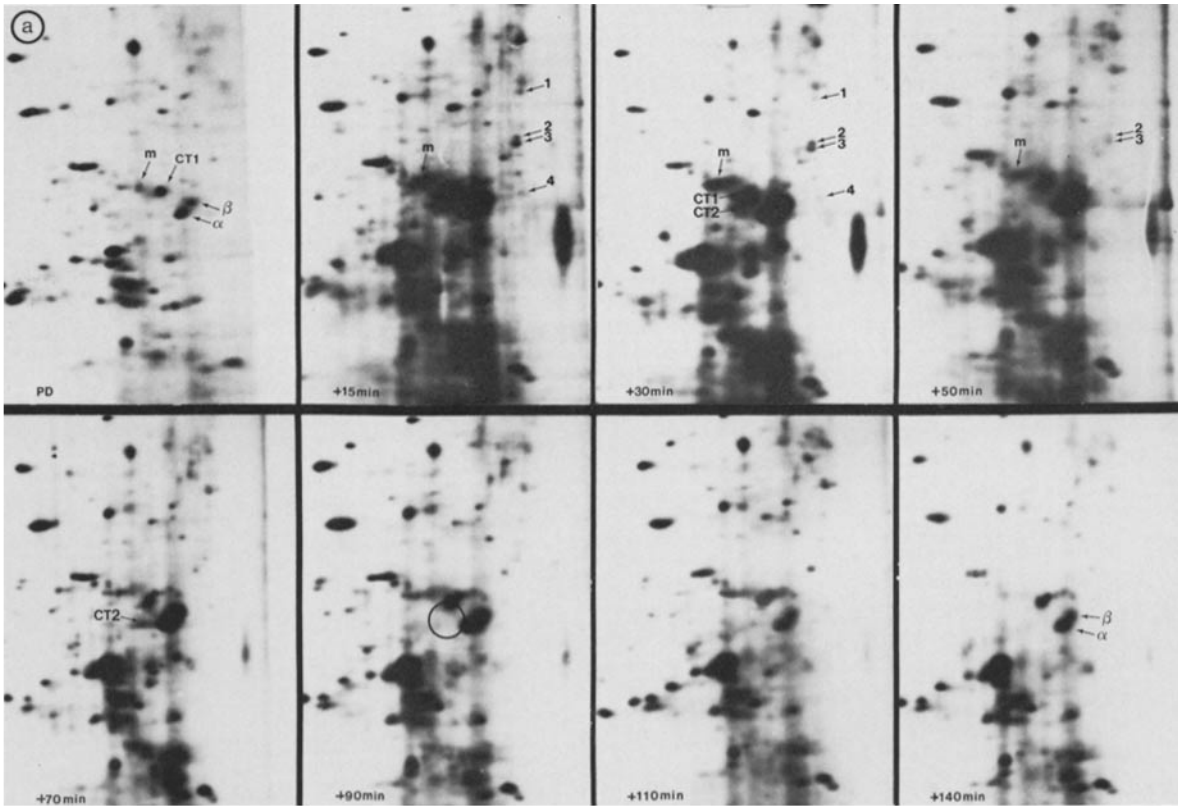
The 65,000-mol wt membrane protein reached a maximum rate of slightly more than fourfold its basal level of synthesis at ~ 30 min into regeneration. It subsequently showed a more gradual decline in labeling than did the tubulins. The rates of synthesis of radial spoke proteins 1–4 peaked by 15 min and declined by 30 min. Because the amount of label recovered from the radial-spoke proteins at the other times approached background levels, the rate of synthesis of these proteins before deflagellation and late in regeneration could not be accurately estimated. However, the data do indicate that after deflagellation their rate of synthesis rapidly increased to at least 10-fold the predeflagellation rate, and then rapidly decreased to relatively low levels.

Incorporation of label into the central tubule protein CT2 increased fourfold to a peak by 15 min and then dropped rapidly, approaching predeflagellation levels by 90 min into regeneration. Uptake of label into CT1 increased fivefold to a maximum by 30 min and gradually declined.

In contrast, the actinlike protein showed slightly more than a twofold increase in labeling after deflagellation. This was by far the smallest increase observed for any flagellar protein. The possibility exists that other actin species are contained in this spot and contribute to the basal level of synthesis; one species might then be elevated several-fold during regeneration, but the increase in the spot as a whole would appear much smaller. In any case, other studies (see below) indicate that nondeflagellated cells contain a relatively large pool of actin available for flagellar formation, and that new synthesis makes a negligible contribution to the actin used in assembly of a new flagellum.

This analysis of protein synthesis in whole cells indicated that at least several flagellar proteins, including α - and β -tubulin, the 65,000-mol wt membrane protein, and CT1, were continuously synthesized at low levels in nonregenerating gametes. After deflagellation, the synthesis of the majority of flagellar proteins was stimulated at least four- to tenfold; for most proteins, this enhancement began at the same time (within the limits of our time resolution, which was ~ 15 min). Proteins contained within any one type of axonemal structure (such as the radial spokes) usually had similar kinetics of synthesis, but

FIGURE 10 (a) Portions of fluorographs of two-dimensional gels of whole cell proteins processed before deflagellation (PD) and at the indicated times during regeneration. Cells were pulsed with $^{35}\text{S}\text{O}_4^{-2}$ for 10 min immediately before isolation. $\sim 1.25 \times 10^8$ cells containing 10^8 cpm were loaded per gel; 24-h exposure. α , β : α - and β -tubulin, respectively; m: 65,000-mol wt membrane protein; CT1, CT2: proteins of the central tubule–central sheath complex; arrows 1–4: (15- to 50-min gels), radial-spoke proteins 1–4. The circle in the 90-min gel indicates the position of CT2, which is not visible beyond 70 min. Because a large number of cpm



were loaded on the gels (10^6 cpm vs. 5.5×10^4 cpm in Fig. 9) to permit detection of minor components, some regions of the fluorographs are not well resolved. This loss of resolution was due to saturation of the emulsion and the resulting coalescence of spots in the fluorographs, and was not a problem inherent in the gels themselves; at shorter exposures the major components were well resolved. This smearing thus did not affect our ability to quantitate the gels. (b and c) Quantification of label in flagellar proteins in whole-cell samples from predeflagellation and regenerating cells. Data is shown for the α - and β -tubulins (α , β), the 65,000-mol wt membrane protein (m), the radial-spoke proteins ($R1-R4$), central-tubule proteins 1 and 2 ($CT1$, $CT2$), and the actinlike protein (A) probably associated with the 12s I dynein ATPase. (b) also shows the means (X , X') and standard deviations (I) for the pooled data from this and another experiment where the α - and β -tubulins were quantitated.

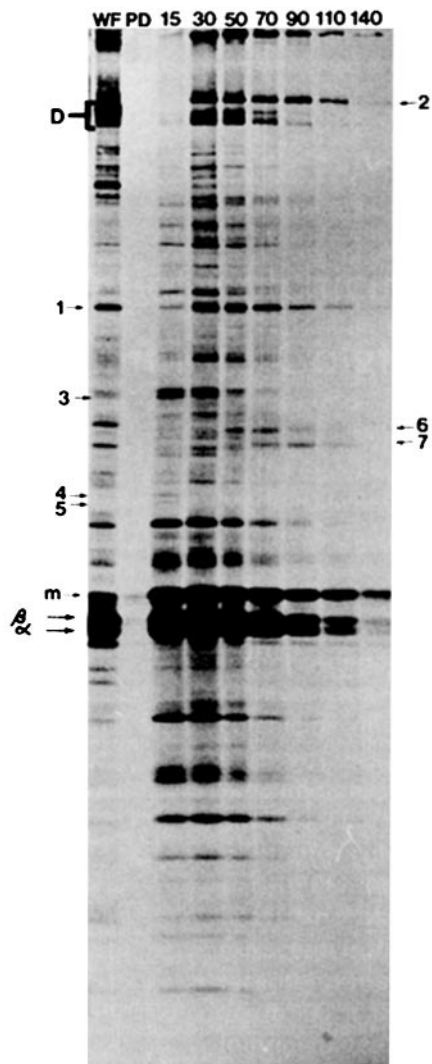


FIGURE 11 Fluorograph of a gel of whole flagella proteins isolated before deflagellation (*PD*) and at the indicated times during regeneration. The cells were pulsed with $^{35}\text{SO}_4^{-2}$ for 10 min immediately before isolation of the flagella. Equal numbers of flagella were loaded in channels *PD* through *140*, with a maximum loading of 100,000 cpm for the 30 min time point and the whole flagella standard (*WF*), which was labeled to steady-state. The gel was exposed for 24 h. A shorter exposure of the tubulin region is shown at the right to clarify the labeling pattern for the 65,000-mol wt membrane protein (*m*), and the α - and β -tubulins (α , β). *D*, high molecular weight dynein bands. Proteins designated 1–7 are discussed in the text.

groups of proteins contained within different structures were often synthesized with dissimilar kinetics.

Transport of Newly Synthesized Proteins into the Flagellum

The above experiments established the timing and rates of synthesis of a number of flagellar proteins. To determine the timing and rates of transport of these newly synthesized proteins into the flagellum, cells were pulsed at various times prior to and during regeneration and the flagella were isolated and prepared for electrophoretic analysis.

Fig. 11 shows a fluorograph of a one-dimensional gel of these samples. Label appeared in the α - and β -tubulins at the earliest time period and peaked by 30 min into regeneration. Most of the proteins followed the tubulin pattern. However, in a number of proteins the appearance of isotope either: (a) was delayed relative to the tubulins (e.g., proteins 1 and 2 [the high molecular weight membrane component], and the high molecular weight dynein components) and declined more slowly (protein 1); (b) peaked earlier (proteins 3, 4, and 5); or (c) peaked later (proteins 6 and 7) during regeneration. Label was taken up into the 65,000-mol wt membrane protein in much-greater amounts than would be expected from the relative amount of label incorporated into it under steady-state condi-

tions; this may indicate that the cytoplasmic pool for this component is relatively small compared to those for most other proteins. Note that the high molecular weight membrane protein (protein 2) shows a later uptake of label during regeneration than does the 65,000-mol wt membrane component.

These flagellar samples were also analyzed in two-dimensional gels. Equal numbers of flagella were run on the gels; Fig. 12 shows portions of the resulting fluorographs. Label appeared in most of the previously identified proteins at one time or another during regeneration. Duplicate gels were quantitated as previously described (Fig. 12 *b-d*).

NONREGENERATING FLAGELLA: The first panel of Fig. 12 *a* shows the transport of labeled proteins into nonregenerating flagella. Label appeared in the 65,000- and 125,000-mol wt membrane proteins and in components which comigrated with the α - and β -tubulins. As previously mentioned, this uptake was not due to formation of new flagella in a subpopulation of the cells nor to continued elongation of previously formed flagella. The appearance of label in proteins under these conditions must therefore represent *turnover* of these proteins in the flagellum. Further evidence for this comes from the fact that the spot pattern in the tubulin region is different in nonregenerating and regenerating flagella (see below).

To determine whether this turnover was confined entirely to the membrane and matrix, or whether some turnover occurred in the axoneme, nonregenerating cells were pulse labeled for

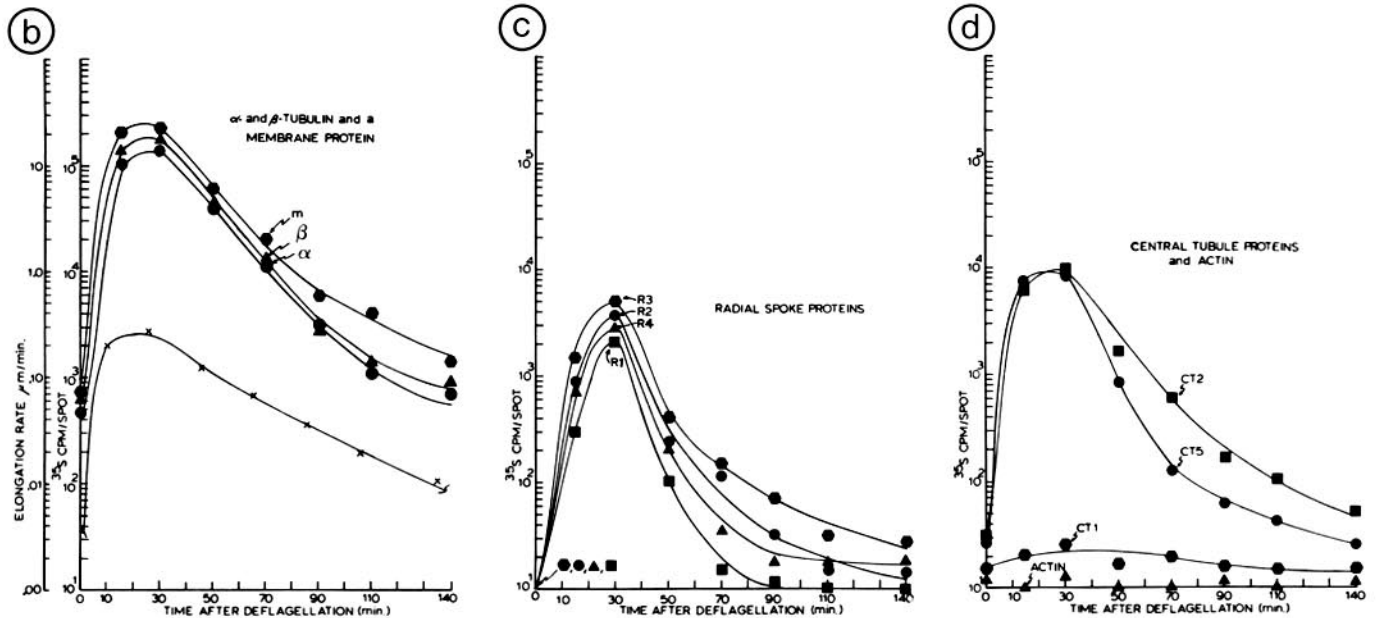
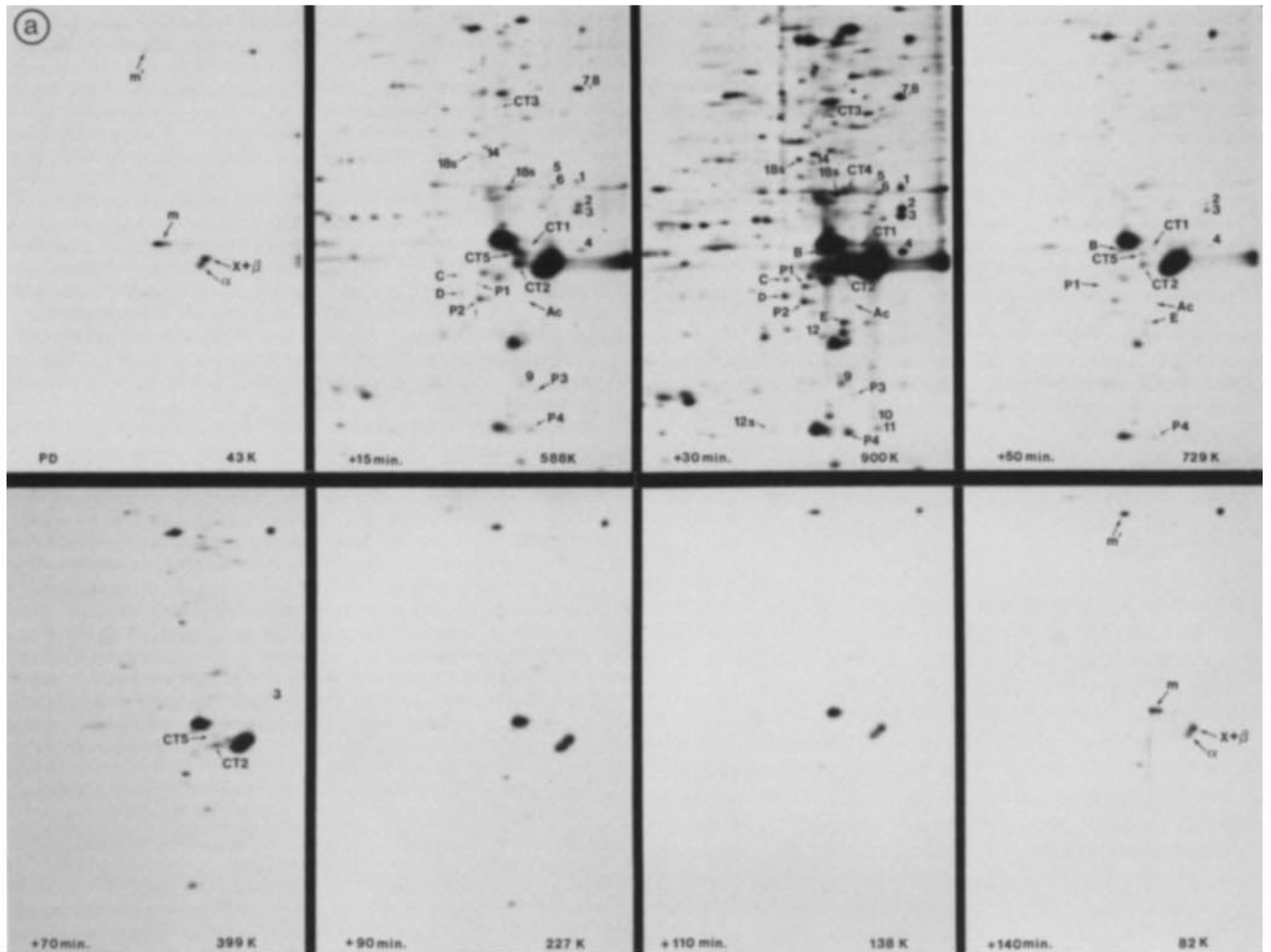


FIGURE 12 (a) Portions of fluorographs of two-dimensional gels of whole flagella isolated before deflagellation (PD) and at the indicated times during regeneration. Equal numbers of flagella ($\sim 2 \times 10^6$) were loaded on each gel; the total cpm loaded are shown in the lower right corner of each panel. The gels were exposed for 2 d. α , β : α - and β -tubulin, respectively; *m* and *m'*, 65,000 and 125,000-mol wt membrane components, respectively; CT2–CT4: central-tubule-complex proteins; 1–14: radial-spoke proteins; P1–P4: proteins associated with the partition protofilaments; 12s, 18s: 12s I and 18s dynein components, respectively; B–E: unidentified proteins discussed in the text; Ac, CT1: positions where the actinlike component and CT1 were expected; X: axonemal component with mobility similar to β -tubulin in gels run with Bio-Rad SDS. (b–d) Quantification of the appearance of label into specific proteins within the flagellum during regeneration. α , β : α - and β -tubulin, respectively; *m*: 65,000-mol wt membrane component; R1–R4: radial-spoke proteins; CT1, CT2, CT5: central tubule complex proteins; A: the actinlike component; x—x, the average rate of flagellar growth ($\mu\text{m}/\text{min}$) during each of the pulse periods.

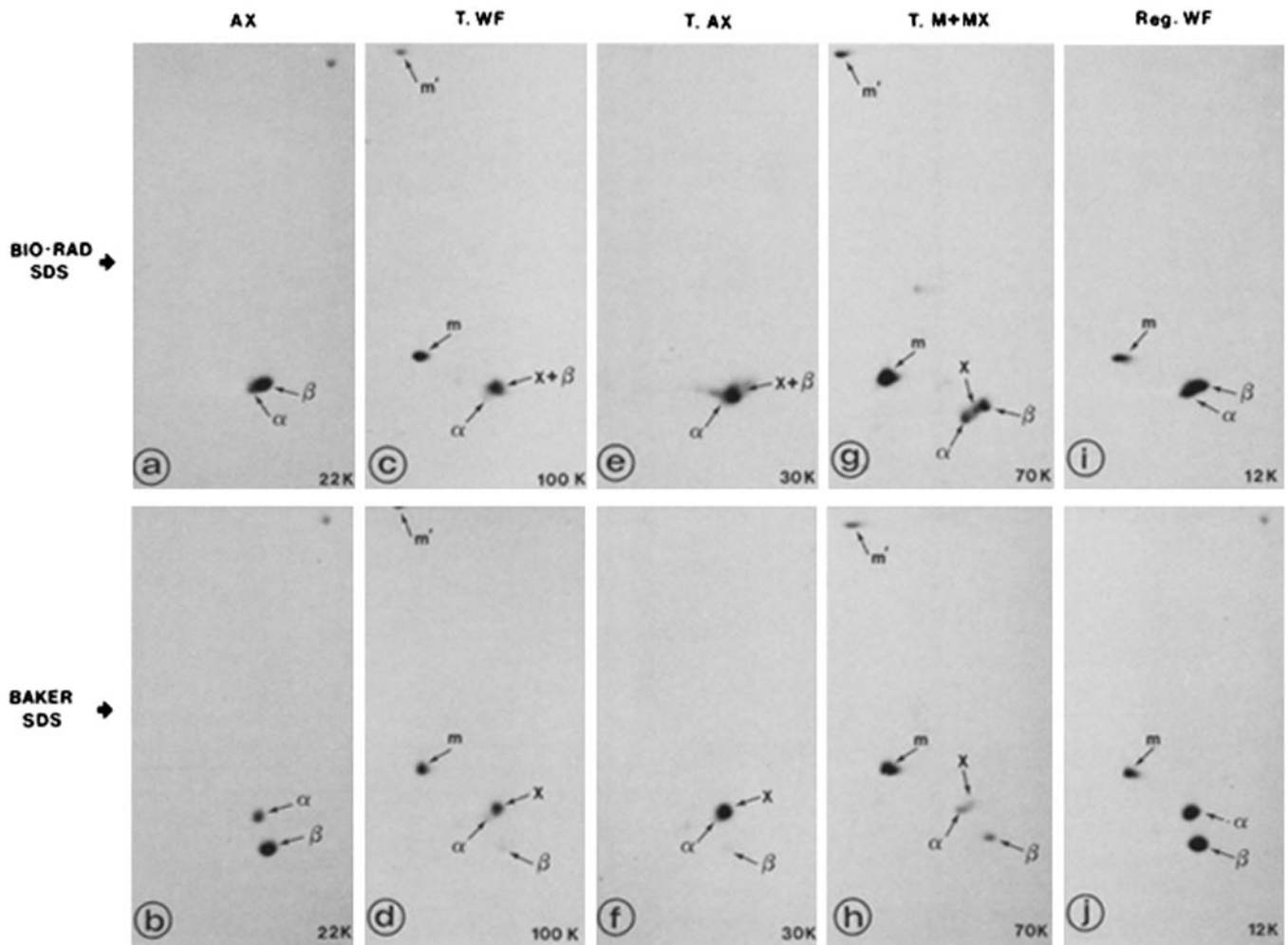


FIGURE 13 Portions of fluorographs of two-dimensional gels prepared using SDS from Bio-Rad Laboratories (panels *a*, *c*, *e*, *g*, and *i*) or the Baker Chemical Co. (panels *b*, *d*, *f*, *h*, and *j*). *T. WF*, *T. AX*, and *T. M + MX*: whole flagella, axoneme, and membrane and matrix fractions respectively. All were isolated from nondeflagellated cells immediately after a 10-min pulse with ^{35}S . *AX*, steady-state ^{35}S -labeled axonemes; *Reg. WF*, whole flagella isolated from regenerating cells immediately after an ^{35}S pulse from 5–15 min after flagellar amputation (similar to 15-min time point, Figure 12 *a*). α , β , α - and β -tubulins, respectively; *m* and *m'*, 65,000 and 125,000-mol wt membrane proteins, respectively; *X*, component with a mobility similar to β -tubulin in the presence of Bio-Rad SDS. Material recovered from approximately equal numbers of flagella were loaded on gels corresponding to panels *c*–*h*; exposures in cpm·d are shown in the lower right hand corner of each panel.

10 min and whole flagella, axoneme, and membrane and matrix fractions were isolated and analyzed in two-dimensional gels (Fig. 13 *c*, *e*, and *g*). As expected, all of the labeled membrane components were in the membrane and matrix fraction (Fig. 13 *g*); this fraction also contained a substantial amount of labeled α - and β -tubulin. Most of the label in the axonemal fraction (Fig. 13 *e*) comigrated with β -tubulin. However, we questioned whether this label was actually in β -tubulin, because (*a*) our synthesis studies suggested that α -tubulin was preferentially synthesized in nonregenerating cells, and (*b*) it seemed unlikely that there would be turnover of assembled β -tubulin but not α -tubulin in the nongrowing axoneme. Therefore, to resolve β -tubulin from any components which might be comigrating with it, the samples were electrophoresed on similar gels containing SDS from the Baker Chemical Co. (Phillipsburg, NJ) rather than from Bio-Rad Laboratories. In our gel system, β -tubulin migrates considerably more rapidly than α -tubulin when Baker SDS is used (cf. Fig. 13 *a* and *b*, and see reference 2).

Comparison of the spot patterns obtained for the whole

flagella sample in the presence of the two brands of SDS (Fig. 13 *c* and *d*) showed that, although α - and β -tubulins were present in the preparation, the majority of the label comigrating with β -tubulin in the presence of Bio-Rad SDS did not shift mobility in the presence of Baker SDS and therefore was not β -tubulin but rather some other component (component "X"). An even greater proportion of the label incorporated into the axonemes was in this component and did not shift mobility in the presence of Baker SDS (cf. Fig. 13 *e* and *f*); only a very small amount of label was present in α - and β -tubulin, and this label was evenly distributed between the two tubulins. In the membrane and matrix fraction, some label was contained in component X, but much more was present in α - and β -tubulin (cf. Fig. 13 *g* and *h*). Component X was not observed in steady-state-labeled axonemes (Fig. 13 *a* and *b*), indicating that it is present in the axoneme in very small amounts relative to the tubulins. Furthermore, it was not observed in regenerating whole flagella isolated from cells pulsed from 5–15 min (Fig. 13 *i* and *j*) or from 20–30 min (not shown) after deflagellation, indicating that the majority of the label comigrating

with α - and β -tubulin in regenerating flagella (see below) is in authentic α - and β -tubulin.

REGENERATING FLAGELLA. In the regenerating flagellum, uptake of labeled α - and β -tubulin and the 65,000-mol wt membrane protein peaked at 30 min (Fig. 12*a* and *b*). The relative amounts of newly synthesized 65,000-mol wt membrane component and α - and β -tubulins transported into the flagellum did not reflect the relative rates of synthesis for these components (compare Figs. 10*b* and 12*b*); as noted previously, the comparatively high rate of transport for the 65,000-mol wt membrane component suggests that there is a smaller usable pool for this protein. The uptake of labeled 65,000-mol wt membrane protein decayed at a slightly slower rate than that of the tubulins; these kinetics are probably due to the fact that synthesis of the membrane component did not drop off as rapidly during regeneration as did that of the tubulins (see Fig. 10*b*). Although the maximum rate of tubulin synthesis occurred at 30–50 min, the maximum rate of transport of labeled tubulin into the regenerating flagellum occurred at 15–30 min, apparently reflecting the rate of growth of the organelle, which was greatest during the pulse period ending at 30 min (Fig. 12*b*).

All of the components of the partition protofilaments were most heavily labeled at 30 min (Fig. 12*a*). However, there were differences in the kinetics of appearance of label into these components: between 15 and 30 min, there was a more rapid increase in the amount of label in P1 and P4 than in P2 and P3

(quantification not shown). These results suggest that the partition components are either noncoordinately synthesized early in regeneration or that there are considerable differences in their relative pool sizes.

Most of the radial-spoke proteins showed a maximum uptake of label at approximately 30 min into regeneration. The relative amounts of label in components R1–R3 were similar to the amounts present under steady-state-labeling conditions (cf. Fig. 4; $R4 > R2 \geq R3 > R1$); however, component R4 contained comparatively low levels of label (Fig. 12*c*). This reflects the low rate of synthesis (Fig. 10*b*) and relatively large pool for component R4 (see below).

Uptake of label into central tubule proteins CT2, CT3, CT4, and CT5 also was greatest at \sim 30 min (Fig. 12*a* and *d*). In contrast, CT1 showed a nearly constant low level of labeling throughout regeneration.

Several components of the 12s I and 18s dyneins showed a maximum uptake of label at 30 min into regeneration (Fig. 12*a*). However, at no time during regeneration was significant label detected in the actinlike protein associated with the 12s I dynein.

The very low level of isotope in CT1 and the absence of isotope in the actinlike component of regenerating flagella was not due to lack of synthesis of either of these proteins (Fig. 10*c*), nor to insufficient amounts of newly synthesized 12s I dynein or central tubule material being transported into the flagellum (Fig. 12*a*, 30- and 50-min gels). Moreover, both the actinlike component and CT1 were observed in Coomassie

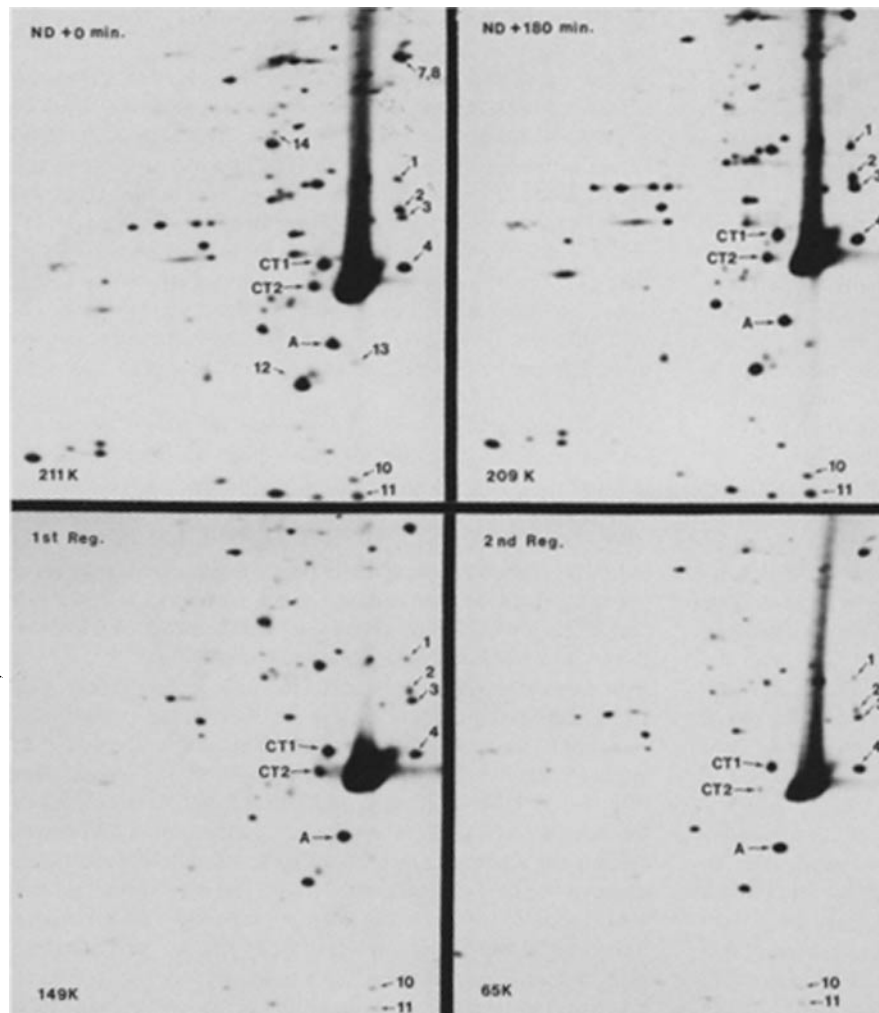


FIGURE 14 ND + 0 min, ND + 180 min, portions of fluorographs of axonemes from nondeflagellated cells isolated at the beginning of the chase and after 180 min, respectively. 1st Reg., 2nd Reg., portions of fluorographs of axonemes from regenerating flagella isolated 140 min after the first and second deflagellation, respectively. 1–4, 7, 8, 10–12, and 14, radial-spoke proteins; CT1, CT2, central-tubule components; A, actinlike component. <1% of the cells failed to regenerate flagella after each amputation, and the protein recovered in each of the samples was proportional to the mean flagellar length in each of the populations from which the samples were isolated. Gels were loaded with an amount of protein proportional to the mean flagellar length in each population to insure that protein from an equal number of axonemes was loaded on each gel. The mean flagellar lengths before deflagellation and at the termination of the first and second regenerations were 11.1 (\pm 0.86 SD), 10.8 (\pm 0.57 SD), and 11.5 (\pm 0.68 SD) μ m respectively. The cpm loaded are indicated in the lower left-hand corner of each fluorograph. 2.5 d exposure.

Blue stained gels of regenerating axonemes (38; results not shown), indicating that both components were transported into the flagellum in an unlabeled form during regeneration. These results suggest that there is a relatively large pre-existing pool for each of these components in nondeflagellated cells (see below).

Spots B-E (Fig. 12a) represent unidentified proteins which were synthesized and transported into the flagellum during regeneration, but were not observed in fluorographs of steady-state labeled whole flagella or in Coomassie Blue stained flagellar preparations. Pulse-chase studies in which cells were pulsed from 15–25 min after deflagellation and their newly formed flagella isolated at 25, 140, and 200 min showed that components C and D were labeled at 25 min and underwent neither an increase nor decrease in labeling at the later times, even though the amount of isotope in total flagellar protein and most individual flagellar components increased fivefold between 25 min and completion of regeneration at 140 min (38; results not shown). Proteins C and D therefore appear to be stable components of the flagellum, but are synthesized and transported into the organelle early in regeneration and in such small amounts that they are not readily detected in steady-state-labeled flagella. By 140 min, the flagella had reached their steady-state length and the pattern of labeling had returned to that seen before deflagellation.

These studies indicated that most newly synthesized flagellar proteins are transported into the regenerating flagellum with kinetics which reflect the rate of growth of the organelle, and that the kinetics of appearance of a labeled protein in the flagellum does not necessarily coincide with the time course of its synthesis in the cytoplasm. Exceptions included CT1 and the actinlike protein, which appeared to be transported into the flagellum from pre-existing, unlabeled pools.

Pool Sizes for Specific Axonemal Proteins

The results of our studies on the transport of newly synthesized proteins into the regenerating flagellum suggested that for certain axonemal proteins there were substantial differences in the relative sizes of their pools in the cell body. To obtain more information on the sizes of the predeflagellation pools of these proteins, isotope dilution experiments were carried out in which vegetative cells were grown in low SO_4^{-2} minimal medium in the presence of $^{35}\text{SO}_4^{-2}$, and transferred to low SO_4^{-2} nitrogen free medium (NFM) containing the same specific activity of $^{35}\text{SO}_4^{-2}$ (see Materials and Methods). The next day the resulting gametes were placed in NFM containing the normal concentration of unlabeled SO_4^{-2} . The cells were then immediately deflagellated and allowed to regenerate flagella for 140 min. A portion of these cells were processed to give isolated axonemes (1st Reg.); the remainder were deflagellated a second time, allowed to regenerate for 140 min, and the newly regenerated axonemes then isolated (2nd Reg.). As controls, axonemes were isolated from a portion of the initial nondeflagellated population of gametes (ND + 0 min), and from a nondeflagellated population after a 3 h chase (ND + 180 min).

With each deflagellation and regeneration, the specific activity (cpm/ μg TCA precipitable protein) of the isolated axonemes decreased by an amount equal to 35–40% of the specific activity of the controls (dashed line in Fig. 15a and b). If there were no cytoplasmic pools for any of the flagellar proteins, this decrease would indicate that the chase was 35–40% effective during the first regeneration and 70–80% effective during the

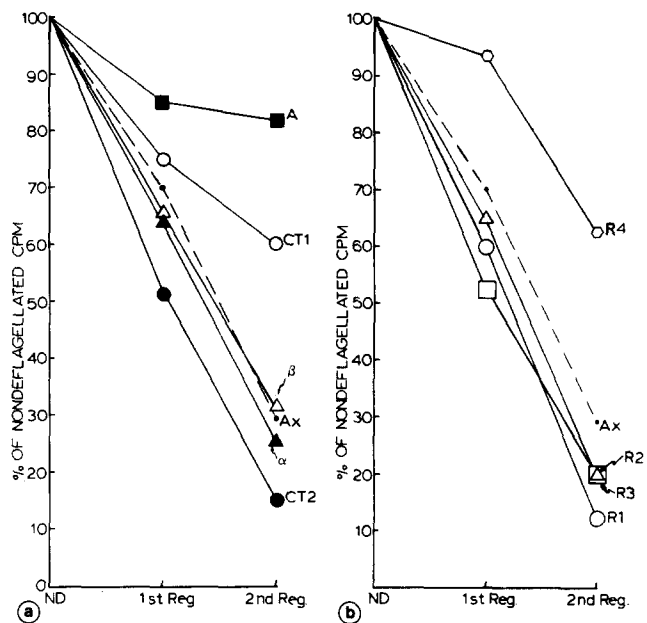


FIGURE 15a and b Quantification for the isotope dilution experiment of Fig. 14. The cpm recovered for specific proteins in axonemes from the first (1st Reg.) and second (2nd Reg.) regeneration were plotted as a percentage relative to those in axonemes from nondeflagellated cells (ND + 0 min). α , β , α -, and β -tubulins, respectively; CT1, CT2, central-tubule proteins; R1–R4, radial-spoke proteins; A, actinlike component. Ax, specific activity (cpm/ μg TCA precipitable protein) of the isolated axonemes plotted as a percentage of the specific activity of the nondeflagellated axonemes.

second regeneration. However, gametic cells are known to contain cytoplasmic pools sufficient to form at least 1–2 μm of flagella in the absence of new protein synthesis (23); these values represent the pool size for the limiting protein, so the total amount of unassembled flagellar protein in the cytoplasm could be considerably greater. Therefore, the chase was probably even more effective than the percentages given above. For example, the decrease in specific activity of the axonemes in this experiment could have resulted from a chase which was 80% efficient, if the average pool size was equivalent to two three-quarter length flagella and 80% of this pool was used during each regeneration.

Equal numbers of isolated axonemes were then loaded on two-dimensional gels. Fig. 14 shows portions of the resulting fluorographs; Fig. 15 shows the quantitation for some of the proteins.

The controls (Fig. 14, ND + 0 min and ND + 180 min) indicated that most of the axonemal proteins are stable components which do not undergo much turnover during a 3-h period. Any proteins undergoing a rapid turnover would have shown a loss of label in the chased controls.

In axonemes regenerated after the first deflagellation (Fig. 14, 1st Reg.), the amount of label in most of the components was visibly less than in the controls. This difference was even greater in axonemes regenerated after the second deflagellation (Fig. 14, cf. ND + 180 min. and 2nd Reg.). Quantification of similar gels indicated that, with each deflagellation and regeneration, the amount of label in the α - and β -tubulins and radial spoke proteins 1–3 decreased 35–40% relative to the control values (Fig. 15a and b); the sizes of the pools of these proteins (relative to their stoichiometries in the axoneme) must therefore be approximately the same. Such a decrease in the α - and β -tubulins was expected in as much as these proteins make up a

substantial proportion of the total axonemal protein.

In contrast, relatively high levels of label persisted in radial spoke protein 4, central-tubule protein 1, and the actinlike protein, even after two deflagellations and regenerations (Fig. 15*a* and *b*). This indicates that comparatively large pools of these proteins must be present in nondeflagellated gametes, and is in good agreement with the results of our studies on the transport of these proteins into the flagellum.

DISCUSSION

Paralyzed mutant and flagellar fractionation studies have allowed a large number of *Chlamydomonas* flagellar proteins to be identified with specific structures. This report has examined the synthesis and transport of some of these proteins in nondeflagellated and regenerating cells to determine whether the synthesis of flagellar proteins is coordinately stimulated and regulated after amputation, and to determine the extent to which new protein synthesis and pre-existing protein pools are involved in flagellar regeneration.

Several proteins, including α - and β -tubulin and the 65,000-mol wt membrane protein, were found to be synthesized at low levels in nonregenerating gametes; these same proteins were also the major species transported to the flagellum of nonregenerating cells. Flagellar fractionation studies showed that most of these labeled proteins were contained in the membrane and matrix fraction. Turnover of components found in this fraction probably represents replacement of proteins lost by blebbing of membrane vesicles from the flagellar tip (3). There are two possible explanations for the presence of α - and β -tubulins in this class of proteins: (*a*) tubulin may be directly associated with the *Chlamydomonas* flagellar membrane, as has been reported for ciliary membranes of *Tetrahymena* (9) and *Aequipectin* (44), or (*b*) unassembled tubulin may be present in the flagellar matrix and become trapped within the membrane vesicles as they are blebbed from the flagellum; the tubulin lost might then be replaced by newly synthesized tubulin. With respect to the latter possibility, it should be noted that Levy (25) has proposed a model in which flagellar growth control is related to the continuous loss of components from the flagellar tip; as the flagellum elongates, this loss, coupled with the increasingly longer time required for diffusion of components down the flagellum, would result in slowing and eventually cessation of growth as the concentration of an essential component decreased and finally dropped below a critical value at the growing tip.

The only protein which turned over rapidly in the axoneme was component X, a protein which comigrated with β -tubulin in the presence of Bio-Rad SDS but was resolved from the tubulins in gels containing Baker SDS. This was a very minor component which could not be detected in steady-state-labeled axonemes and consequently would not have been noticed in our isotope dilution experiments. It would be of considerable interest to know more about this component and its role in the flagellum. Lefebvre et al. (23) have provided evidence that *Chlamydomonas* can "sense" the shortening or absence of its flagella and responds by initiating flagellar protein synthesis; component X could be involved in this "signaling" process if flagellar loss results in its rapid accumulation in the cell body.

In deflagellated cells, synthesis of most flagellar proteins was stimulated at least four- to tenfold above predeflagellation levels; in the case of some of the minor components, such as the radial-spoke proteins, it could not be determined whether this increase represented an induction from a true "off" state

or an elevation from basal levels so low they could not be detected in our experiments. In any case, for most proteins this stimulation appeared to be initiated coordinately (within the limits of our time resolution) during the first 15 min after deflagellation. This is in accord with the observation of Lefebvre et al. (23) that a number of components comigrating with unidentified flagellar proteins were induced within 30 min after deflagellation. However, we also observed a few flagellar proteins which were not synthesized until late in regeneration (see Fig. 9).

Synthesis of the α - and β -tubulins increased rapidly after deflagellation and peaked between 30 and 50 min at a rate at least 10-fold the predeflagellation rate. This 10-fold increase in the level of tubulin synthesis is in good agreement with in vitro studies which have shown that the amount of tubulin mRNA available for in vitro translation is \sim 10-fold higher in regenerating *Chlamydomonas* than in nonregenerating cells (41). Moreover, the kinetics of tubulin synthesis after flagellar amputation closely parallels changes in the levels of mRNA coding for tubulin; hybridization studies using cloned tubulin probes have revealed that the amount of tubulin mRNA in total or polyadenylated RNA begins to increase 5–8 min after deflagellation, and peaks after 30–50 min at levels \sim 25- to 40-fold that found in nondeflagellated cells (30, 41). The close correspondence between the timing of tubulin synthesis and tubulin mRNA production strongly suggests that tubulin synthesis is controlled at the transcriptional level. That the increase in tubulin mRNA appears to be several fold greater than the increase in tubulin synthesis may indicate that at least tubulin mRNA is translated at a lower efficiency during flagellar regeneration. A general decrease in translational efficiency, such as might occur due to crowding of the translational machinery by a large number of newly synthesized mRNAs coding for flagellar proteins, could account for the observed reduction in synthesis of many nonflagellar proteins after deflagellation (23; see Fig. 9). Confirmation of this will require further study to resolve the apparent discrepancy between the measured values for translatable vs. total tubulin mRNA in cells before and after deflagellation.

At first examination, the induction pattern for most other flagellar proteins appeared to follow that of the tubulins, but quantification of these patterns revealed significant differences. For example, synthesis of the radial-spoke proteins peaked at about 15 min after deflagellation (vs. 30–50 min for the tubulins), and synthesis of central tubule protein 1 increased only fivefold above predeflagellation levels (vs. a 10-fold increase for the tubulins) and subsequently did not drop off as rapidly as did that of the tubulins. These findings indicated that production of proteins contained in different parts of the flagellum or axoneme is not coordinately regulated. However, the kinetics of synthesis of certain proteins contained within an axonemal structure (e.g., the α - and β -tubulins, or the radial-spoke proteins R1–R4) were very similar, suggesting that coordinate regulation may occur for certain proteins which are topographically very closely related.

The fact that the synthesis of most flagellar proteins is not coordinate beyond the initial induction implies that different mechanisms must be involved in regulating the production of the tubulins and at least some of the minor components—or groups of minor components. This conclusion is supported by the observation that the compound APM (amiprofos methyl) selectively inhibits tubulin synthesis in regenerating *Chlamydomonas* but appears to have no effect on the production of several other flagellar proteins (7). Whether the apparent dif-

ferences in the kinetics of synthesis for the different proteins are due to differential transcription or translation of specific messages, to different half-lives for the messages, or to some post-translational phenomenon such as rapid degradation of specific polypeptides, remains to be determined.

Interestingly, the pattern of protein synthesis during flagellar regeneration in *Chlamydomonas* is quite similar to that which occurs during heat shock at intermediate temperatures in *Drosophila*. In *D. melanogaster*, heat shock markedly stimulates the synthesis of a number of proteins which are produced at very low levels or not at all at the normal temperature (31). The induction of all heat-shock polypeptides begins within 10 min after temperature shift (26, 31). When the shift is to the relatively high temperatures of 35°C or 37°C, all heat-shock polypeptides have similar patterns of induction, but when the temperature is increased to only 33°C, individual heat-shock proteins have quite different kinetics of synthesis, reaching maximum rates of synthesis at different times and declining at different times (27). The regulation of production of the heat-shock proteins must therefore be quite complex, rather than a case of simple coordinate control as once believed.

In our studies on protein synthesis, we observed that incorporation of label into the high molecular weight dynein polypeptides began at a slightly later time than did that for the tubulins and most other flagellar proteins. (In an earlier study it was reported that the times of synthesis of the dynein polypeptides closely paralleled that of the tubulins [23]; however, the 30-min pulse periods used in that study would not have revealed the differences which we observed). Similarly, in our studies on the transport of newly synthesized proteins into the flagellum, we found that the appearance of label in most high molecular weight proteins, including the high molecular weight dynein polypeptides and the high molecular weight membrane component, was slightly delayed relative to that for most smaller proteins, including the tubulins, the 65,000-mol wt membrane protein, and the 71,000-mol wt polypeptide of the 18s dynein. If synthesis of the high molecular weight membrane and dynein polypeptides is dependent on *de novo* transcription of their respective mRNAs, then the observed lag may reflect the longer times required for transcription and translation of the mRNAs coding for these larger polypeptides. The α - and β -tubulin mRNAs in *Chlamydomonas* are estimated to range from approximately 1840 to 2300 nucleotides in size (41); mRNAs coding for the high molecular weight membrane and dynein polypeptides (molecular weights >220,000) would have to be considerably larger than this. It is thus possible that induction of these larger components actually began at the same time as that for the tubulins and the majority of other flagellar proteins.

Most newly synthesized flagellar components were transported into the flagellum with kinetics which simply reflected the rate of growth of the organelle. However, in addition to the high molecular weight components discussed above, a few proteins had a different pattern of appearance in the flagellum. Some, such as polypeptides 4 and 5 in Fig. 11, were synthesized and transported into the flagellum only at very early times during regeneration; these may be components of structures assembled only at the beginning of flagellar growth—e.g., the central microtubule cap (8). Similarly, components C and D of Fig. 12, which were synthesized and rapidly transported into the flagellum only during a slightly later period in regeneration, may be components of some structure formed only during that period of growth, and therefore having a very limited distribution along the length of the flagellum. Alternatively, such

components may be accumulated in the flagellum at the early times for use throughout flagellar regeneration.

Other exceptions to the general pattern of appearance of newly synthesized proteins in the regenerating flagellum were the central tubule protein 1 and the actinlike protein. These components were synthesized at low to moderate levels throughout much of regeneration. However, during our pulse periods neither protein was transported into the regenerating flagellum in significant amounts in a labeled state. This was not due to a lack of transport of these components into the regenerating flagellum, as both were clearly visible in Coomassie-Blue-stained gels of axonemes isolated from the regenerating organelle. Neither is it likely that a delay in transport, such as might have been caused by posttranslational processing, prevented the newly synthesized polypeptides from reaching the flagellum during the relatively short period of the pulse. In studies in which cells were labeled from 15–25 min after deflagellation (the peak periods of synthesis for these components) and then chased for 115 and 175 min before isolation of the flagella, still no label was detected in these spots (38). The results strongly suggested that large pools of these components are present in the cell body and supply most of the actin and CT1 used during flagellar assembly.

This conclusion was confirmed by our isotope dilution studies which indicated that nondeflagellated gametes contain relatively large cytoplasmic pools of central tubule protein 1 and the actinlike component. Nondeflagellated cells also appeared to contain a large pool of radial-spoke component 4, a finding in good agreement with our data on the rates at which this component is synthesized and transported into the flagellum. All other components quantitated, including α - and β -tubulin, radial spoke components R1–R3, and central-tubule protein 2, had smaller cytoplasmic pools which were all approximately the same size relative to their stoichiometries in the axoneme. For these latter proteins, a minimum of 35–40% of the polypeptide chains used in constructing a new axoneme were newly synthesized; an even larger percentage was used if the chase in these experiments was <100% efficient.

A somewhat different result was obtained by Stephens (44) in studies on the synthesis of axonemal proteins and their transport into growing cilia of sea urchin embryos. Deciliation of *Strongylocentrotus droebachiensis* embryos stimulates the synthesis of many axonemal proteins; however, the majority of these proteins are not used during the subsequent formation of a new axoneme, but rather go to replenish cytoplasmic pools depleted during the regeneration event. These pools appear to be quite large; Stephens (44) estimated that the pools for most minor components and for the tubulins of the B-tubule are 5–10 times larger than needed to form a new set of cilia. In contrast, we found that in *Chlamydomonas* gametes a large amount of the protein used to construct a new axoneme is synthesized during regeneration. Moreover, for most proteins a maximum of only 10–30% of the polypeptide chains used in assembling a new axoneme after two deflagellations were present in the cytoplasm before the first deflagellation, suggesting that existing pools are largely depleted by formation of one set of new flagella. Therefore, compared to sea urchin embryos, *Chlamydomonas* maintains small pools of flagellar proteins and relies quite heavily on protein synthesis for the formation of new flagella. These differences are probably due to the particular requirements of each organism. As Stephens hypothesized (44), it may be energy efficient for a sea urchin embryo to synthesize and store ciliary proteins prior to ciliogenesis for use not only in the initial formation of cilia by the

blastomeres, but also in later elongation of apical cilia and for replacement of lost cilia. However, the flagella of *Chlamydomonas* do not continue to elongate after their initial period of growth, and loss of the flagella in gametes of *Chlamydomonas* is probably a rare event in nature. It would, therefore, be most efficient for *Chlamydomonas* to maintain minimal cytoplasmic pools of flagellar proteins and synthesize a substantial amount of these proteins *de novo* should production of new flagella become necessary.

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REFERENCES

1. Abrue, S. L., and R. L. Brinster. 1978. Synthesis of tubulin and actin during the preimplantation development of the mouse. *Exp. Cell Res.* 114:135-141.
2. Baxandall, J., R. A. Forsman, and T. Bibring. 1979. Electrophoretic patterns with β -tubulin trailing in SDS-urea polyacrylamide gels. *J. Cell Biol.* 83(2, Pt. 2):339a (Abstract).
3. Bergman, K., U. W. Goodenough, D. A. Goodenough, J. Jawitz, and H. Martin. 1975. Gametic differentiation in *Chlamydomonas reinhardtii*. II. Flagellar membranes and the agglutination reaction. *J. Cell Biol.* 67:606-622.
4. Bonner, M., and R. A. Laskey. 1974. A film detection method for tritium-labeled proteins and nucleic acids in polyacrylamide gels. *Eur. J. Biochem.* 46:83-88.
5. Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248-254.
6. Brown, D. L., and K. Rogers. 1978. Hydrostatic pressure-induced internalization of flagellar axonemes, disassembly, and re-utilization during flagellar regeneration in *Polytomella*. *Exp. Cell Res.* 117:313-324.
7. Collis, P. S., and D. P. Weeks. 1978. Selective inhibition of tubulin synthesis by aminoprophos methyl during flagellar regeneration in *Chlamydomonas reinhardtii*. *Science (Wash. D. C.)* 202:440-444.
8. Dentler, W. L., and J. L. Rosenbaum. 1977. Flagellar elongation and shortening in *Chlamydomonas*. II. Structures attached to the tips of flagellar microtubules and their relationship to the directionality of flagellar microtubule assembly. *J. Cell Biol.* 74:747-759.
9. Dentler, W. L., M. M. Pratt, and R. E. Stephens. 1980. Microtubule-membrane interactions in cilia. II. Photochemical cross-linking of bridge structures and the identification of a membrane-associated dynein-like ATPase. *J. Cell Biol.* 84:381-403.
10. Fay, R. B. 1980. The flagellar ATPases of *Chlamydomonas reinhardtii*. PhD Thesis. Princeton University.
11. Fay, R. B., and G. B. Witman. 1977. The localization of flagellar ATPases in *Chlamydomonas reinhardtii*. *J. Cell Biol.* 75(2, Pt. 2):286a (Abstr.).
12. Fulton, C., and J. Kowitz. 1975. Programmed synthesis of flagellar tubulin during cell differentiation in *Naegleria*. *Ann. N. Y. Acad. Sci.* 253:318-332.
13. Gattass, C. R., and G. B. Witman. 1979. Protein composition of the partition protofilaments of *Chlamydomonas* flagellar outer doublet microtubules. *J. Cell Biol.* 83(2, Pt. 2):345a (Abstr.).
14. Gitelman, S. L., and G. B. Witman. 1980. Purification of calmodulin from *Chlamydomonas*: calmodulin occurs in cell bodies and flagella. *J. Cell Biol.* 87:764-770.
15. Guttman, S. D., and M. A. Gorovsky. 1979. Cilia regeneration in starved *Tetrahymena*: an inducible system for studying gene expression and organelle biogenesis. *Cell.* 17:307-317.
16. Guttman, S. D., C. V. C. Glover, C. D. Allis, and M. A. Gorovsky. 1980. Heat shock, deciliation, and release from anoxia induce the synthesis of the same set of polypeptides in starved *T. pyriformis*. *Cell.* 22:299-307.
17. Howell, S. H., J. W. Posakony, and K. R. Hill. 1977. Cell cycle program of polypeptide labeling in *Chlamydomonas reinhardtii*. *J. Cell Biol.* 72:223-241.
18. Huang, B., G. Piperno, and D. J. L. Luck. 1979. Paralyzed flagellar mutants of *Chlamydomonas reinhardtii* defective for axonemal microtubule arms. *J. Biol. Chem.* 254:3091-3099.
19. Jarvik, J., and J. Rosenbaum. 1980. Oversized flagellar membrane protein in paralyzed mutants of *Chlamydomonas reinhardtii*. *J. Cell Biol.* 85:258-272.
20. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* 227:680-685.
21. Laemmli, U. K., and M. Favre. 1973. Maturation of the head of bacteriophage T4. I. DNA packaging events. *J. Mol. Biol.* 80:575-589.
22. Laskey, R. A., and A. D. Mills. 1975. Quantitative film detection of ^3H and ^{14}C in polyacrylamide gels by fluorography. *Eur. J. Biochem.* 56:335-341.
23. Lefebvre, P. A., S. A. Nordstrom, J. E. Moulder, and J. L. Rosenbaum. 1978. Flagellar elongation and shortening in *Chlamydomonas*. IV. Effects of flagellar detachment, regeneration, and resorption on the induction of flagellar protein synthesis. *J. Cell Biol.* 78:8-27.
24. Lefebvre, P. A., C. D. Silflow, E. D. Wieben, and J. L. Rosenbaum. 1980. Increased levels of mRNAs for tubulin and other flagellar proteins after amputation or shortening of *Chlamydomonas* flagella. *Cell.* 20:469-477.
25. Levy, E. M. 1974. Flagellar elongation: an example of controlled growth. *J. Theor. Biol.* 43:133-149.
26. Lewis, M., P. J. Helmsing, and M. Ashburner. 1975. Parallel changes in puffing activity and patterns of protein synthesis in salivary glands of *Drosophila*. *Proc. Natl. Acad. Sci. U. S. A.* 72:3604-3608.
27. Lindquist, S. 1980. Varying patterns of protein synthesis in *Drosophila* during heat shock: implications for regulation. *Dev. Biol.* 77:463-479.
28. Mans, R. L., and G. D. Novelli. 1961. Measurement of the incorporation of radioactive amino acids into protein by a filter paper disk method. *Arch. Biochem. Biophys.* 94:48-53.
29. May, G. S., and J. L. Rosenbaum. 1980. Induction and synthesis of heat shock proteins in *Chlamydomonas reinhardtii*. *J. Cell Biol.* 87(2, Pt. 2):272a (Abstr.).
30. Mizami, S. H., P. S. Collis, E. E. Young, and D. P. Weeks. 1981. Tubulin induction in *C. reinhardtii*: requirement for tubulin mRNA synthesis. *Cell.* 24:89-95.
31. Mirault, M. E., M. Goldschmidt-Clermont, L. Moran, A. P. Arrigo, and A. Tissiers. 1978. The effect of heat shock on gene expression in *Drosophila melanogaster*. *Cold Spring Harbor Symp. Quant. Biol.* 42:819-827.
32. O'Farrell, P. H. 1975. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* 250:4007-4021.
33. O'Farrell, P. Z., H. M. Goodman, and P. H. O'Farrell. 1977. High resolution two-dimensional electrophoresis of basic as well as acidic proteins. *Cell.* 12:1133-1142.
34. Piperno, G., B. Huang, and D. J. L. Luck. 1977. Two-dimensional analysis of flagellar proteins from wild-type and paralyzed mutants of *Chlamydomonas reinhardtii*. *Proc. Natl. Acad. Sci. U. S. A.* 74:1600-1604.
35. Piperno, G., and D. J. L. Luck. 1979. An actin-like protein is a component of axonemes from *Chlamydomonas* flagella. *J. Biol. Chem.* 254:2187-2190.
36. Piperno, G., and D. J. L. Luck. 1979. Axonemal adenosine triphosphatases from flagella of *Chlamydomonas reinhardtii*. *J. Biol. Chem.* 254:3084-3090.
37. Randall, Sir John, T. Cavalier-Smith, A. McVittie, J. R. Warr, and J. M. Hopkins. 1967. Development and control processes in the basal bodies and flagella of *Chlamydomonas reinhardtii*. *Dev. Biol. Suppl.* 1:43-83.
38. Remillard, S. P. 1981. Flagellar regeneration and morphogenesis in *Chlamydomonas*: synthesis of specific flagellar proteins during flagellar regeneration in *Chlamydomonas*. PhD Thesis. Princeton University.
39. Rosenbaum, J. L., J. E. Moulder, and D. L. Ringo. 1969. Flagellar shortening and elongation in *Chlamydomonas*. *J. Cell Biol.* 41:600-619.
40. Sager, R., and S. Granick. 1953. Nutritional studies with *Chlamydomonas reinhardtii*. *Ann. N. Y. Acad. Sci.* 56:831-838.
41. Silflow, C., and J. L. Rosenbaum. 1981. Multiple α - and β -tubulin genes in *Chlamydomonas* and regulation of tubulin mRNA levels after deflagellation. *Cell.* 24:81-88.
42. Stephens, R. E. 1970. Thermal fractionation of outer doublet microtubules into A- and B-subfiber components: α - and β -tubulins. *J. Mol. Biol.* 47:353-363.
43. Stephens, R. E. 1972. Studies on the development of the sea urchin *Strongylocentrotus drobachensis*. III. Embryonic synthesis of flagellar proteins. *Biol. Bull.* 142:489-504.
44. Stephens, R. E. 1977. Differential protein synthesis and utilization during cilia formation in sea urchin embryos. *Dev. Biol.* 61:311-329.
45. Valenzuela, P., M. Quiroga, J. Zaldivar, W. J. Rutter, M. W. Kirschner, and D. W. Cleveland. 1981. Nucleotide and corresponding amino acid sequences encoded by α - and β -tubulin mRNAs. *Nature (Lond.)* 289:650-655.
46. Warr, J. R., A. McVittie, J. Randall, and J. M. Hopkins. 1966. Genetic control of flagellar structure in *Chlamydomonas reinhardtii*. *Genet. Res.* 7:335-351.
47. Weeks, D. P., and P. S. Collis. 1976. Induction of microtubule protein synthesis in *Chlamydomonas reinhardtii* during flagellar regeneration. *Cell.* 9:15-27.
48. Witman, G. B., K. Carlson, J. Berliner, and J. L. Rosenbaum. 1972. *Chlamydomonas* flagella. I. Isolation and electrophoretic analysis of microtubules, matrix, membrane, and mastigonemes. *J. Cell Biol.* 54:507-539.
49. Witman, G. B. 1975. The site of *in vivo* assembly of flagellar microtubules. *Ann. N. Y. Acad. Sci.* 253:178-191.
50. Witman, G. B., R. B. Fay, and J. Plummer. 1976. *Chlamydomonas* mutants: evidence for the roles of specific axonemal components in flagellar movement. In *Cell Motility: Book C. R. Goldman, T. Pollard, and J. Rosenbaum, editors. Cold Spring Harbor Laboratory. 969-986.*
51. Witman, G. B., J. Plummer, and G. Sander. 1978. *Chlamydomonas* flagellar mutants lacking radial spokes and central tubules. *J. Cell Biol.* 76:729-747.