Properties of Microtubule Sliding Disintegration in Isolated *Tetrahymena* Cilia

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ABSTRACT Properties of the sliding disintegration response of demembranated *Tetrahymena* cilia have been studied by measuring the spectrophotometric response or turbidity of cilia suspensions at a wavelength of 350 nm relative to changes in the dynein substrate ($MgATP^{2-}$) concentration. The maximum decrease in turbidity occurs in 20 μM ATP, and 90% of the decrease occurs in ~5.9 s. At lower ATP concentrations (1–20 μM), both the velocity and magnitude of the turbidity decreases are proportional to ATP concentration. The velocity data for 20 μM ATP permit construction of a reaction velocity curve suggesting that changes in turbidity are directly proportional to the extent and velocity of disintegration. At ATP concentrations >20 μM (50 μM to 5 mM), both velocity and magnitude of the turbidimetric response are reduced by ~50%. This apparent inhibition results in a biphasic response curve that may be related to activation of residual shear resistance or regulatory components at the higher ATP concentrations. The inhibitory effects of elevated ATP can be eliminated by mild trypsin proteolysis, whereupon the reaction goes to completion at any ATP concentration. The turbidimetric responses of the axoneme-substrate suspensions are consistent with the extent and type of axoneme disintegration revealed by electron microscope examination of the various suspensions, suggesting that the turbidimetric assay may prove to be a reliable means for assessing the state of axoneme integrity.

The $MgATP^{2-}$-induced sliding disintegration of demembranated flagella, introduced by Summers and Gibbons in 1971 (15), has been used in several recent studies directed at the cross-bridging behavior of the dynein arms (11, 12, 16, 18). However, the kinetic aspects of the disintegration response as measured by changes in optical absorbance at the 350 nm wavelength have not been described, and, except for potential high speed ciné analysis of sliding, the turbidimetric assay is at present our only means of analyzing microtubule interactions without the study being encumbered by the overlap of major mechanisms such as occurs in the study of beating cilia. The assay is, therefore, particularly useful for analyzing physiological conditions having a direct effect on sliding, in contrast to conditions known to affect the overall beat pattern of intact cilia.

The sliding forces generated during the course of ciliary motion must be resisted by internal mechanisms for propagated bending to occur. Summers and Gibbons (15) showed that the restraint mechanisms could be perturbed by mild proteolysis of demembranated flagella, in which case the nine doublet microtubules would slide apart to the full extent of their original overlap. This procedure uncoupled the sliding from the bending mechanism, and Summers and Gibbons concluded that the latter was probably mediated by one or both of the two structures perturbed by proteolysis: the interdoublet links or the radial spokes.

The present study is directed at the sliding disintegration response of isolated, Triton X-100-demembranated *Tetrahymena* cilia to various concentrations of $MgATP^{2-}$. *Tetrahymena* cilia are presently the only known cilia or flagella that disintegrate spontaneously in the presence of $MgATP^{2-}$ without first having been subjected to proteolysis (16). This feature permits study of the system in the absence of obvious structural damage and allows direct turbidimetric comparisons of the disintegration response before and after proteolysis. Our study has revealed several surprising features of the response, features neither revealed by nor predicted from studies of beating cilia.

Most importantly, sliding disintegration in the absence of external proteolysis is inhibited by elevated ATP concentrations to the extent that sliding fully activated in 20 μM ATP is completely inhibited in 1 mM ATP. Limited proteolysis removes this inhibition, and the cilia are then capable of complete sliding disintegration at any ATP concentration. This result suggests that the elements that normally regulate sliding and

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enable propagated bending are activated by ATP, and hence probably are coupled with the sliding mechanism to produce bending. This feature of *Tetrahymena* cilia may provide a useful means for studying the physiological mechanism behind such regulatory components.

MATERIALS AND METHODS

Cilia from *Tetrahymena pyriformis* were isolated as described previously (16) and demembranated in 0.2% Triton X-100. Demembranated axonemes were resuspended in 40 mM HEPES buffer, 2 mM $MgSO_4$, pH 7.4. For experiments in which it was necessary to control the free Ca^{2+} concentration, cilia were isolated in the presence of either 0.1 mM EDTA or EGTA and eventually resuspended in 40 mM HEPES buffer containing 0.1 mM EGTA. Axonemes were reactivated by the addition of NaOH neutralized ATP (vanadate-free, equine disodium ATP, Sigma Chemical Co., St. Louis., Mo.) at final concentrations of 1 μM to 5 mM. When the ATP concentrations to be studied exceeded 100 μM, $MgSO_4$ was added at a concentration 2 mM in excess of the ATP concentration.

Spontaneous sliding disintegration of *Tetrahymena* cilia (16) was monitored by changes in optical absorbance at a wavelength of 350 nm (ΔA350 nm), as originally described by Summers and Gibbons (15). The turbidity changes were traced by a recorder coupled to a Beckman model 24 double-beam spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.). The disintegration response was initiated by adding 10–20 μl of ATP at the appropriate concentration to a 1.0-ml suspension of cilia. The protein concentration of the axoneme suspensions were routinely 100–200 μg/ml (0.1–0.25 absorbance units), as measured by a Coomassie Blue dye binding technique (1).

For some experiments, demembranated axonemes were subjected to trypsin proteolysis before reactivation by ATP. Trypsin was added to the sample cuvette at a final concentration of 0.2–0.4 μg/ml, and the axonemes were digested to a 10% decrease in the original absorbance value. It should be noted that the major effects of proteolysis on the disintegration response are apparent after <1% digestion, but, for uniformity between cilia preparations, 10% digestion was found to be most useful. Trypsin proteolysis was stopped with soybean trypsin inhibitor added simultaneously with ATP at a final concentration of 10–20 μg/ml (50–100-fold excess inhibitor). The inhibitor has no discernible effect on the disintegration response other than to block further decreases in turbidity not associated with the ATP-activated response.

The tracing speed of the recorder is critical in terms of both visual inspection of the response and subsequent velocity measurements. A tracing speed of 0.5 inches/min was found to be ideal for visual inspection and rapid comparison between reactivation conditions. However, for velocity measurements (see Results), a tracing speed of 2–5 inches/min was necessary for adequate resolution within the response curve.

For electron microscopy, ATP-reactivated axonemes were sampled during the disintegration response and negatively contrasted with uranyl acetate at pH 4.5, or fixed and embedded for thin-section electron microscopy, as described elsewhere (18).

RESULTS

The Disintegration Response (ΔA350 nm)

As described in an earlier paper (16), isolated *Tetrahymena* cilia spontaneously slide apart when exposed to $MgATP^{2-}$ without first having been subjected to external proteolysis. This disintegration response can be monitored as a decrease in optical absorbance at 350 nm (15). The magnitude of the response as measured by apparent changes in turbidity of the axoneme suspension normally varies from a 29 to 38% decrease in absorbance units between different preparations of cilia. Because of the variable turbidity decreases, to combine and compare data from different preparations of cilia, we often assign a value of 100% to the turbidity decrease (ΔA350 nm) that occurs in 20 μM ATP, 2 mM $MgSO_4$, pH 7.4. The turbidimetric response under all other experimental conditions is then calculated relative to this standard (as in Fig. 2). Similarly, it is sometimes necessary to express data in terms of the absolute turbidity decrease (e.g., 37% ΔA350 nm), in which case direct comparisons between experimental conditions can be made for only a single preparation of cilia (as in Fig. 2).

The Turbidimetric Response and Dynein Substrate Concentration

The optimum turbidimetric response defined here as a maximum decrease in optical absorbance coupled with a maximum reaction velocity occurs in a buffer consisting of 40 mM HEPES, 2 mM $MgSO_4$, 20 μM ATP, at pH 7.4, 15°C. Under these conditions ~90% of ATP^{4-} is available as substrate or $MgATP^{2-}$ for the dynein ATPase (14). Protein concentration over a considerable range (50–500 μg/ml) does not significantly influence the reaction.

When standardized turbidity decreases are determined for ATP concentrations over the range 1 μM to 5 mM, it becomes apparent that the magnitude of the response of *Tetrahymena* cilia is very sensitive to the ATP concentration. In fact, ATP concentrations >50 μm appear to inhibit the turbidity decreases by ~50%. For example, Fig. 1 shows ΔA350 nm graphed against ATP concentration (1 μM to 5 mM) at a common exposure to ATP of 15 s (in 20 μM ATP the reaction is 90% complete after an ~6-s exposure). If, instead of the 15-s measurement, ΔA350 nm is measured after a 3-min exposure to ATP, the shape of the response curve will change slightly so that a 100% response will then be apparent for both 50 μM and 100 μM ATP. Continued exposure to even higher ATP concentrations (0.2–5 mM) will result in further but very gradual turbidity decreases, but, as we will demonstrate, the reaction is clearly inhibited by ATP concentrations >30 μM when it is measured in terms of velocity.

The relationship of the substrate concentration to the turbidity decreases is also evident in the original recorder tracings of the response, as is shown in Figs. 2 and 3. In 20 μm ATP the response goes to completion in 20–30 s, in 50 μM ATP it requires 60–90 s, and in 100 μM ATP it requires 2–3 min. At ATP concentrations >100 μM, the reaction does not go to completion within any meaningful time period (<4 min, Fig. 3). At ATP concentrations <20 μM the reaction is also reduced,

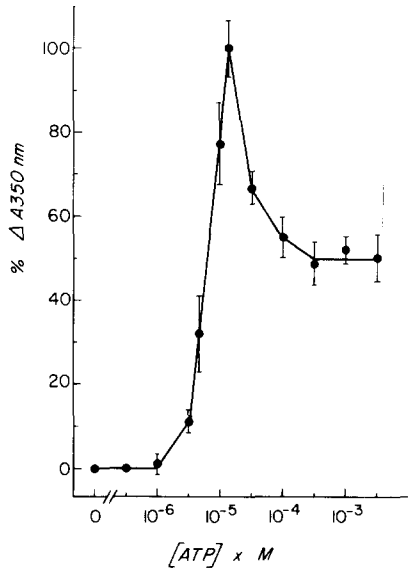

FIGURE 1 The graph depicts the relationship of absorbance decreases at 350 nm in response to ATP concentration. All values were measured at 15-s exposure to ATP and are standardized against the ΔA350 nm (100%) that occurs in 20 μM ATP. Each data point represents the mean and standard deviation of four measurements from two preparations of cilia. At ATP concentrations >100 μM, Mg^{2+} was maintained at 2 mM in excess of the ATP concentration.

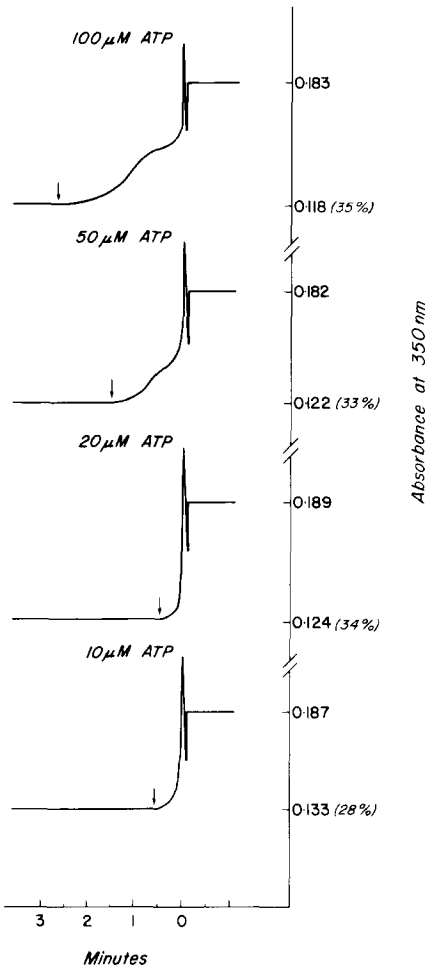

FIGURE 2 Original spectrophotometric tracings of the absorbance decreases of cilia in response to the addition of 10–100 μM ATP. The tracing speed was 0.5 inches/min, and the responses are read from right to left. The absolute absorbance changes are indicated on the vertical axis along with the percent reduction in parentheses. The down and up spikes in the tracings are optical artifacts associated with the addition of ATP. The extent of the response (34% ΔA350 nm) for 20, 50, and 100 μM is the same, but the tracings acquire a distinct biphasic character at ATP concentrations >20 μM, and the time required for the reaction to go to completion (arrows) is greatly extended.

in terms of both magnitude (Fig. 2) and velocity (see next section).

The recorder tracings of the turbidimetric response also reveal that the shape of the curves and the velocity of the response changes dramatically at ATP concentrations >30 μM. The tracings take on a distinct biphasic character particularly evident in 50–100 μM ATP (Fig. 2). The biphasic slope of the tracing can only be described accurately in terms of reaction velocity (see below), but in general it first becomes apparent at about 40% ΔA350 nm, regardless of to what level the ATP concentration is raised. And, although the apparent velocity of both phases is diminished as ATP is increased, it is the second phase of the response that shows the greatest decrease, until it is eliminated entirely at about 0.5 mM ATP (Fig. 3).

In summary, it is apparent that the optimal turbidimetric response of *Tetrahymena* cilia takes place in 20 μM ATP and is measured in seconds. Increasing the ATP concentration effectively decreases the magnitude of the turbidity changes ~50% by greatly extending the time required for the reaction to go to completion. This phenomenological account is described below in terms of systematic changes in reaction velocity.

Velocity of the Turbidimetric Response

In addition to measuring the magnitude of the turbidity changes as a function of time and ATP concentration, the decrease in absorbance at 350 nm appears to be a good measure of reaction velocity. Velocity can be expressed as the rate of decrease in optical absorbance units, but to express the data in more meaningful units, we have also converted the turbidimetric response (%ΔA350 nm) to micrometers of disintegration. We need only to assume that the turbidimetric assay represents a direct measure of the extent of disintegration, and the decrease in turbidity will be directly proportional to the extent of sliding or increase in length that the axoneme has undergone. If we also assume that the overall population of axonemes has an average length of 5.5 μm, measured from the cilium base to the point of radial spoke termination along the doublet A subfiber, the axoneme must undergo 9 × 5.5 μm sliding for a final length of 49.5 μm at 100% ΔA350 nm. The value does not take into consideration potential differences in axoneme length

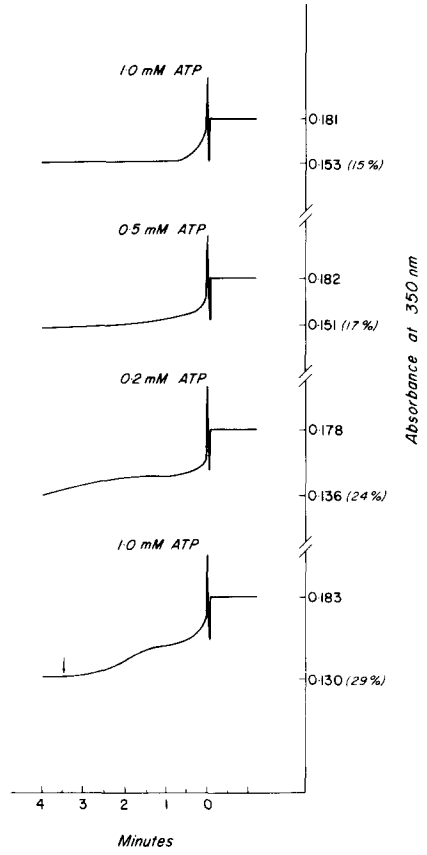

FIGURE 3 Original spectrophotometric tracings of the absorbance decreases of cilia in response to the addition of 0.1–1 mM ATP. The tracing speed was 0.5 inches/min, and the responses are read from right to left. The tracings were made from a preparation of cilia different from that illustrated in Fig. 2, and, hence, the absolute absorbance decrease (29%) is different but the shape of the response is the same at corresponding ATP concentrations. Increasing ATP concentration to >0.1 mM causes a progressive inhibition of the reaction apparently associated with the second phase of the response until only ~50% of the potential maximum response occurs, similar to the data illustrated in Fig. 1.

resulting from either natural variation or breakage of intact cilia that may occur during isolation. Nevertheless, velocity data based on these assumptions show that the assumptions are reasonably correct and that changes in optical absorbance are a potentially useful measure of the extent of disintegration vs. time.

DETERMINATION OF REACTION VELOCITY IN 20 μM ATP: To calculate the velocity of $MgATP^{2-}$-induced turbidity changes, the velocity of the reaction can be expressed as changes in absorbance units per second or micrometers per second. The latter is a function of the sliding distance interval (%ΔA350 nm × 49.5 μm) divided by the corresponding time interval measured in seconds. This gives velocity calculations for successive time intervals along the reaction curve (Fig. 4). The best resolution of velocity would be possible if the time duration of the turbidimetric response were divided into 0.1-s intervals; however, because of the quickness of the response, such fine division of the time scale is not practical. Instead, we routinely divide the absorbance scale into 10% units of ΔA350 nm. The calculated velocities thus represent an average velocity for the various time intervals, which between 10 and 60% ΔA350 nm are of 0.2–0.4-s duration but become progressively longer as the reaction nears completion (Figs. 5 and 6).

Velocity data were derived from four different preparations of cilia and four separate measurements from each preparation: each data point in Fig. 5 thus represents the mean of 16 measurements. Within a single preparation of cilia, maximum reaction velocity (at 20% ΔA350 nm) does not vary by more than 3.6 μm/s; however, when common points are compared among different cilia preparations, the velocity is seen to vary over a considerable range. For example, at 20% ΔA350 nm, the velocity of the four preparations varied between a low of 12.87 μm/s and a high of 44.64 μm/s: the mean velocity was 27.35 μm/s (Fig. 5).

When cilia are reactivated by 1–15 μM ATP, both the magnitude and velocity of the turbidity changes increase in proportion to increasing ATP concentration, reaching a maximum at 20 μM ATP. The velocity data for 20 μM ATP describes the behavior of the axonemes from the time of ATP addition to the time of maximum decrease in optical absorbance (Fig. 5). The curve has two important features. Most of the data points fit a straight line and clearly show that the decrease in reaction velocity over time is directly proportional to the change in turbidity at any given time interval. The linear

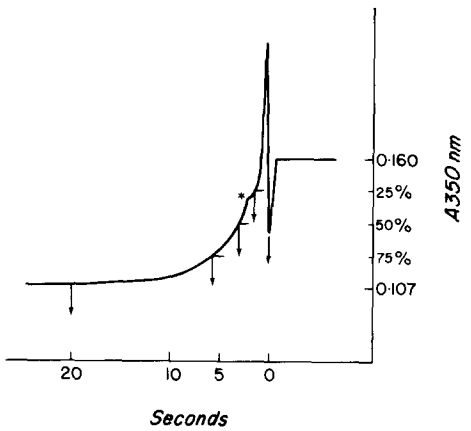

FIGURE 4 An original spectrophotometric tracing at 5 inches/min of cilia reactivated in 2 mM $MgSO_4$ and 20 μM ATP. The response is read from right to left, and the addition of ATP or t_0 is marked by the apex of the downward spike. The shoulder in the tracing (*) is an artifact associated with closing the optical chamber after ATP addition. Such tracings were used to calculate reaction velocity. It is apparent that the turbidimetric response is very quick with 75% ΔA350 nm occurring in ~5 s for this particular preparation of cilia.

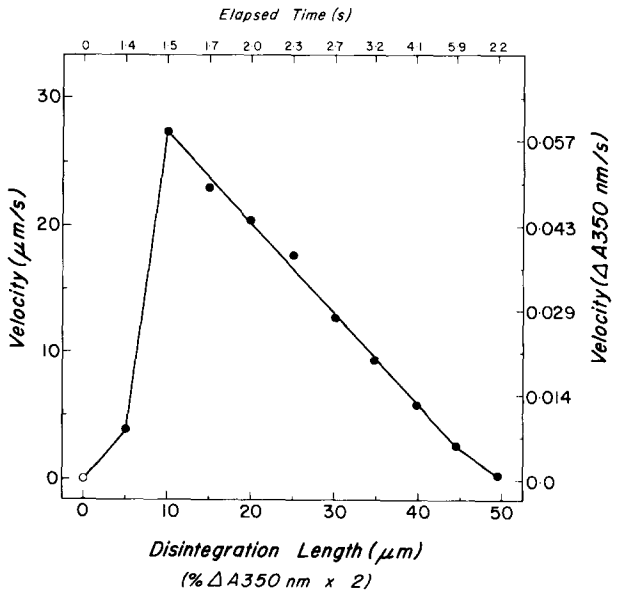

FIGURE 5 The graph depicts the disintegration or turbidimetric response velocity for cilia reactivated by 2 mM $MgSO_4$ and 20 μM ATP. Each data point represents the mean of four measurements from each of four separate preparations of cilia. The elapsed time intervals are not linear, but each interval corresponds to a 10% decrease in absorbance at 350 nm. Thus, 90% ΔA350 nm occurs at a mean elapsed time of 5.9 s. For the purpose of constructing the curve, we assume that disintegrated cilia have an extended length of 49.5 μm at 100% ΔA350 nm. Maximum mean velocity occurs at 20% ΔA350 nm or 1.5 s. The progressive decline in velocity with respect to the increasing disintegration length is linear for the most of the response curve (20–90% ΔA350 nm). See Results for details and interpretation.

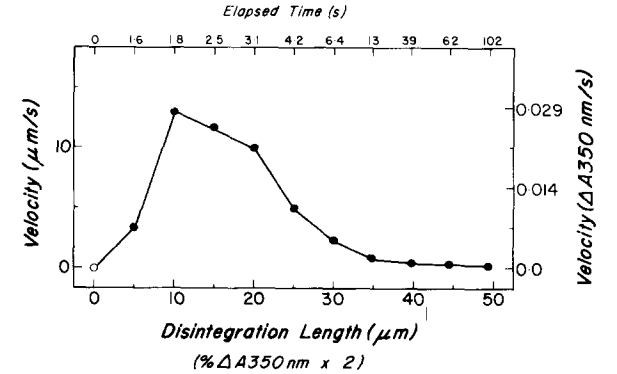

FIGURE 6 The graph depicts the disintegration or turbidimetric response velocity for cilia reactivated in 2 mM $MgSO_4$ and 50 μM ATP. Each data point represents the mean of four measurements from each of the same four cilia preparations used in Fig. 5, and for the purpose of comparison the curves are constructed on the same scale. Note the major differences in mean velocities and the nonlinear time intervals. The response in 50 μm ATP (first part of the curve, 10–40% ΔA350 nm) is similar to the response in 20 μM ATP but occurs with about half velocity of the latter. However, at 40–50% ΔA350 nm (3–4 s), the second phase of the turbidimetric response (Fig. 3) results in a nonlinear decrease in reaction velocity that at higher ATP concentrations effectively inhibits the reaction from going to completion. See Results for details and interpretation.

decline in velocity clearly is not related to decreases in the substrate concentration. This is easily demonstrated either by increasing the substrate concentration or by varying the protein concentration in 20 μM ATP (the decline is unaffected). The first two data points on the curve fall well below the main velocity slope. The reaction is occurring at a velocity substantially slower than maximum velocity at 20% ΔA350 nm. This 1.5-s lag in the response may result from the time required for added ATP to come to concentration equilibrium in the sample volume and the time required for generated force to overcome initial resistance to disintegration, which may be high because the microtubules are temporarily restrained by the basal region of the axoneme.

DETERMINATION OF REACTION VELOCITY IN 50 μM ATP: Using the same reasoning used in calculating the velocity of the turbidimetric changes in 20 μM ATP, we similarly characterized reaction velocity at 50 μM ATP because it is at this concentration (40–50 μM) that the biphasic nature of the absorbance changes first becomes apparent (Fig. 2). The most obvious feature of the response is that, whereas 100% ΔA350 nm occurred at a mean elapsed time of 22 s in 20 μM ATP, in 50 μM ATP the reaction required 102 s to reach completion, which is a nearly fivefold decrease in the overall reaction velocity.

When the velocity data are graphed against the decrease in absorbance units or change in axoneme length to construct a velocity curve, the biphasic nature of the velocity response is readily apparent (Fig. 6). The velocity values illustrated in Fig. 6 represent the mean values for the same four cilia preparations used in Fig. 5, but they were measured from recorder tracings at 2 inches/min. The first part of the response (10–40% ΔA350 nm) is similar to the response in 20 μM ATP, except that mean maximum velocity (at 20% ΔA350 nm) is only 12.96 μm/s. The slope of the velocity decrease between 20 and 40% ΔA350 nm projects on a straight line; however, at about 40% ΔA350 nm, the velocity drops sharply as the second phase in the tracings becomes apparent. This velocity decrease is nonlinear and the reaction approaches zero velocity at ~70% ΔA350 nm, although the turbidity will continue to slowly decrease until the reaction comes to completion at ~100 s. Further increases in ATP concentration up to 1–2 mM show similar reaction velocity curves. The second phase of the reaction, however, is progressively damped, until it nearly disappears in 0.5 mM ATP, and the overall response goes to only ~50% completion (as in Figs. 2 and 3).

Effects of Trypsin Digestion on the Turbidimetric Response

If *Tetrahymena* cilia are first subjected to limited proteolysis by trypsin before activation by ATP, the turbidimetric response exhibits very different characteristics. The magnitude of the turbidity decrease is increased to a range of 35–46% ΔA350 nm, and within any given preparation of cilia the turbidity decrease is uniform (±1.1%) and independent of the ATP concentration in the range of 20 μM to 5 mM (Fig. 7).

Trypsin digestion clearly eliminates the biphasic response curves (cf. Figs. 7 and 3), and hence relieves the constraints on the turbidity changes normally activated by the higher ATP concentrations. The velocity of the reaction is comparable to the response of nondigested axonemes in 20 μM ATP (Fig. 5), except that maximum reaction velocity is reduced, often requiring 60–90 s to go to completion.

We have also tested the effects of the enzyme elastase (2.5 μg/ml) on the turbidimetric response because Brokaw (4) has suggested that it may have a more selective action, possibly attacking only the interdoublet links, as opposed to the more generalized action of trypsin (6). In 20 μM ATP, the effect of elastase on the active turbidity change is not distinguishable from that of trypsin. However, at higher ATP concentrations the reaction after elastase digestion (10%) is still inhibited from going to completion in a way similar to the response of axonemes not exposed to trypsin proteolysis. The results of these experiments are summarized in Table I.

Microscopic Analysis of the Disintegration Response

Spontaneous disintegration of isolated *Tetrahymena* cilia takes numerous visible forms, including ATP-dependent fraying (16). Axoneme disintegration strictly depends upon the presence of substrate for the dynein ATPase, and hence we presume hydrolysis of the substrate. No disintegration occurs when ATP is added in the absence of Mg^{2+} (Fig. 8*a*). Active

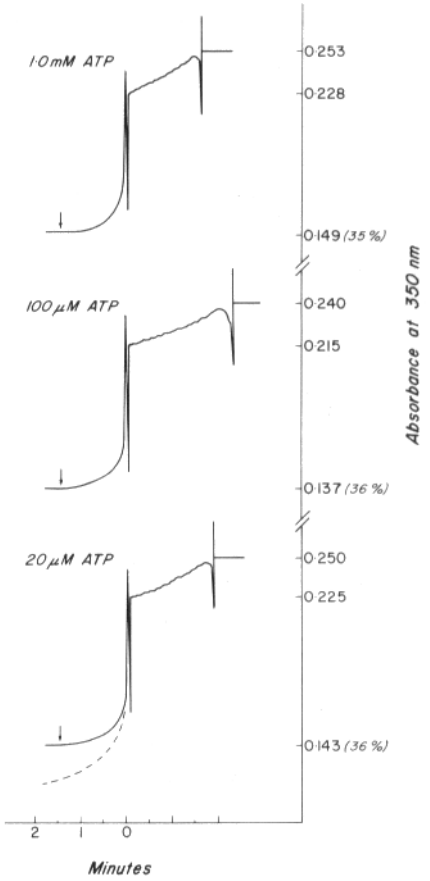

FIGURE 7 Original spectrophotometric tracings of the turbidity decreases of cilia digested with trypsin before the addition of 20 μM, 100 μM and 1 mM ATP. The tracing speed was 0.5 inches/min and the responses are read from right to left. The first spike in the tracings results from the addition of trypsin, and the second spike results from the simultaneous addition of ATP and trypsin inhibitor. The magnitude of the turbidity decrease in response to ATP is identical at each ATP concentration, although the response goes to completion (~90 s) more slowly than when compared with nondigested axonemes in 20 μM ATP (~20 s; Fig. 2). The dashed line shows the shape of the ATP response curve in the absence of trypsin inhibitor.

TABLE I

Effects of Proteolysis on the Turbidimetric Response of Isolated Tetrahymena Cilia *

ATP	No enzyme	Trypsin	Elastase
μM	%	%	%
20	100	108	106
100	57	113	93
1,000	51	112	69

All percent values are the means of two measurements from each of two preparations of cilia. Maximum standard deviation was ± 7%.

* Percent turbidity decrease (ΔA350 nm) is expressed as percent of the maximum decrease (100%) occurring in 20 μM ATP, 2 mM $MgSO_4$, pH 7.4. All values were measured after a 60-s exposure to ATP.

disintegration occurs in two forms, as seen by both dark field light microscopy and negative contrast electron microscopy. Disintegration activated by <30 μM ATP occurs strictly by active sliding (Fig. 8*b*). Sliding occurs in one of two modes: it appears to be either sequentially activated, as characterized by doublets having been individually extruded from an otherwise intact axoneme (Fig. 9*a*), or it appears to be simultaneously activated as characterized by all nine doublets having undergone approximately equal amounts of sliding (Fig. 9*b*). At present, we have no means of determining which of the two sliding modes predominates because, when examined at 100% ΔA350 nm, the two cannot be readily distinguished (Fig. 8*b*).

When disintegration is activated by ATP concentrations >30 μM, active fraying of the axonemes increases significantly until, in 0.5–1 mM ATP, little if any sliding is observed, and fraying is clearly seen to be the predominant form of disintegration (Fig. 10*a*). Many intact axonemes also remain in these preparations. However, when axonemes are treated with trypsin before active disintegration, activation by ATP at any concentration results in disintegration, predominantly by sliding (Fig. 10*b*), although some fraying is evident at the higher ATP concentrations. Optical inspection of cilia reactivated in 1–5 mM ATP shows them to be mainly intact and sometimes weakly motile, forming random twitches and bends.

Whereas it is not convenient to quantitate the disintegration response by microscopy, these observations agree well with the described effects of ATP on disintegration as measured by the turbidimetric assay.

DISCUSSION

The study of spontaneous sliding disintegration of demembranated *Tetrahymena* cilia permits both a general description of the response and its characterization in terms of reaction velocity. The velocity curves reveal that at a given ATP concentration, reaction velocity decreases in direct proportion to changes in turbidity. Reaction velocity is also sensitive to ATP concentration such that the reaction is effectively inhibited at concentrations >100 μM.

With regard to our reaction velocity calculations, an important note of caution must be interjected. The use of the spectrophotometric assay for monitoring absorbance changes in a nonideal particle population such as cilia can be challenged on several grounds. The turbidimetric assay is influenced by numerous factors, including particle mass, shape, and size, and all of these factors probably change as the cilium disintegrates. Nevertheless, and without attempting to determine which factor contributes most to the observed changes in turbidity, we feel that the assay is a reliable measure of the magnitude and velocity of the reaction in response to the addition of $MgATP^{2-}$.

Considerations of Axoneme Disintegration

In constructing the velocity curves illustrated in Figs. 5 and 6 we are measuring only the velocity of the turbidity decrease associated with ATP hydrolysis-dependent disintegration, but we also are assigning to that velocity a micrometer scale. The reaction velocity curves clearly represent an accurate description of the turbidity changes, but until we can be certain what the spectrophotometric assay is actually measuring, our results should be interpreted and used with discretion. The resulting curve for experiments run in 20 μM ATP (Fig. 5) is representative of the turbidity changes when the reaction is neither limited by low ATP (<20 μM) nor inhibited by high ATP (>30 μM). The curve is distinguished by data points that fit a straight line, indicating that reaction velocity decreases in constant proportion to ΔA350 nm. The linear decrease is consistent with an interpretation that changes in A350 nm are directly proportional to the extent of axoneme disintegration. This interpretation is also supported by our microscope observations and is used as the basis for ensuing discussion.

We presume that disintegration velocity, as it reflects sliding in otherwise intact cilia, is determined by the internal force: resistance ratio, as it is in striated muscle (9). This ratio should remain constant as the cilia disintegrate. Therefore, the decrease in reaction velocity can be explained only if the external resistance to disintegration is simultaneously increasing. The constant units of internal force:resistance must be pushing progressively greater numbers of units of microtubule surface area in the form of n, $n + 1$, $n + 2$, $n + 3$ and so on, groups of doublets, which in a fluid medium should cause increased viscous drag and, hence, apparent resistance in proportion to the increase in surface area.

The dynein arms must generate force to create sliding shear, but at the same time the cilium must also generate a variable resistance to that shear to enable propagated bending to take place. The absolute velocity of sliding as it may be related to arm activity during normal beating probably does not vary significantly inasmuch as adjacent doublets undergo very little absolute displacement (30–40 nm). Because sliding (dynein activity) does not appear to be activated or deactivated by an obvious and direct control mechanism, the system is left with only potential manipulations of the substrate concentration or interaction with a dynamic resistance mechanism to control the rate of sliding and, hence, beat frequency. In *Tetrahymena* cilia and trypsin-treated cilia or flagella, this resistance mechanism is either wholly or partially inactivated, and microtubules can presumably slide apart in a manner in which the velocity of the reaction will be proportional to the external resistance encountered by the disintegrating axonemes and sensitive to substrate conditions.

Control of Sliding Disintegration

The active disintegration response of *Tetrahymena* cilia reveals several features that are not revealed by corresponding studies of beating cilia. At ATP concentrations >20 μM, velocity of the turbidimetric decrease under conditions that we assume to be a minimal or low internal resistance load does not increase with the substrate concentration. Maximum reaction velocity occurs in only 20 μM ATP, which is well below

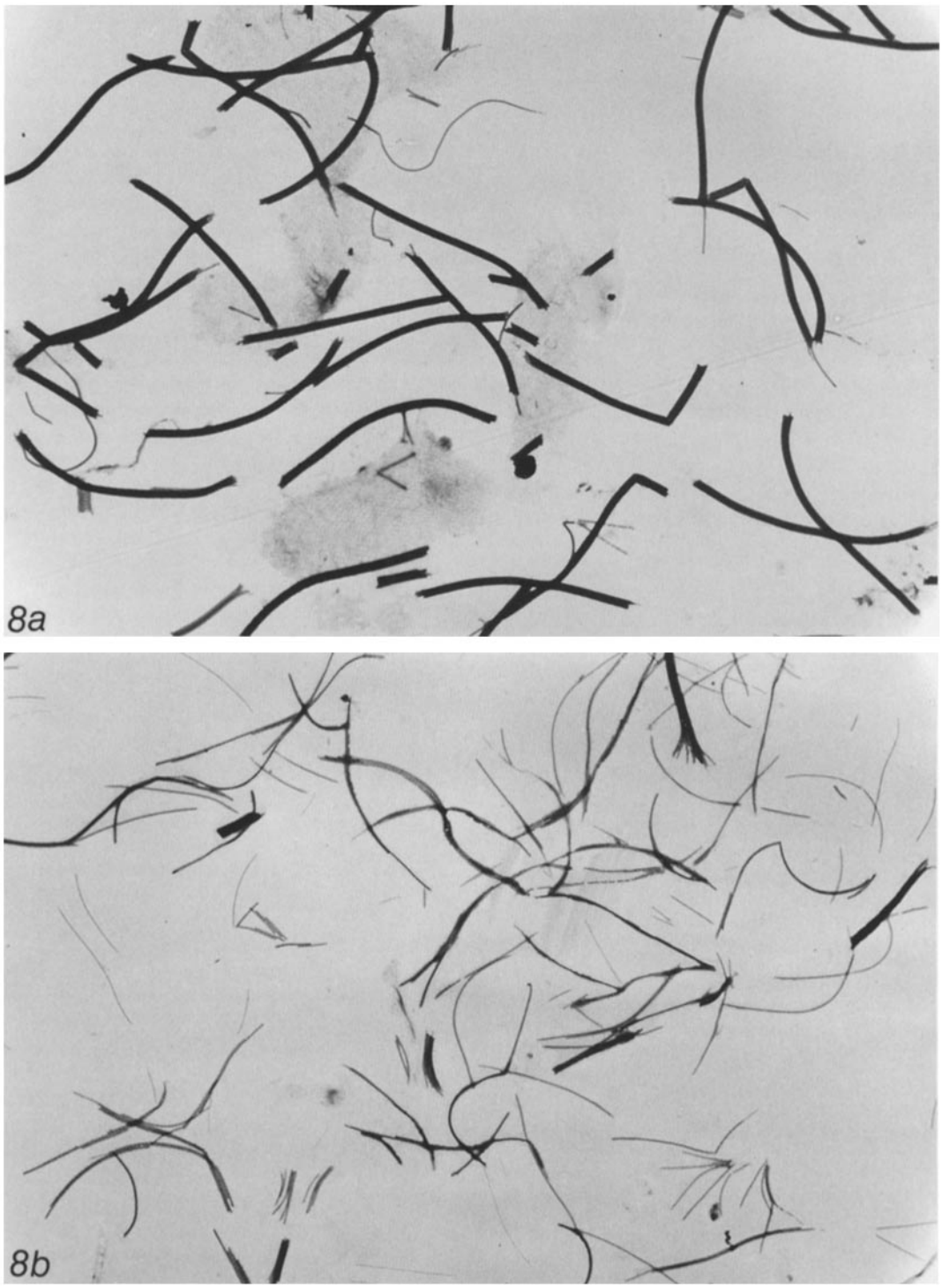

FIGURE 8 Demembranated *Tetrahymena* cilia negatively contrasted for electron microscopy. *a* shows cilia in 20 μM ATP and no Mg^{2+}. *b* shows cilia in 20 μM ATP and 2 mM $MgSO_4$. $MgATP^{2-}$ induced disintegration is nearly complete and has occurred predominantly by sliding (see Fig. 10). × 6,000.

the millimolar concentration of ATP assumed to exist in the native cilium. Most importantly, the experiments show velocity to be sharply inhibited if the ATP concentration is raised much above 20 μM. This observation is perplexing inasmuch as several studies of reactivated cilia or flagella have shown beat frequency to be linearly dependent upon the ATP concentration to a level of ~1 mM ATP (3, 5). In reactivated flagella, the product of bend angle and beat frequency are proportional to the shear rate or sliding velocity (2). Conditions such as substrate concentration, which directly influence beat frequency, must, therefore, be acting upon either dynein activity (sliding velocity) or those features of the system that affect bending. However, dynein activity is not known to be inhibited by the substrate concentrations used in this study (cf. references 3 and 7), and, therefore, our observed inhibition of the disintegration response at ATP levels >30 μM may be related to factors that are otherwise involved in bending.

In principle, disintegration velocity in the absence of any

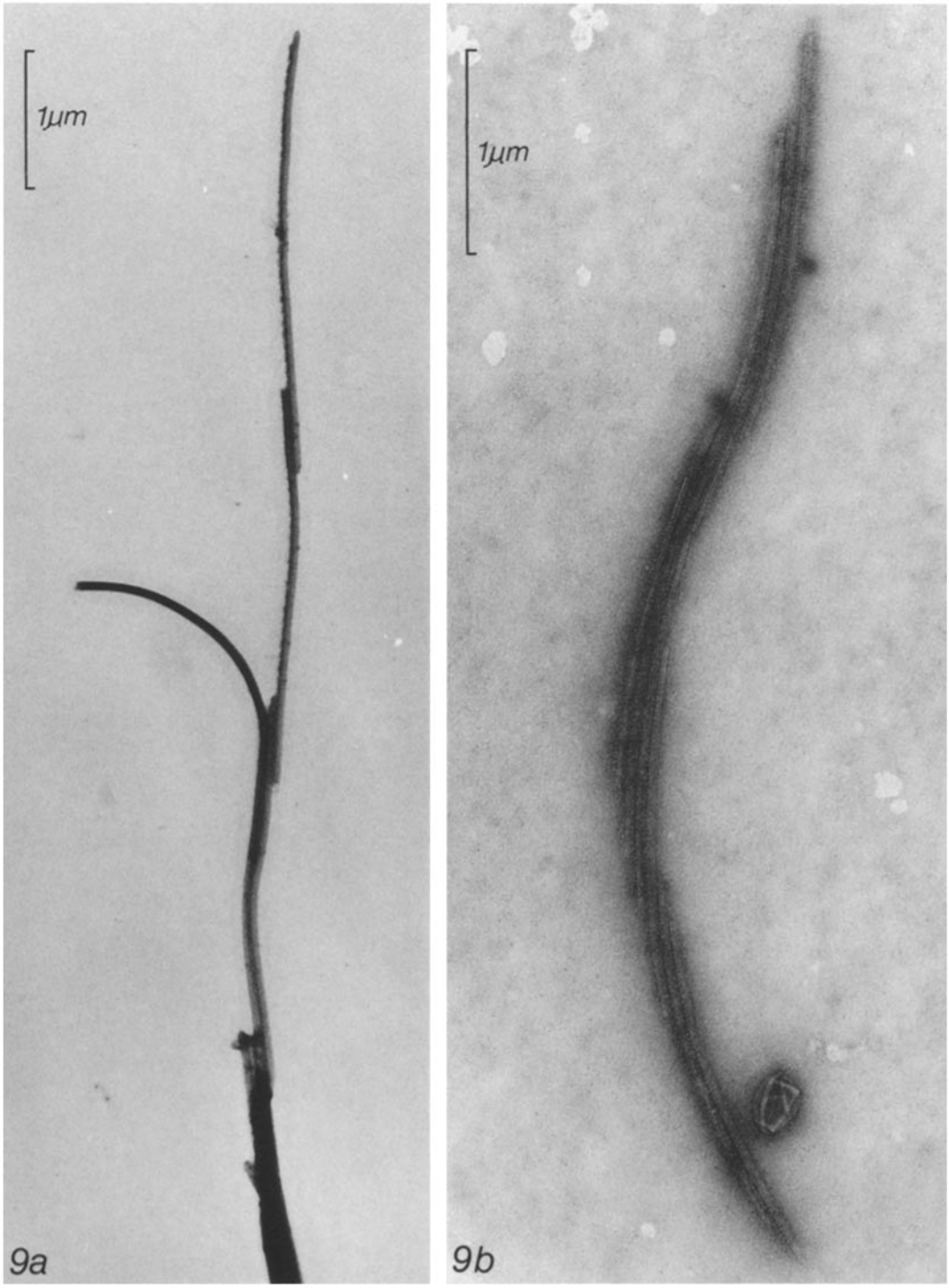

FIGURE 9 Broken segments of *Tetrahymena* cilia that illustrate the two predominant modes of sliding after reactivation in $MgATP^{2-}$. *a* illustrates apparent sequential activation of sliding where doublet microtubules appear as if they were individually extruded from an otherwise intact axoneme. × 21,000. *b* illustrates apparent simultaneous activation of sliding where all nine doublets appear to have undergone approximately equal amounts of sliding. × 31,000.

internal constraints should be directly proportional to the rate of ATP hydrolysis by the dynein arms, and, hence, also should be proportional to the substrate concentration consistent with Michaelis-Menten or saturation kinetics. In practice, however, double reciprocal plots of reaction velocity vs. substrate concentration show that reaction velocity in nondigested axonemes does not obey saturation kinetics. The resulting curve (not illustrated) suggests that at least two ATP-sensitive sites may be activated at different ATP concentrations, and that one of these sites has a marked inhibitory effect on the disintegration response. Activation of simultaneous and potentially interacting mechanisms is also suggested by the biphasic nature of the response tracings (Figs. 2 and 3), and it is reasonable to think, for example, that some residual part of an ATP-dependent control mechanism having a very different binding affinity for ATP may be activated along with the cross-bridge cycling of the dynein arms. However, whereas control of sliding in a beating cilium may be biphasic in association with the effective

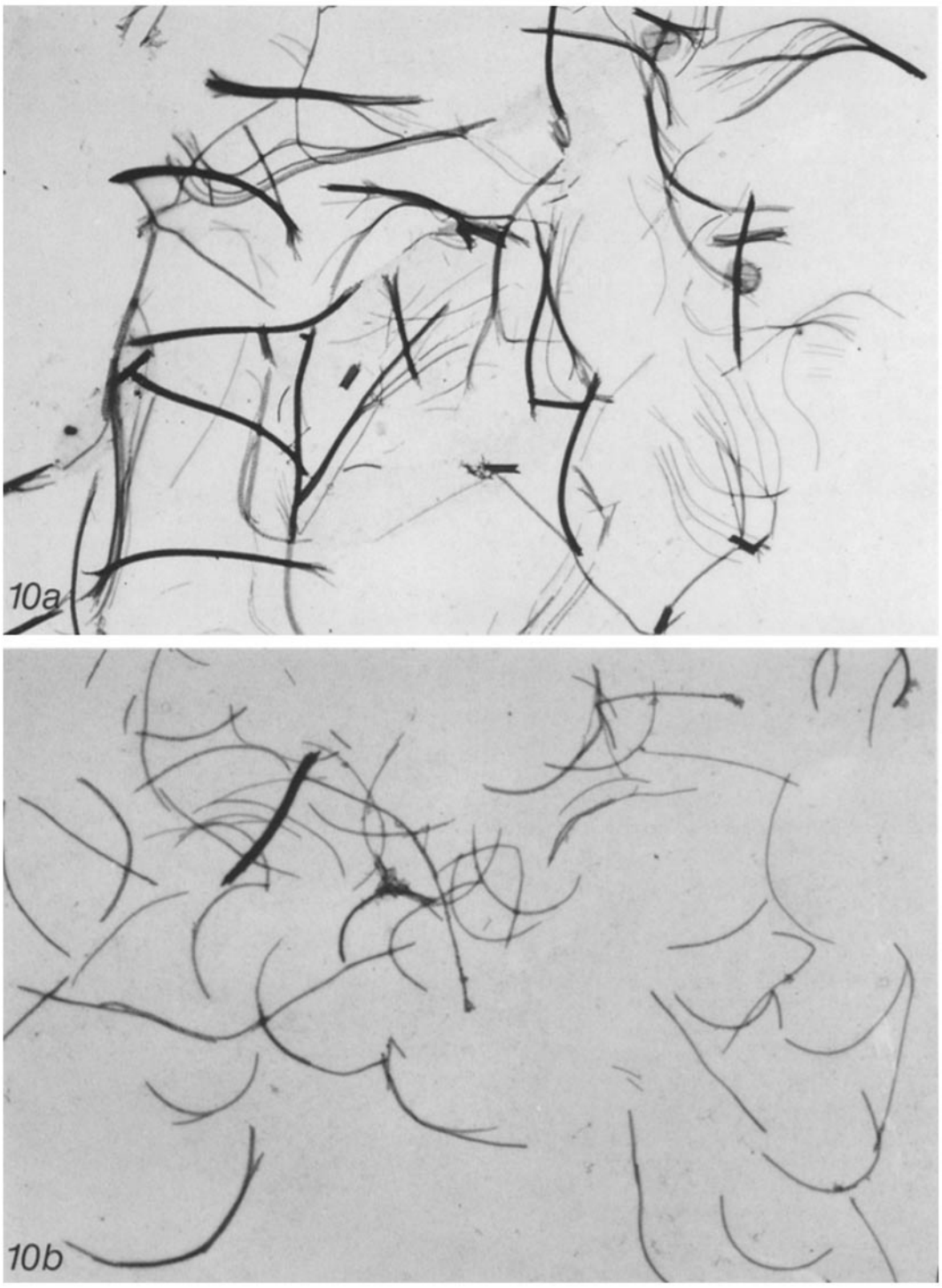

FIGURE 10 Demembranated *Tetrahymena* cilia negatively contrasted for electron microscopy. The axonemes were reactivated in 1 mM ATP before (*a*) and after (*b*) limited proteolysis by trypsin. Nondigested axonemes disintegrate predominantly by fraying, but many intact axonemes are also present. Digested axonemes disintegrate by sliding comparable to the nondigested axoneme response in 20 μM ATP (Fig. 8 *b*). × 6,000.

and recovery strokes, it is unlikely that this control would be expressed as a major effect on sliding velocity, particularly when the effective and recovery strokes span approximately equal lengths of time and bend angles (13).

The simplest explanation for the biphasic kinetics and inhibition of disintegration may be related to residual activation of constraints or regulatory mechanisms that are otherwise related to the conversion of sliding into bending. Though it has often been suggested that these mechanisms may be energy dependent, dependence on ATP has never been demonstrated but would be consistent with our observation that both the extent of axoneme disintegration and the apparent velocity of the reaction are progressively inhibited as ATP is elevated. Stated differently, at low ATP concentrations only dynein arm cycling may be activated and the doublets slide apart. But at higher ATP concentrations, constraints may be simultaneously acti-

vated that prevent complete disintegration and in normal cilia enable propagated bending to take place. This analysis does not distinguish between strictly elastic constraints (interdoublet links) that may serve to hold doublets together (4) as opposed to more dynamic constraints (radial spokes) that may be involved in bend regulation (17), nor does it distinguish between constraints that may be activated independently by ATP as opposed to constraints that may be activated by shear rate or sliding velocity. However, the general interpretation is strengthened by our observation that ATP-inhibition of disintegration is eliminated by mild trypsin proteolysis, whereupon the disintegration reaction goes to completion independent of the substrate concentration. This observation is also consistent with Brokaw and Simonick's (6) finding that flagellar beat frequency increases in proportion to the extent of tryptic digestion of the axoneme.

If constraints on sliding are being activated at the higher ATP concentrations, they would also obscure potential increases in disintegration velocity that might otherwise occur with increased ATP. Summers and Gibbons (15) and Gibbons (8) reported increased disintegration velocities between 10 μM and 1 mM ATP, and Hata et al. (10) have measured sliding velocity increases in 4 μM to 5 mM ATP from ciné analyses of the disintegration response. Thus, our finding that reaction velocity in undigested axonemes does not increase beyond 20 μM ATP may not strictly be correct. The reaction velocity of our trypsin-digested axonemes may well increase with respect to the substrate concentration, but precise characterization is difficult because the velocity is simultaneously diminished in trypsin-digested axonemes when compared with the velocity of nondigested axonemes at comparably low ATP concentrations (<30 μM).

Okuno and Brokaw (20) have recently described inhibitory effects on flagellar beat frequency by ATP^{4-}, ADP^{3-} and P_i, all of which are competitive for the dynein substrate or $MgATP^{2-}$. However, under the experimental conditions used in this study, ~90% of ATP^{4-} should be in the form of $MgATP^{2-}$, and, hence, free ATP and reaction products are not likely to have interfered with the disintegration response. Interestingly, however, Okuno and Brokaw suggest that beat frequency rather than sliding velocity may be the reaction variable more fundamentally dependent upon $MgATP^{2-}$ concentration, which is consistent with our interpretation that constraints on sliding that may regulate bending and, hence, beat frequency are activated at substrate concentrations higher than are needed to cause normal sliding disintegration.

Gibbons and Gibbons (19) have recently found that flagellar beat quiescence is caused by 10^{-4} M free Ca^{2+}, but only when flagella are reactivated by >0.6 mM $MgATP^{2-}$. Quiescence is reversed by reducing free Ca^{2+}, by reducing $MgATP^{2-}$, or by mild trypsin proteolysis. These effects are complementary to the results reported in this paper if free Ca^{2+} is a regulatory cation that, when present, inhibits the forces that otherwise restrain sliding and enable bending to occur.

Calcium modulation of these forces may prove to be the unifying principle needed to explain such apparently diverse phenomena as Ca^{2+}-mediated ciliary arrest and reversal and flagellar quiescence and beat asymmetry.

We thank D. R. Mitchell for providing Fig. 9*b*.

This study was supported by research grant GM20690 from the National Institutes of Health.

Received for publication 3 December 1979, and in revised form 24 March 1980.

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