AN EFFECT OF ADENOSINE TRIPHOSPHATE ON THE LIGHT SCATTERED BY SUSPENSIONS OF CILIA

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ABSTRACT

Cilia were isolated from *Tetrahymena pyriformis* by an ethanol-calcium method. Addition of adenosine triphosphate to a suspension of intact or digitonin-extracted cilia caused a decrease of about 20 per cent in turbidity. Study of fractionated cilia showed that the presence of two distinct axonemal components, the outer fibers and the 30S dynein (the axonemal ATPase protein), was necessary for this effect on turbidity to occur. The decrease in turbidity is interpreted as a result of a specific interaction of ATP with these protein components causing an effective increase in hydration. The high nucleotide specificity suggests that the change in hydration is closely related to the processes responsible for motility. The outer fibers themselves swell when suspended in media of very low ionic strength. The concentration of salt needed to prevent this swelling (2 mM $MgSO_4$ or 30 mM KCl) is about the same as that needed to keep dynein bound to the fibers. The recombination of purified 30S dynein with the outer fibers can be followed by the rise in turbidity resulting from increased dry mass of the particles.

In 1962 Tibbs (9) reported that ATP[1] causes a reduction of 15 to 20 per cent in the turbidity of suspensions of perch sperm tails, and interpreted this as due to a swelling of the tails. The present paper describes a similar effect in cilia isolated from *Tetrahymena*. Since fractionation procedures for these cilia are available (3, 4), it has been possible to study this effect of ATP in more detail and to identify the protein components of the cilium which are involved. Two other examples of turbidity changes are described in order to illustrate the general usefulness of turbidimetry in the study of ciliary fractions.

[1] The following abbreviations are used in this paper: Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediamine tetraacetate; ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, 5′-adenosine monophosphate; ITP, inosine triphosphate.

METHODS

Cilia were isolated from *Tetrahymena pyriformis*, strain W, by an ethanol-calcium method slightly modified from that of Watson and Hopkins (10, 4), and stored in TMSK solution (30 mM Tris buffer, 2.5 mM $MgSO_4$, 0.2 M sucrose, 40 mM KCl, pH 8.3 at 0°C). Cilia isolated by this method are structurally intact and retain their ATPase activity, but, for reasons which are not yet clear, they do not become motile when treated with ATP. Digitonin-extracted cilia were prepared by extracting with 0.5 per cent digitonin in Tris-Mg solution (30 mM Tris-HCl buffer, 2.5 mM $MgSO_4$, pH 8.3 at 0°C). The digitonin-extracted cilia were fractionated by dialyzing them against Tris-EDTA-5KCl solution (0.1 mM EDTA, 5 mM KCl, 1 mM Tris-thioglycolate buffer, pH 8.3 at 0°C). All operations were carried out at 0–4°C. A more detailed account of the fractionation procedures has been given elsewhere (4). Solutions of nucleotides were titrated to approximately pH 7.8 before use.

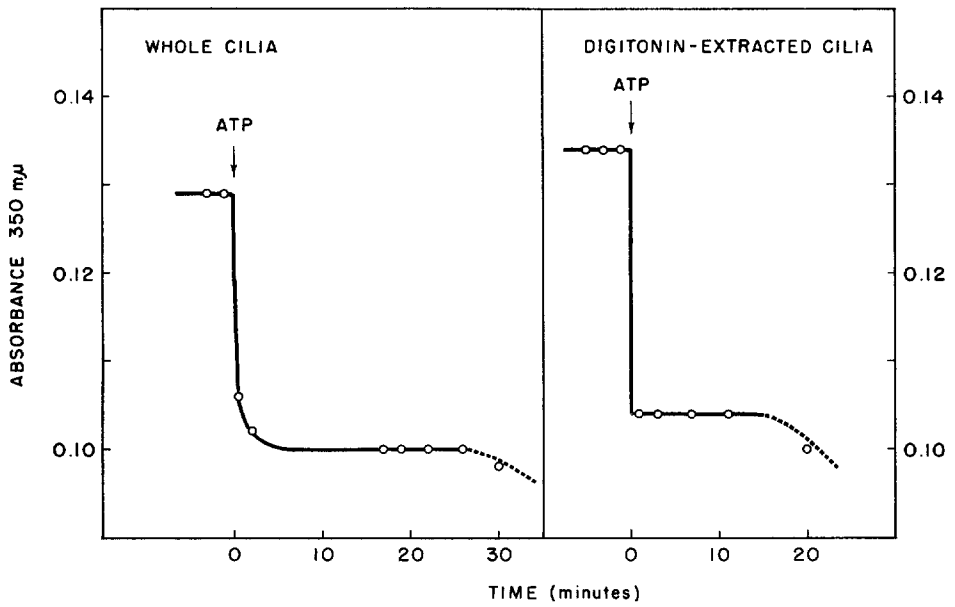

FIGURE 1 Turbidity of whole cilia and of digitonin-extracted cilia suspended in TMSK solution. ATP (1.6×10^{-4} M) was added at time 0 as indicated.

Turbidity measurements were usually made at 350 mμ using 1-cm cuvettes in a Zeiss PMQ II spectrophotometer at room temperature. The semiacceptance angle of the photomultiplier was approximately 3°. Except where otherwise noted, the preparations were suspended in either Tris-Mg or TMSK solution in a concentration such that the initial absorbance lay between 0.1 and 0.2. Control experiments showed that under these conditions the absorbance was accurately proportional to the concentration of cilia in suspension.

RESULTS

The addition of ATP to a suspension of intact, freshly isolated cilia causes an immediate decrease of 15 to 20 per cent in turbidity (Fig. 1). A similar effect is obtained when ATP is added to a suspension of digitonin-extracted cilia, but in this case the magnitude of the decrease is 20 to 30 per cent. Since the percentage decrease in turbidity is usually larger for digitonin-extracted cilia, which lack membranes (3), it is clear that the ATP is acting on the axonemal protein of the cilium and that the presence of the outer membrane is unnecessary. Accordingly, experiments to study the effect in more detail were carried out on digitonin-extracted cilia.

With freshly prepared digitonin-extracted cilia, the magnitude of the decrease in turbidity is independent of the concentration of ATP in the range 1×10^{-5} to 3×10^{-3} M. Within this range of concentration the decrease appears permanent; that is, there is no recovery within 20 minutes.[2] However, at lower concentrations of ATP the effect is partly reversible; for example, a concentration of 6×10^{-6} M gave an immediate decrease of 9 per cent, but the turbidity then slowly increased over a period of about 10 minutes, until the net decrease was only 3 per cent. The addition of ADP had an effect similar to that obtained with ATP, although there were some differences in detail. However, the effect

[2] Observations on turbidity are limited to about 20 minutes since ATP causes a gradual aggregation of the digitonin-extracted cilia. The early stages of this aggregation cause no measurable change in turbidity, as is shown by the fact that turbidity remains unchanged during its slow development in the period from 2 to 20 minutes after adding ATP (Fig. 1). After about 20 minutes, however, when aggregation has proceeded to the point where small flocculi are visible to the eye, the readings of turbidity tend to decrease and become somewhat unreproducible. After 1 to 2 hours the flocculi usually settle toward the bottom of the cuvette.

The different rate of development with time indicates that the initial decrease in turbidity is unrelated to this aggregation.

was specific for ATP and ADP, since addition of AMP (1.6×10^{-4} M), ITP (up to 3×10^{-3} M), sodium pyrophosphate (6×10^{-4} M), or EDTA (1.6×10^{-4} M) caused no change in turbidity. The high specificity is shown particularly clearly by the fact that ITP had no effect, even when added in a concentration 300-fold higher than was necessary for ATP. A similar specificity for ATP or ADP was described for perch sperm tails by Tibbs (9).

A suspension of digitonin-extracted cilia in 2.5 mM $CaCl_2$, 30 mM Tris buffer gave the usual decrease in turbidity when treated with ATP. On the other hand, ATP had no effect on the turbidity of a suspension in 1 mM EDTA, 25 mM KCl, 30 mM Tris buffer. These results support Tibbs' conclusion (9) that activation of the ATPase is necessary for the change in turbidity to occur.

The variation in the turbidity of a suspension of cilia with the wavelength of incident light was measured at several points in the range 350 to 1000 mμ. A log-log plot of absorbance against wavelength yielded a straight line of slope −2.3, showing that the relationship $A \propto 1/\lambda^{2.3}$ held for the range studied. With only slight changes in the power exponent this relationship held for both whole and digitonin-extracted cilia, before and after adding ATP.

So far only the turbidity of the intact digitonin-extracted cilia has been considered. Further information can be obtained by studying the effect in different subfractions. When digitonin-extracted cilia are dialyzed against Tris-EDTA-5KCl solution, the resultant soluble fraction (Fraction 1) consists of 14S and 30S dynein (the axonemal ATPase protein), together with some non-ATPase protein having a sedimentation constant of about 4S. Examination by electron microscopy of the residue insoluble in Tris-EDTA-5KCl solution (Fraction 2) has shown that only the ciliary outer fibers remain (3, 4). The three components of Fraction 1 can be separated by density gradient centrifugation.

The intrinsic turbidity of Fraction 1 in solution was too small to be measured with the apparatus used. The turbidity of the particles of Fraction 2, suspended in Tris-Mg solution, was unaffected by addition of ATP (1.6×10^{-4} M).

In previously published work involving chemical analysis for protein and ATPase activity it has been shown that part of Fraction 1 recombines with the particles of Fraction 2 when excess magnesium is added back to the dialyzed preparation (3). It has now been found that this recombination can be followed turbidimetrically upon addition of Fraction 1 (in the same proportion as that present in intact cilia) to a suspension of Fraction 2 particles in Tris-Mg solution. In one such experiment, the turbidity of the suspension increased 63 per cent over a period of about 15 minutes and then remained constant (Fig. 2). This increase in turbidity can be interpreted as due to the increase in anhydrous mass of the particles as the soluble protein of Fraction 1 becomes bound (see Discussion). Addition of ATP to the suspension after recombination was complete caused a decrease of about 15 per cent in turbidity.

The three components of Fraction 1 were next tested separately to see which of them would recombine and restore sensitivity to ATP. The results of these experiments (Table I) showed clearly that only 30S dynein was capable of both recombining and restoring sensitivity. The 14S dynein also recombined to some extent, but it failed to restore sensitivity. The 4S component was completely inactive. Thus it seems clear that the presence of both Fraction 2 and the 30S dynein is necessary for sensitivity to ATP. Moreover, the recovery of sensitivity argues that these

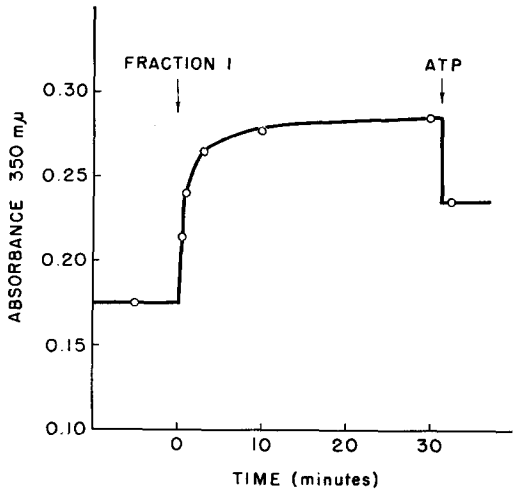

FIGURE 2 Turbidity of Fraction 2 particles suspended in Tris-Mg solution. Fraction 1 was added at time 0, in same proportion as is present in intact cilia. ATP (1.6×10^{-4} M) was added at time 31 minutes.

TABLE I

Recombination with Fraction 2 by the Various Components of Fraction 1

	4S	14S	30S
Initial absorbance of Fraction 2 in Tris-Mg solution	0.155	0.160	0.155
Absorbance 20 min. after addition of component shown	0.155	0.171	0.198
Absorbance after subsequent addition of 1.6×10^{-4} M ATP	0.153	0.170	0.175

The amounts of 4S, 14S, and 30S components added were equal to 58, 8, and 21 per cent, respectively, of the amount of Fraction 2 present. These are approximately the proportions in which they are obtained from the cilia.

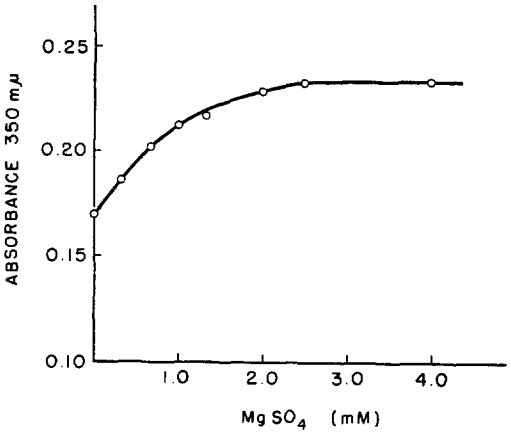

FIGURE 3 Particles of Fraction 2 were suspended initially in 3 mM KCl, 1 mM Tris buffer, pH 7.8. Small aliquots of $MgSO_4$ were added successively at intervals of 5 minutes. The turbidity was read 4 minutes after each addition.

two fractions recombine in a manner similar to their original relationship in the native cilium.

It was noticed that the turbidity of pure Fraction 2 particles was decreased when they were suspended in a medium of very low salt concentration instead of the usual Tris-Mg solution. The effect of adding $MgSO_4$ to a suspension of Fraction 2 is shown in Fig. 3. As the magnesium concentration was increased from 0 to 2 mM, the turbidity increased by about 40 per cent; higher concentrations of magnesium (up to 4 mM) had little further effect. In producing this effect, $MgSO_4$ could be replaced by approximately equimolar concentrations of $CaCl_2$ or by 15-fold higher concentrations of KCl. This effect of salt concentration on turbidity is considered to mean that the outer fibers become swollen in the absence of sufficient salt (see Discussion). As mentioned above, ATP has no specific effect on the turbidity of Fraction 2 particles alone.

DISCUSSION

There is as yet no completely rigorous theory which enables one to calculate the intensity of light scattered by particles of the size of cilia, in terms of their dimension and refractive index. Suitable approximations have been considered in some detail by Koch (7), who has applied them to calculate the turbidity of bacteria and mitochondria. The Jobst approximation (6) is the most amenable, and will be adopted here as a basis for discussion.

This approximation can be written (7)

$$A = \frac{27}{4 \times 2.3} \cdot \sqrt[3]{\frac{\pi}{6}} \cdot \left(\frac{1}{n_0}\frac{dn}{dc}\right)^2 \cdot \frac{q^2\nu}{V^{2/3}\lambda'^2} \qquad (1)$$

where A is absorbance, q is anhydrous mass of a scattering particle, V is volume of a particle, ν is number of particles per unit volume, λ' is wavelength of light in the medium, n_0 is refractive index of the medium, and dn/dc is the differential refractive index of the substance forming the particle. For our purpose equation (1) can be modified to

$$A \propto \frac{q^2\nu}{V^{2/3}\lambda^{2.2}} \qquad (2)$$

where λ is wavelength of the light in air. The variation of n_0 and dn/dc with wavelength (8) has been incorporated directly into the wavelength term.

The Jobst approximation is a limiting case for large spheres, and the validity of applying it to cilia may be considered as open to some question. However, use of equation (2) as an approximation in considering *changes* in turbidity is justified empirically by its agreement with the experimental data. It has been shown that the absorbance is proportional to the number of cilia per unit volume, and that the wavelength power

dependence is 2.3 experimentally, in good agreement with the calculated value of 2.2.

If aggregation of the particles occurs, equation (1) predicts that the absorbance will vary as $k^{1/3}$, where kq is the anhydrous mass of an aggregated particle. In fact, the effect of aggregation of cilia is even smaller, probably because their asymmetric shape tends to make them form very loose flocculent aggregates. As shown in Fig. 1, there is essentially no change in absorbance during the slow aggregation that occurs in the period from 2 to 15 minutes after ATP is added to a suspension.

Under the usual experimental conditions the concentration of cilia and the wavelength are constant, and equation (2) can be used to calculate the approximate change in turbidity to be expected for a given change in anhydrous mass or in degree of hydration. Use of this equation assumes that the particles do not change appreciably in over-all shape, and that changes in mass and hydration are homogeneous throughout a particle.

When Fraction 1 of ciliary protein recombines with the particles of Fraction 2, the increase in turbidity to be expected can be calculated, since the amount of protein that becomes bound is known (Table II). The reasonably close agreement between the calculated and observed values suggests that this increase in turbidity can be satisfactorily explained by the increase in anhydrous mass of the particles.

The addition of ATP decreases the turbidity of digitonin-extracted cilia by 20 to 30 per cent. If this decrease were to be attributed to anhydrous mass, then it would correspond to a loss of 11 to 17 per cent. However, a preliminary chemical analysis showed that the actual loss of protein into solution under these conditions corresponds to only about 5 per cent of the mass (see Table 2 of reference 3), and this can account for only a small part of the decrease in turbidity. It seems probable, therefore, that most of the decrease in turbidity induced by ATP is the result of an effective increase in hydration of the ciliary structure. A similar interpretation, although based on different evidence, was made by Tibbs in his study of the effect of ATP on perch sperm tails (9).

This interpretation receives indirect support from the experiments on the effect of salt concentration on the turbidity of Fraction 2 particles alone. In this case there is no possibility of a significant increase in anhydrous mass, so that there can be no doubt that the changes in turbidity are due to changes in hydration. The results indicate that the particles of Fraction 2, *i.e.* the outer fibers, become highly swollen at very low salt concentration. The presence of about 2 mM $MgSO_4$ or 30 mM KCl is sufficient to prevent this swelling, and higher salt concentrations produce little further change in hydration. It is interesting to note that the minimum concentration of KCl needed to keep dynein bound to the outer fiber (25 mM) (4) is about the same as that needed to prevent the fiber from swelling.

The study of fractionated cilia has shown that the presence of two distinct protein components, the outer fibers and the 30S dynein, is necessary for the effect of ATP on hydration to occur. Details of the underlying changes in molecular organization remain to be elucidated. However, the nucleotide specificity and the low concentration of ATP needed to give full effect indicate that the increased hydration is caused by a specific interaction of the nucleotide with the ciliary protein. The conditions for inducing motility in glycerinated cilia show the same specificity for ATP or ADP, and the minimum concentration of ATP needed is also about 10^{-5} M (1). This similarity in conditions suggests that the effect of ATP on hydration is closely related to the processes responsible for motility.

TABLE II

Comparison of Calculated and Observed Turbidity Changes due to Recombination of Fractions 1 and 2

	Δq	$\Delta A_{calc.}$	$\Delta A_{obs.}$
Whole Fraction 1	8.3	49	63
Purified 30S dynein	5.4	30	28

Δq is percentage of total ciliary protein which became rebound to the particles of Fraction 2, as determined by protein and enzyme analysis. $\Delta A_{calc.}$ is the percentage increase in turbidity calculated from $A \propto q^2$, assuming Fraction 2 to be 38 per cent of the total ciliary protein. $\Delta A_{obs.}$ is the observed percentage increase in turbidity 20 minutes after adding a stoichiometric amount of whole Fraction 1, or of the purified 30S dynein, to a suspension of Fraction 2 in Tris-Mg solution.

See Table I for amounts of Fraction 1 and of 30S dynein added.

The action of ATP in increasing the hydration of isolated cilia may be contrasted with its action on isolated myofibrils and on gel particles of purified actomyosin, where it characteristically causes a *decrease* in hydration (corresponding to contraction or syneresis) (2). If this comparison is taken at face value, it would suggest that the direct effect of ATP on cilia is to produce expansion of the active elements responsible for motility. This hypothesis makes an interesting parallel with Hoffmann-Berling's finding that ATP causes extension of the motile stalk of the ciliate *Vorticella* (5). However, other possible explanations need to be studied further before it can be accepted that the basic mechanism of motility in cilia is so different from that thought to exist in muscle.

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