SOME ENZYMATIC PROPERTIES OF AXONEMES FROM THE CILIA OF *PECTEN IRRADIANS*

R. E. STEPHENS and E. E. LEVINE. From the Department of Biology, Brandeis University, Waltham, Massachusetts 02154

INTRODUCTION

Ciliary and flagellar axonemes have been fractionated into several well defined protein components. As in muscle, the proteins of these organelles correspond to enzymatic and structural elements, but their interactions are still quite uncertain.

The ATPase dynein, identified as "arms" in the 9 + 2 structure (Gibbons, 1963), can be obtained in two enzymatically active forms. Cilia from *Tetrahymena pyriformis* yield both a 14S "monomer" and a 30S polymeric form (Gibbons and Rowe, 1965), while sea urchin flagella yield only the 14S particle (Gibbons, 1965 *a*). The enzymology of the two forms of dynein has been studied in detail by Gibbons (1966). Allen (1968) has recently presented evidence that the two arms of *Tetrahymena* cilia are structurally asymmetric, but it is still unclear whether the two enzymatic forms of dynein have different locations or are simply related as monomer and polymer, associating differently to form the unique arms.

The protein of the outer fibers of cilia and flagella, *tubulin* (Mohri, 1968), has been shown to contain bound guanine nucleotides, occurring in a ratio of 1 mole of total guanosine di- or triphosphate per 60,000 g (1 mole) of outer fiber tubulin in sea urchin flagella, or the same amount of guanine or guanosine in *Tetrahymena* or *Pecten* cilia (Stephens, Renaud, and Gibbons, 1967; Stephens and Linck, 1969). These results have been confirmed in principle for sea urchin outer fibers by Yanagisawa, Hasegawa, and Mohri (1968) and, in addition, these workers have presented evidence that the terminal phosphate of ATP is transferred to the bound guanine nucleotide of the outer fibers during active flagellar beat, presumably as a result of dynein ATPase activity.

A great deal has been learned over the last several decades about the chemistry of muscle contraction through detailed ultrastructural studies of the sarcomere during contraction, the fractionation of the sarcomere into its constituent proteins, a study of the properties of these proteins, the reconstitution of the natural form that these proteins take in the filaments of the sarcomere, and finally a comparison of the enzymatic properties of the myofibril with those of its constituent parts (cf. Huxley, 1969). It is with this philosophy in mind that a detailed study of the cilium and its constituent proteins has been undertaken. This present report attempts to outline some of the basic enzymatic properties of the ciliary axoneme, i.e. the dynein tubulin complex without the presence of complicating membranexATPase. A subsequent report (R. Linck, manuscript in preparation) will describe the properties of dynein obtained from this same system, the gill cilia of *Pecten irradians*.

MATERIALS AND METHODS

Mass quantities of cilia were prepared from the gills of the pelecypod mollusc *Pecten irradians* by the procedure of Stephens and Linck (1969). Excised gills,

washed three times with cold filtered seawater, were suspended in 10 times their volume of double-strength sea water (30 g of NaCl per liter of normal seawater) at room temperature and agitated gently for 10 min. The deciliated gills were removed by filtration through cheese cloth, and the filtrate was centrifuged for 5 min at 1000 *g*. Pure cilia were recoved from the resulting supernatant fluid by centrifugation at 10,000 *g* for 5 min. Axomemes were then prepared by removal of the ciliary membrane by extracting the preparation twice in the cold with 25 volumes of 1% Triton X-100 detergent in a solution of 30 mM Tris·HCl, pH 8.0, 3 mM $MgCl_2$, and 0.1 mM dithiothreitol. The resulting axonemes were routinely washed with 10 mM Tris, pH 8.0, by centrifugation and then resuspended in either the same buffer or in the above Tris-magnesium buffer, to a concentration of 3–5 mg of protein per ml. The preparation was used within 24 hr for the procedures described below.

Aliquots of the axoneme suspension (400–800 μg of total protein) were pipetted at zero time into 10 ml of an assay mixture consisting 30 mM Tris·HCl, pH 8.0, 0.15 mM EDTA, 1.5 mM $MgCl_2$, and 1 mM ATP at 20°C. Samples (3 ml) were taken at 0, 10, and 20 min and the enzymatic action was stopped by addition of 0.3 ml of 50% trichloracetic acid. Protein was removed by centrifugation and the amount of inorganic phosphate in the supernatant fluid was determined by the method of Taussky and Schorr (1952). Protein concentration in the axoneme suspension was determined by the method of Lowry et al. (1951), using bovine serum albumin as a standard. Enzymatic activity was expressed in micromoles of inorganic phosphate released per milligram of axonemal protein per minute (μM P_i/mg-min). Appropriate substitutions of nucleotide, divalent cation, buffer, or salt were made in a fashion obvious from the experimental determinations to follow.

For the electron microscopic observations, samples of whole cilia and Triton-washed axonemes were fixed as pellets for 1 hr at 0°C with 3% glutaraldehyde in 30 mM Tris·HCl, pH 8.0, containing 3 mM $MgCl_2$ and 0.1 mM dithiothreitol. The preparation was washed three times with the above buffer, and then postfixed with 1% OsO_4 in Veronal-acetate buffer for 1 hr. Following dehydration in a graded series of alcohols, the preparations were embedded in Araldite resin. Sections of 600 A thickness were cut perpendicular to plane of the pellets with a diamond knife, stained with uranyl acetate and lead citrate, and observed in an RCA EMU 3G electron microscope at 50 kv.

RESULTS AND DISCUSSION

The isolated whole cilia were quite free of gross contamination when observed by electron microscopy (Fig. 1). As is typical of isolated cilia subjected to centrifugal force, many membranes of adjacent cilia were fused. The Triton X-100-washed cilia were virtually free of ciliary membranes but showed some membrane remnants, apparently of cellular origin, not removable with further detergent washing (Fig. 2). These axonemes consistently showed the characteristic 9 + 2 structure and arms, but secondary fibers and spokes were seldom obvious. With the use of the tilting and rotation technique devised by Allen (1968) to enhance ninefold symmetry in cross-sectioned cilia, only the arms showed any appreciable enhancement. As first described by Allen (1968), but apparent also in much earlier micrographs (cf. Gibbons and Grimstone, 1960), the two dynein arms are clearly asymmetric (Fig. 3).

The isolated gill cilia of *Pecten irradians* provide an almost ideal experimental system for biochemical study. They may be conveniently prepared in mass quantities and require no further purification. Whether stored as whole cilia or as axonemes, the ATPase properties are retained indefinitely in 50% glycerol at −20°C, or for many days as a pellet on ice. Removal of the ciliary membrane is easily done with Triton X-100, leaving an axoneme whose ATPase properties are not adversely affected by the extraction. Whole cilia have an ATPase activity of 0.09 ± 0.02 μM P_i/mg-min, measured at 20°C with magnesium activation. About two-thirds of the ciliary protein is accounted for by the axonemal fraction, which has an ATPase activity of 0.11 ± 0.01 μM P_i/mg-min and represents 88% of the ciliary ATPase. The remaining 12% can be accounted for as a membrane-bound ATPase, removable with detergent extraction. Thus essentially all of the original ATPase can be recovered, either as axonemal or detergent-extracted enzyme, agreeing in principle with the fractionation results of Gibbons (1965 *a*) for cilia of *Tetrahymena pyriformis*.

With variation in temperature, the logarithm of the ATPase activity decreases linearly with the reciprocal of the absolute temperature in the range of 0°–35°C. An Arrhenius plot of the data is given in Fig. 4; the slope of the function corresponds to an activation energy of +12,300 kcal, characteristic of a reaction which doubles with every 10°C increase in temperature. The deviation above 35°C may be indicative of structural breakdown, since the B-subfiber protein in flagellar outer fibers becomes soluble under such conditions of time and

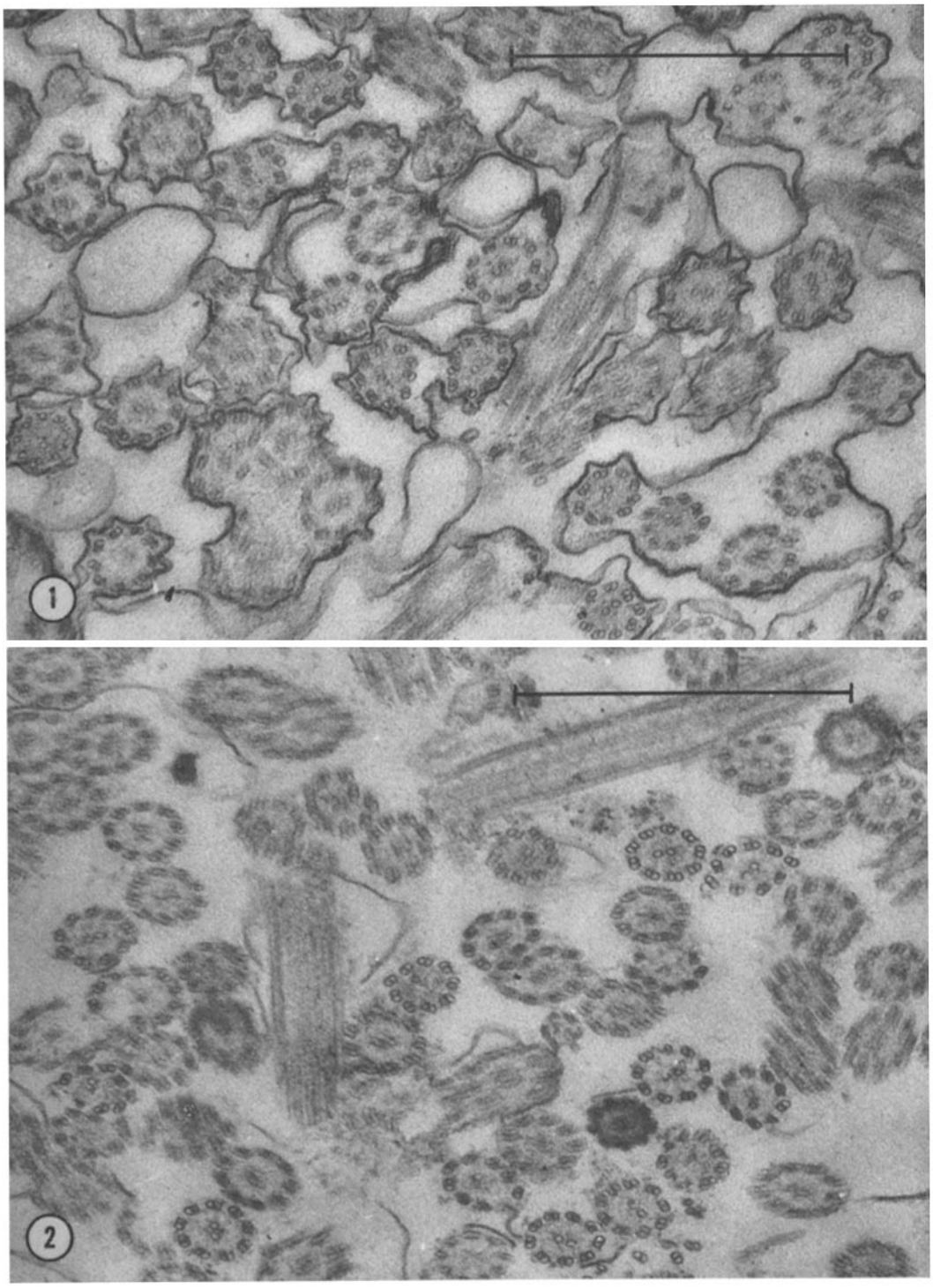

FIGURE 1 Cilia isolated from the gills of *Pecten irradians*. Membranes are fused due to repeated centrifugation. Scale = 1 μ. × 50,000.

FIGURE 2 Ciliary axonemes prepared by extraction of whole cilia with Triton X-100. Scale = 1 μ. × 50,000.

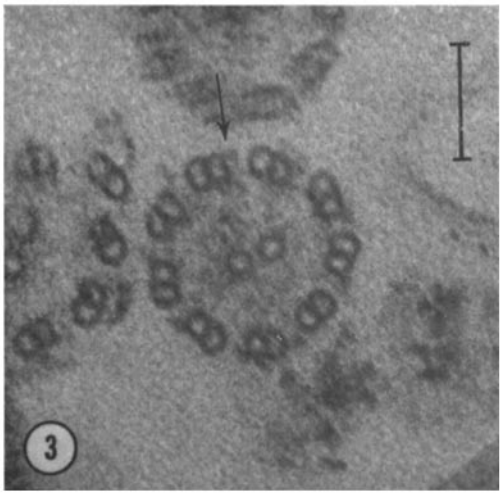

FIGURE 3 High magnification view of a typical cross-section of a ciliary axoneme. Arrow indicates the asymmetric arms characteristic of dynein. Scale = 1000 A. × 150,000.

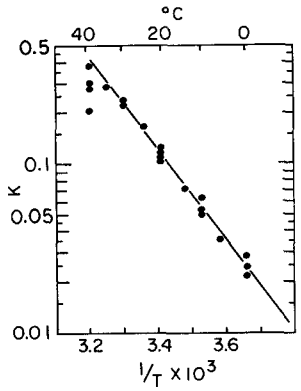

FIGURE 4 Arrhenius plot of the log of ATPase activity (μM P_i/mg-min) vs. the reciprocal absolute temperature ($°K^{-1}$). The ATPase was measured in the presence of magnesium.

temperature (Stephens, 1970), though generally not at such an ionic strength.

The enzymatic properties found for *Pecten* axonemes were generally parallel to those determined earlier for whole cilia or flagella (Brokaw, 1961; Child, 1959; Mohri, 1958; Nelson, 1954, 1955; Tibbs, 1959), but in this case the values were not complicated by either membrane or mitochondrial ATPases.

Ciliary axonemes possessed a remarkably high degree of specificity for ATP. Under identical conditions, the nucleotide triphosphates GTP, ITP, UTP, and CTP were hydrolyzed, on the average, at about 7% of the rate at which ATP was split. ADP was hydrolyzed at roughly 25% of the ATPase rate, while AMP and GDP were not hydrolyzed significantly. The nucleotide specificities for ciliary axonemes presented here are virtually identical to those of either 14S or 30S *Tetrahymena* dynein (Gibbons, 1966). Thus, association of dynein with guanine nucleotide-containing outer fiber microtubules appears not to affect the specificity; neither GTP nor GDP is a significant substrate for the enzyme-microtubule complex, though the former nucleotide is hydrolyzed at a slightly greater rate than the other nucleotide triphosphates studied. The apparent ADPase activity has been explained as an accompanying adenylate kinase (Brokaw, 1961; Gibbons, 1965 *a*). In preparations of *Pecten* axonemes, it was present in a consistent amount, assuming of course that ADP hydrolysis is an accurate reflection of adenylate kinase alone.

In the absence of added divalent cation, EDTA-washed axonemes show no significant ATPase activity, i.e. less than 0.005 μM P_i/mg-min. As mentioned above, the magnesium-activated ATPase was 0.11 ± 0.01 μM P_i/mg-min. That for calcium under the same conditions (1.5 mM) was found to be 0.08 ± 0.01 μM P_i/mg-min. Reduction of the concentration of cation through a range down to 10^{-4} M reduced the activity by no more than 20%, with the magnesium to calcium ratio consistently remaining 3:2. Increase in cation concentration to 10^{-2} M caused only a slight decrease in activity, and no change in the magnesium to calcium ratio. When other divalent cations were employed at 1.5 mM concentrations, manganese was found to activate nearly as well as magnesium, whereas iron, cobalt, and nickel functioned from 20 to 40% as well. Strontium activated at roughly 5% of the maximal magnesium value. There was no appreciable activation with zinc or cadmium. In *Tetrahymena*, 30S dynein shows no preference for either cation, but the 14S form has a magnesium to calcium ratio of about 2:1 (Gibbons, 1966). The axoneme of *Pecten* thus has a magnesium to calcium activation ratio midway between the two forms of dynein. With respect to the other cations tested, the axoneme closely parallels the divalent cation activation profile for the isolated dyneins of *Tetrahymena*.

When the standard assay conditions were used,

but Tris·HCl buffer in the range of pH 5.5–10.0 or sodium acetate buffer in the range of pH 3.5–7.0 was substituted, the pH optimum for the ATPase activity of *Pecten* axonemes was determined. Since some of these pH values are substantially beyond the buffering ranges of the salts in question, the pH was measured before and after reaction but was found not to have changed significantly. The relative variation in ATPase activity with pH is illustrated in Fig. 5. The axoneme has maximum ATPase activity at pH 8.0 ± 0.5, but the data also indicate a possible second maximum around pH 6.0. Judged by comparison with other enzymes, the ciliary axoneme has a rather broad pH "optimum," with both extremes of pH causing denaturation. The pH optimum for *Pecten* axonemes is centered nearly one pH unit below that for either form of *Tetrahymena* dynein. Like the 30S *Tetrahymena* dynein, the *Pecten* axoneme has a second maximum around pH 6.0. Aside from the shift in alkaline pH optimum, the pH behavior of *Pecten* axonemes may be fairly well represented as a composite of the unique pH curves for the two dyneins of *Tetrahymena* (cf. Gibbons, 1966).

Varying amounts of KCl were added to the standard assay mixture, and the ATPase activity of *Pecten* axonemes under conditions of increasing ionic strength was determined. Fig. 6 represents the variation in the axonemal ATPase activity at ionic strengths of KCl up to 1.0. At most, a 30% increase in ATPase activity took place as the concentration of KCl approached 0.4 M, after which the activity decreased until, at 1.0 M, it reached a value nearly comparable to that of no salt addition. In *Tetrahymena*, 14S dynein decreases in activity about threefold when KCl is increased to 0.8 M, whereas 30S dynein increases by a comparable amount (Gibbons, 1966). The axonemal ATPase thus somewhat resembles the latter form. Perhaps combination of dynein with outer fibers minimizes the effect of ionic strength through some stabilization of conformation.

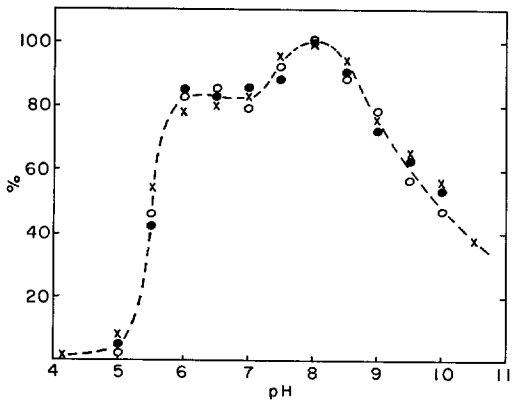

FIGURE 5 Relative ATPase activity of ciliary axonemes at various pH values. The data were normalized by taking the pH optimum as 100%.

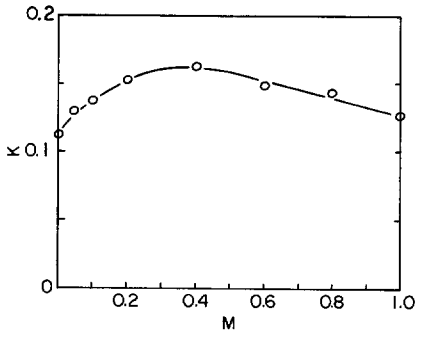

FIGURE 6 The effect of KCl concentration on the ATPase activity of ciliary axonemes. The activity is expressed in μM P_i/mg-min.

From the data presented here, one might speculate that, since the axonemal ATPase properties in *Pecten* are generally "mid-way" between those of the two forms of isolated *Tetrahymena* dynein, perhaps there are two distinct forms of dynein actually present in the ciliary axoneme, as constrasted with the idea that 30S dynein is a simple polymer of the 14S form. One can invoke species differences here, however, since dynein from a ciliate is being compared with the axoneme of a mollusc. But parallel studies on the axonemes of echinoderm flagella (Stephens and Levine, unpublished data) or early work on whole echinoderm sperm tails (Mohri, 1958) indicate properties virtually indistinguishable from those of *Pecten* axonemes, and 14S dynein isolated from *Pecten* behaves in a manner quite like its *Tetrahymena* counterpart (R. W. Linck, manuscript in preparation). Thus, at least as far as these studies have gone, species differences appear to be negligible.

The lack of any significant departure of the enzymatic properties of the axoneme from those of isolated dynein could indicate that the motile force for ciliary movement resides in dynein-dynein interaction alone, with the doublet microtubules serving some sterically-necessary support function. The ATP-specific turbidity changes observed in cilia, axonemes, and in 30S dynein-outer fiber

mixtures (Gibbons, 1965 *b*) demonstrate that the presence of outer fibers is crucial for some sort of enzyme-substrate interaction resulting in a change in either conformation or state of aggregation. It need not imply that the basic rates or specificities of the enzyme must change, contrasted with the case of muscle wherein actin greatly enhances myosin ATPase while troponin modifies its calcium sensitivity. It is, of course, also possible that dynein, as isolated, is already modified by other components; both the 14S and 30S dyneins from *Tetrahymena* have been shown to have additional proteins present (Gibbons, 1963, 1965 *a*).

This work was supported by USPHS Grant GM 15,500.

Received for publication 12 January 1970, and in revised form 28 February 1970.

REFERENCES

ALLEN, R. D. 1968. *J. Cell Biol.* **37**:825.

BROKAW, C. J. 1961. *Exp. Cell Res.* **22**:151.

CHILD, F. M. 1959. *Exp. Cell Res.* **18**:258.

GIBBONS, I. R. 1963. *Proc. Nat. Acad. Sci. U. S. A.* **50**:1002.

GIBBONS, I. R. 1965 *a*. *Arch. Biol.* **76**:317.

GIBBONS, I. R. 1965 *b*. *J. Cell Biol.* **26**:707.

GIBBONS, I. R. 1966. *J. Biol. Chem.* **241**:5590.

GIBBONS, I. R., and A. V. GRIMSTONE. 1960. *J. Biophys. Biochem. Cytol.* **7**:697.

GIBBONS, I. R., and A. J. ROWE. 1965. Science (*Washington*). **149**:424.

HUXLEY, H. E. 1969. Science (*Washington*). **164**:1356.

LOWRY, O. H., N. J. ROSENBROUGH, A. L. FARR, and R. J. RANDALL. 1951. *J. Biol. Chem.* **193**:265.

MOHRI, H. 1958. *J. Fac. Sci. Univ. Tokyo Sec. IV.* **8**:307.

MOHRI, H. 1968. *Nature* (*London*). **217**:1053.

NELSON, L. 1954. *Biochim. Biophys. Acta.* **14**:312.

NELSON, L. 1955. *Biol. Bull.* (*Woods Hole*). **109**:295.

STEPHENS, R. E. 1970. *J. Mol. Biol.* **47**:353.

STEPHENS, R. E., and R. W. LINCK. 1969. *J. Mol. Biol.* **40**:497.

STEPHENS, R. E., F. L. RENAUD, and I. R. GIBBONS. 1967. *Science* (*Washington*). **156**:1606.

TAUSSKY, H. M., and E. SCHORR. 1952. *J. Biol. Chem.* **202**:675.

TIBBS, J. 1959. *Biochim. Biophys. Acta.* **33**:220.

YANAGISAWA, T., S. HASEGAWA, and H. MOHRI. 1968. *Exp. Cell Res.* **52**:86.