most (if not all) early erythroblasts are dividing cells (*4*). A total labeling index of only 40 percent for the late erythroid cells indicates that the presence of nondividing components or slower rates of production, or both, may be a large factor in determining the extent of labeling in these cells.

It is possible from our data to calculate hourly cell production rates for both early and late erythroblasts. This is obtained from the ratio (*20*): labeling index (%)/T_S (hours), giving values of 25 and 5.3 percent per hour for early and late erythroblasts, respectively. For the less mature cells this represents a turnover time of about 4 hours (*21*), and for the more mature cells, of about 20 hours. An hourly production rate of 25 percent in the early erythroblast compartment is far greater than that given by others for the rat (for example, 7 percent per hour) (*17*). The discrepancy lies in the vastly different T_S durations used in calculating production rates, figures between 6 and 8 hours usually being employed (*17*). From our data it now appears that this contention of a constancy of T_S in early and late erythroblasts is no longer valid and that gross underestimations of early cell proliferation kinetics result if it is accepted. Some workers (*16*, *17*) have suggested that T_S could be shorter in earlier stages of differentiation but they made no direct observations to confirm this. Since T_S is basic to models of cell proliferation it will be necessary to determine the extent to which T_S differs among members of a given maturational sequence before such models are constructed.

The greater proliferative "efficiency" of the earlier stages in cell lineages has been noted before (*3*, *4*) and may prove to be a general phenomenon. It is now clear that a pool of dividing cells, belonging to the same cell line, can vary greatly in its kinetic constitution. The presence of nondividing cells and two or more subpopulations in cycle contained within the confines of a single cell lineage decrease the value of average measurements as parameters of kinetic analysis (*1*, *3*).

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Flagellar Adenosine Triphosphatase from Sea Urchin Sperm: Properties and Relation to Motility

Abstract. *Adenosine triphosphatase activated by divalent cations is apparently a component of the motile apparatus in flagella of* Arbacia *sperm, as judged by the activity of this enzyme in intact flagella, glycerol-extracted flagella, and soluble extracts prepared from flagella. However, the variation in the physical properties and in the amount of enzyme obtained after a variety of treatments suggests that additional components are involved in the motile mechanism. These features distinguish the soluble flagellar enzyme from adenosine triphosphatases of other motile cells.*

Adenosine triphosphatase components generally analogous to those of muscle cells are believed to participate in the motility of spermatozoa. Extraction of this enzyme in a soluble form (*1*) has made possible a study of its enzymatic and physical properties and its relationship to flagellar activity.

Male *Arbacia punctulata* were injected with 0.5*M* KCl solution (0.5 ml), and semen was collected in dry, chilled Stender dishes (*2*). All subsequent manipulations were carried out in the cold to minimize metabolic activity. After low-speed centrifugation to remove gross contamination, semen was diluted with ten volumes of cold CMFSW (the calcium- and magnesium-free synthetic seawater prepared by the Marine Biological Laboratory). Sperm were sedimented at 1000*g* for 10 minutes, washed by suspension in CMFSW and sedimented again, resuspended in 20 volumes of CMFSW, and homogenized 3 minutes in a glass homogenizer with Teflon pestle. Nearly quantitative separation of heads and flagella was achieved with very little debris. Heads and intact sperm were sedimented by two or three successive centrifugations at 1000*g* for 10 minutes, until the supernatant was essentially free of heads. The flagellar fraction was sedimented at 10,000*g* for 15 minutes. As indicated in Table 1 (preparation 1), such isolated flagella displayed substantial adenosine triphosphatase activity as well as motility. No measurable activity was extracted by Weber-Edsall solution (*3*) without prior mechanical or chemical disruption of the flagella, indicating the presence of structural barriers to extraction. The membrane nature of these barriers was shown by the facilitation of extraction by freezing and thawing (Table 1, preparation 2) or digitonin (preparation 3); flagella were exposed to digitonin-tris-Mg^{++} solution (*4*) [tris is tris(hydroxymethyl)aminomethane] for two successive 10-minute periods, each terminated by centrifugation at 10,000*g* for 10 minutes. The removal of membranes and cytoplasmic components from the motile apparatus by

glycerol extraction (*5*) of flagella (preparation 6) also revealed high adenosine triphosphatase activity. This enzymatic activity could be solubilized from flagella (as shown by its presence in supernatants from centrifugation at 10,000*g*) by various extraction procedures (Table 1). Preparations 2 and 3 were obtained by extracting flagella, after prior treatment as indicated, with Weber-Edsall solution (*3*) overnight at 4°C. Preparation 4 was obtained, from digitonin-treated flagella, by dialysis against a tris-ethylenediaminetetraacetate (EDTA) solution (*4*) for 30 hours at 4°C, and centrifugation of the contents of the dialysis bag. Preparation 5 was obtained by a single extraction of flagella overnight at 4°C, with a pyrophosphate medium (*6*) to which were added digitonin (2.5 mg/ml), tris (15 mmole/liter) and Mg^{++} (1.25 mmole/liter), *p*H 9.0.

Comparison of the adenosine triphosphatase activity per animal (1.5×10^{10} cells) measured in isolated flagella (preparation 1) with that of several soluble preparations (preparations 2 to 4) shows that they are nearly equal. A similar conclusion has been drawn in studies of isolated cilia and the enzyme obtained in soluble form from the cilia (*7*). (Comparison of the enzymatic activities solubilized in preparations 2 and 3 shows that freezing and thawing or exposure to digitonin were equally effective in facilitating subsequent extraction of adenosine triphosphatase.) However, activities two to three times higher were observed in a soluble preparation (*5*) and in the flagellar structure after glycerol extraction (preparation 6).

Sperm extracted with glycerol under the same conditions were tested for motility; they were virtually nonmotile in EDTA-Mg^{++}-ATP medium (*5*), but were vigorously motile in histidine-Mg^{++}-ATP medium (*5*). The specific activity of preparation 4 was highest because of the small amount of protein extracted. However, specific activities of preparations 5 and 6 were comparable to that of preparation 4 because large amounts of both adenosine triphosphatase activity and protein were recovered.

The enzymatic and physical properties of preparations 3 and 4 were of particular interest. The optimum concentration of either Mg^{++} or Ca^{++} for activation of the enzyme was near 5 m*M* (Fig. 1). Preparation 3 was more active than preparation 4 when no divalent cations were added, and also nearly optimally active over a larger range of

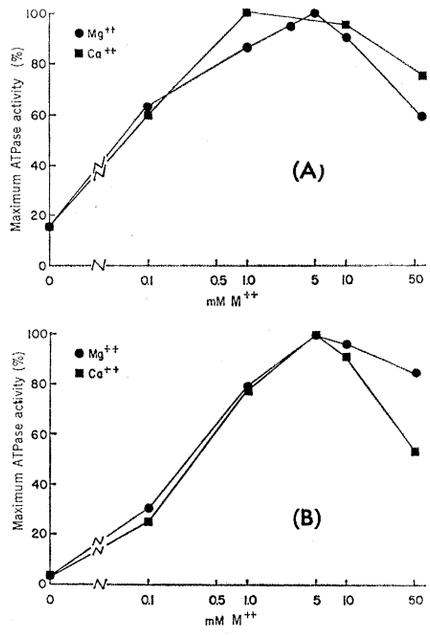

Fig. 1. Specific adenosine triphosphatase activity as a function of added divalent cation concentration (M^{++}), assayed as for Table 1. (A) Preparation 3; (B) preparation 4.

Ca^{++} concentration. [With 5 m*M* Mg^{++}, its activity was the same in either standard buffer or in a glycine buffer at *p*H 9.0 (*8*) developed for myosin.] In several separate preparations, activity with Ca^{++} was about 90 percent of that with Mg^{++} in preparation 3, and 90 to 100 percent of that with Mg^{++} in the case of preparation 4. Over a period of weeks in the Weber-Edsall solution, Mg^{++}-stimulated activity declined very slowly at 4°C; Ca^{++}-stimulated activity declined more rapidly, and activity measured without addition of cations declined still more rapidly. Nonstimulated and Ca^{++}-stimulated activities were also destroyed more quickly by lowered *p*H and exposure to room temperature than

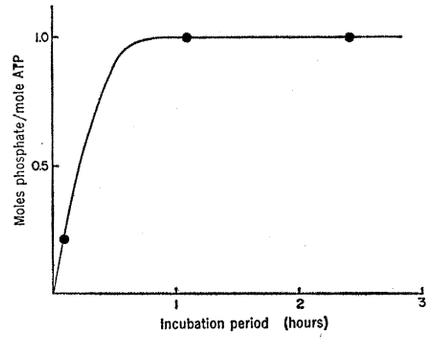

Fig. 2. Time course of the release of orthophosphate from ATP by preparation 3. One milliliter of the standard assay mixture contained 1 μmole of ATP (based on weight), 100 m*M* tris buffer, 5 m*M* Mg^{++}, and 1 mg of protein.

was that stimulated by Mg^{++}. In experiments with preparation 4, 97 percent of the Mg^{++}-activated adenosine triphosphatase activity was retained after 12 hours at 4°C and *p*H 7.0, whereas at *p*H 6.0 only 48 percent of the activity remained. (The original solution retained 70 percent of its activity after 8 days at 4°C.) After 12 hours at *p*H 7, 72 percent of the Ca^{++}-stimulated activity remained, and at *p*H 6, 20 percent remained.

The stoichiometry of the reaction was examined with preparation 3 under standard assay conditions, except that the buffer concentration was increased tenfold. An excess of the enzyme was incubated with adenosine triphosphate (ATP) over an extended period (Fig. 2); true adenosine triphosphatase (ATP phosphohydrolase, E.C. 3.6.1.3) activity was shown by the release of only 1 mole of phosphate per mole of ATP. The negligible slope of the latter portion of the graph indicates that contamination by irrelevant reactions and less specific phosphatases was negligible. [An analogous preparation from cilia (*9*) exhibits an apparent adenosine diphosphatase activity.] Control experiments showed that 20 percent of the initial adenosine triphosphatase activity remained after 2 hours at 25°C.

Preparation 3 had an ultraviolet absorption maximum near 270 nm and an absorbancy ratio (280/260) of 0.90; its spectrum was quite similar to that of actomyosin (*10*). Its intrinsic viscosity, n_{sp}/c, was less than 3 dl/g. The ultraviolet absorption maximum of preparation 4 was 277 nm and its 280/260 ratio was 1.3. After centrifugation in the Spinco model L preparative ultracentrifuge (50 rotor) calculated to precipitate material having a sedimentation constant ($S_{20,w}$) of 50 or greater, all of the adenosine triphosphatase activity remained in the supernatant although 35 percent of the solution protein was precipitated. The same preparation was examined in the Spinco model E analytical ultracentrifuge at 5°C and 59,780 rev/min. Heavy heterogeneous material, a small peak having a sedimentation constant ($S_{20,w}$) of 12, and a somewhat larger peak of sedimentation constant 2.7 were observed. After 135 minutes of centrifugation, when material having an $S_{20,w}$ of 10 or greater was calculated to be completely precipitated, most of the initial adenosine triphosphatase remained in the supernatant.

When preparation 3 was subjected to procedures (*3*) used to precipitate mus-

Table 1. Adenosine triphosphatase from *Arbacia* sperm flagella. Preparations 1 and 6 were suspensions; preparations 2 to 5 were supernatants from centrifugation at 10,000*g*. Protein was estimated by ultraviolet absorption and by the biuret reaction (*15*), and adenosine triphosphatase activity was measured by the method of Nelson (*16*). One milliliter of solution containing tris (10 m*M*), *p*H 8.6, ATP (1 m*M*), activating ions normally at 5 m*M*, and the enzyme was incubated for 5 minutes at 25°C. After addition of 1 ml of 10 percent trichloroacetic acid, chilling, and centrifugation, the supernatant was assayed for orthophosphate (Pi) (*17*). The specific activity is expressed as micromoles of Pi per minute per milligram of protein.

Preparation No.	Extraction		Yields per animal			Specific activity	
	Prior treatment	Method	Protein* (mg)	Pi(μmole min^{-1})			
				Mg^{++}	Ca^{++}	Mg^{++}	Ca^{++}
1	None	None	8.1	0.20		0.025	
2	Freeze-thaw (2 cycles)	Weber-Edsall	1.9	0.18	0.14	0.10	0.075
3	Digitonin	Weber-Edsall	3.3	0.18†	0.16	0.054	0.048
4	Digitonin	Dialysis versus EDTA	0.53	0.135	0.13	0.26	0.24
5	None	Pyrophosphate-digitonin	2.0	0.47‡		0.25	
6	None	Glycerol-KCl (28 days)	3.3	0.59	0.57	0.18	0.17

* Protein recoveries during fractionation: washed sperm 70 mg per animal. First flagellar supernatant (crude): 8.8 mg per animal. † Adenosine triphosphatase activity was observed, in the standard Mg^{++} assay, in the first and second digitonin extracts, equal respectively to 56 percent and 2 percent of that of the final preparation 3. ‡ There was no detectable pyrophosphatase activity in this preparation.

cle actomyosin, only about 3 percent of the protein precipitated at each step (adjustment to *p*H 6.5 and dilution to 0.33 ionic strength); on further dilution to 0.04 ionic strength as in the classical myosin preparation, no further precipitation was observed. At least 85 percent of the initial activity was lost and could not be recovered by combining the two precipitates in their original proportions with the supernatant solution.

The enzyme studied here is probably a major component of the motility apparatus of flagella; both soluble extracts and glycerol-extracted structures show the enzymatic activation by divalent cations characteristic of preparations from muscle (*3*) and cilia (*4*, *7*, *9*). Detailed mechanisms of flagellar motility cannot be specified at this time, but questions to be considered include their resemblance to the multicomponent system (actin, myosin, and other proteins) understood to function in muscle, or to a unique single-component mechanism proposed in cilia (*7*).

The equivalence between adenosine triphosphatase activities of isolated cilia and of soluble preparations from the cilia (*7*) indicated that all of the active protein was extracted from these organelles (as the single component "dynein"). Recoveries of adenosine triphosphatase activities from flagella (preparations 1 to 4) might suggest the same interpretation except that (i) activity greater than that of flagella can be solubilized (235 percent recovery in preparation 5 and 140 percent recovery in preparation 3 when digitonin extracts are included); (ii) glycerol-extracted flagella (preparation 6) have still greater activity (the motility of glycerol-extracted sperm indicating that the motile apparatus remained intact); and (iii) the oxygen consumption of sea urchin sperm (*11*) together with an assumed ratio of phosphorus to oxygen of 3 correspond to orthophosphate production of 0.6 to 4 μmole/hr per 10^8 cells. Since most of the sperm cell's energy is expended through flagellar activity (*12*), the production of 0.08 μmole/hr per 10^8 cells measured in this study suggests that all of the potential adenosine triphosphatase activity of isolated flagella was not expressed.

Two factors which may reduce the adenosine triphosphatase activity of flagella below that of preparation 5 or 6 are structural barriers to diffusion of reactants and enzymatic masking related to normal control mechanisms. The lower activities of preparations 2 to 4 compared with preparations 5 or 6 or with that anticipated from oxygen-consumption data suggest that these solutions may lack a component necessary for full activity. The high specific activities of preparations 5 and 6 support this suggestion, since they probably contain additional inactive structural proteins.

The *Arbacia* sperm adenosine triphosphatase resembles in several aspects that prepared from cilia of *Tetrahymena* (*4*, *7*, *9*), although it appears to be more slowly sedimenting, more stable, and more specific in that adenosine diphosphate is not hydrolyzed. The *Arbacia* sperm enzyme corresponds in some properties (solubility and stability in Weber-Edsall solution, and activation by both Mg^{++} and Ca^{++}) to muscle actomyosin, but differs in other properties (behavior on dilution, and solubility in tris-EDTA solution of ionic strength 0.02) (*13*). A pyrophosphate solution designed for extraction of myosin from muscle was particularly efficient in solubilizing flagellar adenosine triphosphatase; the enzymes may be similarly bound to structural proteins. Isolation of an actin-like material from sperm flagella of another echinoderm, *Asterias forbesii*, has been reported (*14*). The data are compatible with a multicomponent system in these flagella analogous to that of muscle. Nomenclature must remain tentative since some physicochemical properties differ from those of known muscle and ciliary components.

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