

# THE EFFECT OF ANTIDYNEIN 1 SERUM ON THE MOVEMENT OF REACTIVATED SEA URCHIN SPERM

BARBARA H. GIBBONS, KAZUO OGAWA, and I. R. GIBBONS

From the Pacific Biomedical Research Center, University of Hawaii, Honolulu, Hawaii 96822

## ABSTRACT

Rabbit antiserum prepared against an ATPase-containing tryptic fragment of dynein by Ogawa and Mohri (*J. Biol. Chem.* **250**:6476-6483) specifically inhibited the ATPase activity of dynein 1 and not that of dynein 2. Varying amounts of this antidynein 1 serum were added to demembrated sperm while they were swimming in reactivating solution containing 1 mM ATP. The sperm continued to form regularly propagated flagellar bending waves, but the beat frequency decreased gradually with time, the greater part of the change occurring in the first 15 min. The beat frequency after 1 h was a function of the amount of antiserum used, and could be as low as 1 Hz. The waveforms of the treated sperm resembled those of normal reactivated sperm except that the bend angles of both the principal and reverse bends were larger in the proximal portion of the flagellum. The ATPase activity and corresponding beat frequency of sperm which had been pretreated with varying amounts of antidynein 1 serum for 15 min at 0°C and then diluted were both decreased as a function of the amount of antiserum added, the ATPase activity decreasing more steeply than the frequency. The ATPase activity of homogenized, nonmotile sperm also decreased upon pretreatment with antiserum, but the percentage decrease was less than for motile sperm. For moderate to low concentrations of antiserum, the rates of reaction with motile and with rigor sperm were almost identical. The overall results suggest that antidynein 1 inhibits the functioning of the dynein arms, probably by blocking the ATPase sites of the dynein 1.

The general basis of the mechanism of flagellar motility is now well established (3, 10, 18-20). Active sliding movements between doublet tubules of the axoneme are produced by the interaction of the dynein arms with ATP and the neighboring B-tubule, perhaps in a cyclical manner involving a making and breaking of cross-bridges in a manner analogous to that thought to occur in muscle (13). The ATPase protein, dynein, thus plays a key role in movement by mediating the conversion of the chemical energy provided by ATP dephosphorylation into mechanical work.

However, the details of the energy transduction process are not yet understood, nor is it known by what mechanism the sliding movements of the tubules are regulated so as to produce the propagated bending waves characteristic of flagella.

In an attempt to learn more about the involvement of dynein in the mechanism of movement, we have investigated the effects of an antiserum which is known to inhibit dynein ATPase activity *in vitro* on the motility, waveform, beat frequency, and ATPase activity of demembrated sea urchin sperm. The antiserum was prepared by

Ogawa and Mohri (16) against Fragment A, an ATPase-containing tryptic fragment of dynein obtained from sperm flagella of the sea urchin *Anthocidaris crassispina* (14). Fragment A was shown to be homogeneous by sucrose density gradient centrifugation, polyacrylamide gel electrophoresis, and equilibrium centrifugation. Ouchterlony's test showed a single precipitin line between Fragment A and its antiserum. The antiserum specifically inhibited the ATPase activity both of Fragment A and of dynein itself (16). The inhibitory activity of this antiserum preparation has also been tested against different isoenzymes of dynein ATPase isolated and characterized in Gibbons's laboratory (12, 15), and shown to be specific for dynein 1. Therefore, we shall refer to this antiserum as antidynein 1 serum.

Our results provide further evidence for the involvement of the dynein arms in the mechanism of flagellar movement. The results are interpreted in terms of the various forms and possible functions of dynein 1 and dynein 2, and the detailed mechanism of motility. A preliminary account of these experiments has appeared previously (9, 12). Okuno et al. (17) have recently reported some related experiments using antidynein serum with sperm from two species of Japanese sea urchin.

## MATERIALS AND METHODS

Sperm from the sea urchin *Colobocentrotus atratus* were collected and stored as described previously (6). Before use they were diluted with sea water to give the desired concentration and maintained at 0°C.

The antiserum to dynein Fragment A was prepared in Japan by one of us (K. Ogawa) according to the procedure already described (16). For the present work, it was further purified by one or two precipitations from 50% saturated ammonium sulfate at pH 7.0, 0°C, then dialyzed into storage solution containing 0.02% sodium azide, 0.15 M KCl, 2 mM MgSO<sub>4</sub>, 0.5 mM EDTA, and 10 mM Tris-HCl buffer, pH 8.0. The purified preparation of antiserum, which contained approximately 20 mg protein/ml, was then passed through a Millipore filter (Millipore Corp. Bedford, Mass.), stored at 0°C, and used within 5 wks. Pre-immune serum was purified similarly.

Three principal experimental methods were used, all involving demembrated sperm. In the first method, designed to observe the action of antidynein on the beat frequency and waveform of reactivated sperm as a function of time, 25  $\mu$ l of a sperm suspension in seawater (~12 mg protein/ml) were added to 0.3 ml of demembrating solution (0.15 M KCl, 2 mM MgSO<sub>4</sub>, 0.5 mM EDTA, 1 mM dithiothreitol [DTT], 0.04% Triton X-

100 [wt/vol], 10 mM Tris-HCl buffer, pH 8.0) at room temperature (22°C). After gentle mixing for 30 s, 2.5  $\mu$ l of this suspension were transferred to 2.5 ml of reactivating solution (1 mM ATP, 0.15 M KCl, 2 mM MgSO<sub>4</sub>, 0.5 mM EDTA, 1 mM DTT, 2% polyethylene glycol [mol wt 20,000] (PEG), and 10 mM Tris-HCl buffer, pH 8.1). An aliquot of the purified antidynein was then added, and the flagellar beat frequency was measured as a function of time. After an appropriate incubation period, photographs were taken to obtain a record of the flagellar waveform of sperm beating at a known frequency.

In the second type of experiment, designed for measuring both the ATPase activity and the beat frequency of a given sample, 50  $\mu$ l of a sperm suspension in seawater (~20 mg protein/ml) were added to 0.6 ml of weakly buffered demembrating solution (0.15 M KCl, 2 mM MgSO<sub>4</sub>, 0.5 mM EDTA, 1 mM DTT, 0.04% Triton X-100, and 2 mM Tris-HCl buffer, pH 8.1, containing also 1 mM ATP). After 30 s, an aliquot of antidynein 1 serum was added and the suspension was incubated at 0°C for 15 min. The entire sample was then transferred to 15 ml of assay solution (0.15 M KCl, 2 mM MgSO<sub>4</sub>, 0.5 mM EDTA, 2% PEG, 1 mM ATP, 50  $\mu$ l oligomycin solution [3 mg in 2 ml 65% ethanol]), which were being stirred on a pH stat. The rate at which the sperm dephosphorylated ATP was measured at pH 8.0 and 25°C as described previously (6), and measurement of the average beat frequency was made by transferring a small sample of sperm to buffered reactivating solution containing 1 mM ATP in a petri dish. Non-motile sperm were prepared by forcing the sperm suspension in demembrating solution three times through a 30-cm length of 30-gauge plastic tubing (Bolab, Inc., Derry, N. H.) with a 1-ml plastic syringe. Antidynein 1 serum was then added and the incubation carried out as above. Control experiments were performed in which either an aliquot of the serum buffer or an aliquot of pre-immune serum was substituted for the antidynein serum.

A third type of experiment was designed to compare the extent of reaction of reactivated and of rigor wave sperm with antidynein serum. To observe the reaction with motile reactivated sperm, a 25- $\mu$ l aliquot of sperm suspension in seawater (~13 mg protein/ml) was added to 0.3 ml of demembrating solution containing 0.02 mM ATP, in a test tube precoated with egg white. After 30 s, a 5- $\mu$ l aliquot was transferred to 0.25 ml of buffered reactivating solution containing 1 mM ATP, an aliquot of purified antiserum was added, and the sample was incubated at room temperature (22°C) for 30 s to 5 min as described in Results. At the end of this time, the whole sample was transferred to 2.5 ml of buffered reactivating solution containing 1 mM ATP in a petri dish for the measurement of beat frequency. To observe the reaction of the antiserum with sperm in the rigor state, 25  $\mu$ l of sperm suspension in seawater were added to 0.3 ml of demembrating solution containing 0.02 mM ATP. Then, 100  $\mu$ l were diluted into 5 ml of

buffered reactivating solution without ATP, in order to prepare rigor sperm (8). A 0.25-ml aliquot of this suspension was transferred to an egg white-precoated test tube, antidynein serum was added, and the incubation was carried out at room temperature as above. The sperm were then transferred into reactivating solution containing 1 mM ATP for measurement of their beat frequency. This experimental design is such that concentrations of sperm and of antiserum during incubation are the same in both cases.

Observations of sperm swimming were made by dark-field light microscopy in a petri dish. To minimize sticking of the sperm to the glass surface, dishes either were rinsed with egg white, and then with distilled water, or they were coated with 0.05% Formvar in ethylene dichloride. Frequency measurements were made stroboscopically with the  $10 \times 0.22$  NA objective lens, as described earlier (6). For photography of swimming sperm, which required higher magnification, we used a  $40 \times 0.75$  NA water-immersion objective and a 1.2–1.4 NA cardioid dark-field condenser. The light source was a xenon flash lamp, Model No. 71 powered by a Model 136 power supply (Chadwick-Helmuth Co., Inc. Monrovia, Calif.), and triggered by a General Radio Strobotac (General Radio Co., Concord, Mass.).

Sources of chemicals were the same as those reported previously (6). Formvar was obtained from Ladd Research Industries, Inc., Burlington, Vt.

## RESULTS

The effects of antidynein serum on reactivated sperm were determined by adding antiserum directly to demembrated sperm swimming in a petri dish containing ATP, and following the changes which occurred with time. The flagella of the sperm exposed to antiserum continued to produce regular propagated bending waves, but their beat frequency decreased with time and there were some changes in waveform.

The effect of different quantities of antiserum on flagellar beat frequency as a function of time is shown in Fig. 1. The frequency decreased rapidly during the first 5–15 min of incubation, and then more slowly for the rest of the period up to 60 min. The frequency attained after 60 min of incubation was lower with larger amounts of antiserum, and with 400  $\mu$ l of antiserum it was as low as 1 Hz. The percent of motile sperm, which was close to 100% at the start, decreased somewhat with time, averaging about 70% after 45 min in both the experimental and control samples. Two kinds of control experiment were performed: one involved addition of no serum at all to the dish of reactivated sperm, and the second involved addition of 100  $\mu$ l of pre-immune serum to the dish. In

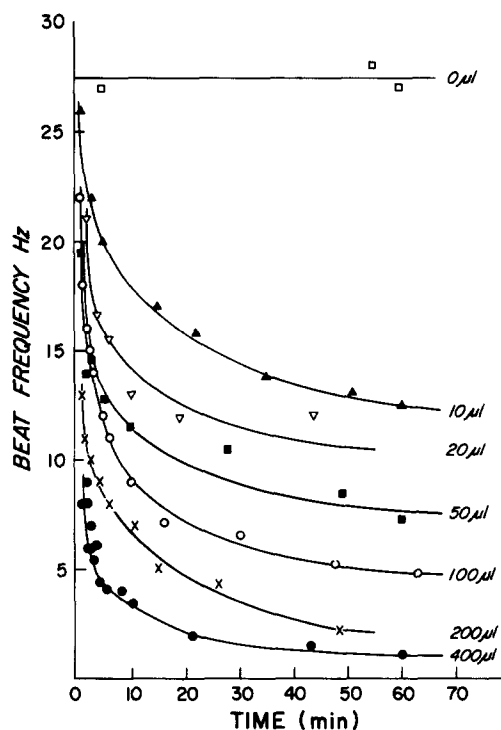


FIGURE 1 Effect of antidynein serum on the beat frequency of demembrated sea urchin sperm. Sperm were reactivated in a petri dish containing 2.5 ml of reactivating solution. Antiserum was added to the petri dish in the quantity indicated at time zero, and the beat frequency was measured as a function of time. Temperature, 21°C.

both cases, the beat frequency after 60 min remained unchanged from its initial value.

In Fig. 2, the waveforms of two typical sperm treated with antiserum are compared with those of a normal reactivated sperm. The waveforms of the treated sperm appear generally similar to those of the untreated control, but there are differences in detail. The principal difference involves the curvature of the bends in the proximal portion of the axoneme. In most preparations of untreated reactivated sperm of *Colobocentrotus*, the curvature of bends in the proximal portion of the axoneme is not constant over the bend, so that the bend cannot be represented as a circular arc (2, 6). This nonuniform curvature largely disappears in preparations of sperm that have been treated with antiserum, and the bends have a greater and more uniform curvature in the proximal region of the axoneme. In the distal portion of the axoneme, the nonuniformity of bend curvature is hardly ap-

parent even in the untreated sperm, and there is little difference between the waveforms of the treated and untreated sperm in this region of the axoneme (see especially the bottom two images of Figs. 2 a and c). Apart from the different curvature of the bends in the proximal portion of the

axoneme, there appears to be little difference between the waveforms of the treated and untreated sperm. The wavelength, measured along the axoneme, appears unchanged. The bend angles of the waves appear to increase somewhat as the frequency decreases, but they are difficult to measure

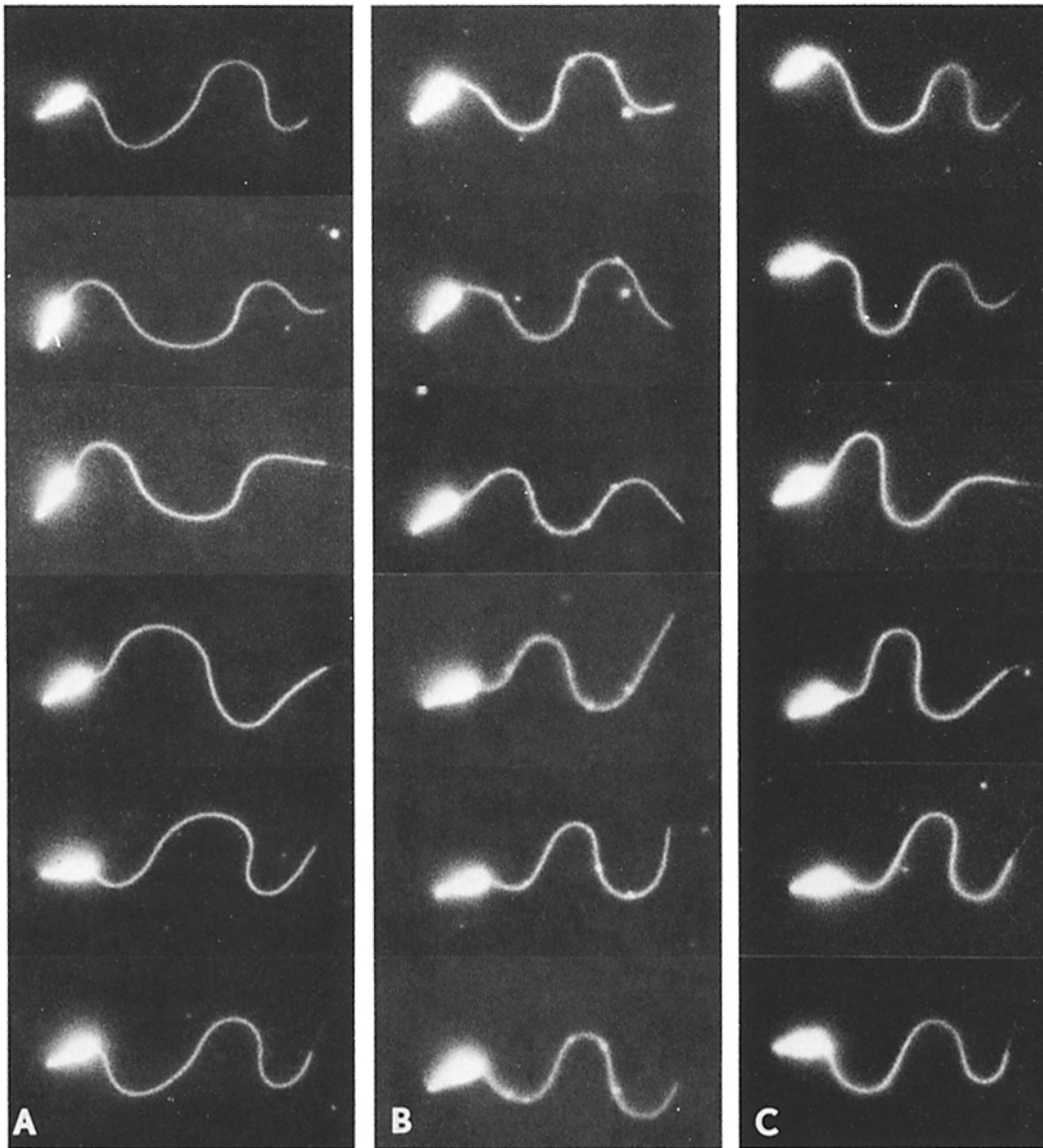


FIGURE 2 (a) Dark-field light micrographs of a control reactivated sperm swimming in buffered reactivating solution at room temperature (22°). The photographs, which are all of the same sperm, have been arranged into a series to illustrate the form of the bending waves as they propagate. The sperm was circling at the bottom surface of a Formvar-coated dish with its tail beating parallel and close to the glass surface. Beat frequency was 26 Hz. Flash exposure; magnification  $\times 1,090$ . (b) Same as (a) but showing a sperm treated with antidynein serum and beating at 10 Hz. (c) Same as (b) but showing a sperm beating at 4 Hz.

accurately when the bends have nonuniform curvature.

In order to measure both the ATPase activity and the beat frequency of a sample of sperm pretreated with antidynein, the second experimental procedure described in Materials and Methods was followed. The results of a typical experiment are presented in Fig. 3. Demembrated sperm were incubated with varying amounts of antidynein serum at 0°C for 15 min, and then diluted approximately 20-fold for assay on the pH stat. The ATPase activity of motile reactivated sperm ( $v_i$ ) pretreated with antidynein decreased with an increasing amount of antidynein used. The ATPase activity of homogenized, nonmotile sperm ( $v_{hl}$ ) also decreased on pretreatment of the sperm with antidynein serum, but less markedly than  $v_i$ . The beat frequencies of the reactivated sperm also fell, but less steeply than either their total ATPase activity ( $v_i$ ) or their movement-coupled ATPase activity ( $v_i - v_{hl}$ ).

Visual observation of the waveform of the motile sperm confirmed the results of the first experiment, namely, that even at the greatly reduced frequencies produced by the largest quantities of antidynein serum there was only a minor change in the waveform. As above, this consisted of an increase in the curvature of the bends in the proximal region of the axoneme. Microscopic observations of samples removed from the pH stat showed

no significant appearance of sperm aggregates during the first 10 min of the measurement of ATPase activity. Decreased motility resulting from aggregation, therefore, cannot be supposed to account for any substantial part of the decrease in  $v_i$ .

Control experiments were performed with either 50  $\mu$ l of the buffer into which the antidynein serum had been dialyzed in the final step of the ammonium sulfate purification, or 50  $\mu$ l of pre-immune serum. Neither the dialysis buffer containing 0.02% sodium azide nor the pre-immune serum had any significant effect on either the ATPase activity or the beat frequency of the sperm.

The third type of procedure described in Materials and Methods was designed to compare the reactivity of antidynein with motile sperm and with rigor sperm under otherwise similar conditions. In the one case antidynein was incubated with demembrated sperm in the presence of 1 mM ATP at room temperature. The sperm presumably were motile during the 30-s to 5-min incubation before they were diluted out of the antidynein serum into more reactivating solution containing ATP, where their beat frequency was recorded as a measure of the extent of reaction of the serum. In the second case antidynein serum was incubated with the same concentration of rigor sperm in the absence of ATP, followed by

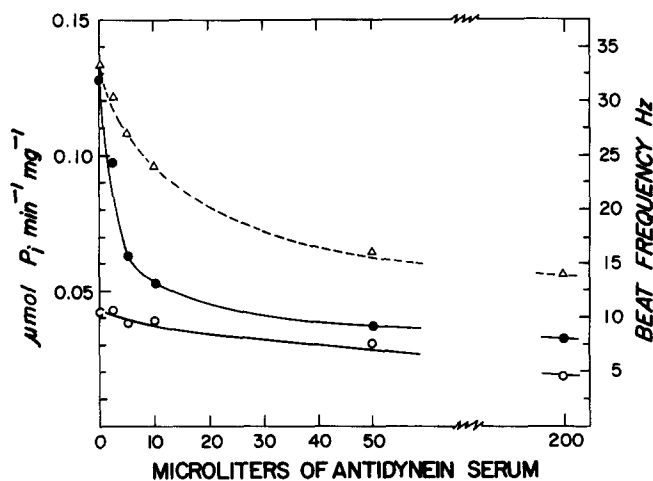


FIGURE 3 Effect of antidynein serum on the rate of hydrolysis of ATP by reactivated sperm (●) and by homogenized sperm (○). Demembrated sperm were pretreated with the stated amount of antidynein serum in 0.6 ml of demembrating solution for 15 min at 0°C and then diluted into assay solution on the pH stat. The pH of the assay was 8.0 and the temperature was 25°C. The corresponding beat frequency of sperm from this same sample is also shown as a function of the amount of antidynein serum used ( $\Delta$ ). These were measured at room temperature (21°C) and corrected to 25°C (6).

dilution into 1 mM ATP and measurement of beat frequency. In both cases two quantities of antidynein serum were used, 10  $\mu$ l and 60  $\mu$ l. The results are shown in Fig. 4. The shape of the curves is similar to that of those in Fig. 1, except that the time scale is shortened, presumably because the sperm and antidynein concentrations are both 20-fold greater. The curve for 10  $\mu$ l of antidynein serum is the same for both reactivated and rigor sperm, suggesting that the availability and number of sites that bind antidynein are the same in the two cases. However, the results with 60  $\mu$ l of antidynein serum were quite different. The curve for reactivated sperm is similar to that for 10  $\mu$ l of antiserum. Rigor sperm, however, were almost completely nonmotile in ATP after treatment with 60  $\mu$ l of antidynein for either 30 s or 2 min; just a very few sperm were twitching feebly at about 10 Hz after the 30-s treatment. There was no further change in the preparation after standing 15 min in the presence of ATP. It may be noted that the concentration of antidynein 1 in this experiment is equivalent to 1,200  $\mu$ l under the con-

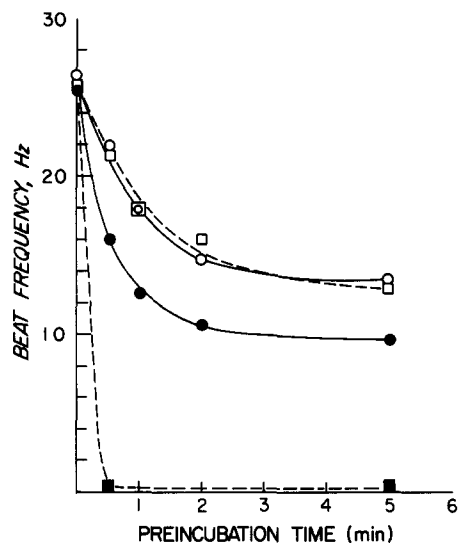


FIGURE 4 Effect of preincubation of reactivated and rigor sperm with antidynein serum on their beat frequency after dilution into reactivating solution containing 1 mM ATP. Reactivated sperm were prepared and treated as described in Materials and Methods with either 10 (—○—) or 60  $\mu$ l (—●—) of antidynein at room temperature (22°C) in the presence of 1 mM ATP before dilution. The other curves show similar data for rigor wave sperm preincubated with either 10  $\mu$ l (---□---) or 60  $\mu$ l (---■---) of antidynein serum in the absence of ATP, followed by dilution into ATP.

ditions of the experiment shown in Fig. 1, although the sperm/antidynein 1 ratio is the same in the two cases.

We tested the reversibility of the binding of antidynein 1 to reactivated sperm. Sperm were treated with 400  $\mu$ l of antidynein as in the first procedure and allowed to swim until the frequency had decreased to approximately 2 Hz. By this time a small percentage of the sperm had stuck by their heads to the bottom surface of the petri dish. This allowed us to gently pour off the reactivating solution containing the antidynein and to wash the sperm twice with fresh reactivating solution. After such treatment, the beat frequency rose to approximately 5 Hz within 5 min, but no further reversal occurred upon standing. This result suggests that about 90% of the inhibition produced by this antiserum was due to the binding of high affinity antibodies which formed stable complexes, while the remaining 10% of the inhibition resulted from low affinity antibodies forming loose complexes that were dissociated upon washing.

#### DISCUSSION

Ogawa and Mohri (16) reported that this preparation of antidynein serum cross-reacted with and inhibited the ATPase activity of dynein from two other species of sea urchin sperm flagella. Here we have found it to be inhibitory to dynein ATPase from a third species of sea urchin *Colobocentrotus atratus*. One may suppose, therefore, that there is great similarity between the active sites of the dynein molecules from these species of sea urchin.

The effect of antidynein 1 serum on reactivated sperm motility is similar to that obtained by extracting the sperm with 0.5 M KCl (7), that is, it markedly reduces the beat frequency while only slightly modifying the waveform (Fig. 2). It has been demonstrated by electron microscopy that salt extraction of demembrated sperm partially removes the dynein arms, and that beat frequency is proportional to the number of dynein arms present. In the work presented here, the change in movement presumably results from the inhibition of the functioning of the dynein arms by their reaction with antiserum, although in this case we cannot see the interference of function directly as in the KCl-extraction experiments.

Antisera to enzymes often contain a population of antibodies differing in their inhibitory capacity, for the catalytic site of an enzyme is not the only possible antigenic determinant on the molecule (1). Fragment A is a large molecule, mol wt

~400,000 (16), and may be expected to stimulate the production of a heterogeneous antibody population. Moreover, dynein 1 is known to consist of two forms having different solubility properties, and perhaps different locations on the axoneme (12). Fragment A, the antigen for our sample of antidynein 1, was prepared by Ogawa and Mohri from a dynein fraction containing both forms of dynein 1 (12, 14, 16).

The data of Fig. 1 may be taken to reflect possible heterogeneity in the antibody preparation or in the existence of two types of dynein 1, differing in their reactivity to our preparation of antidynein 1, for under our conditions only 10  $\mu$ l of antidynein 1 were needed to reduce the beat frequency to one-half its original value, whereas vastly greater amounts were required to lower the frequency further. If our antidynein 1 is highly inhibitory to one form of dynein 1, but only weakly inhibitory to the other, and if it is necessary to inhibit both forms to reduce the frequency below half the initial value, then a set of curves such as those in Fig. 1 would be obtained.

Since antidynein 1 is inactive against dynein 2 ATPase *in vitro* (12, 15), then presumably dynein 2 is still functional in the serum-treated sperm beating at 1 Hz. Moreover, dynein 2 accounts for 15% of the total ATPase activity of the axoneme (12). What then is its function? Warner and Satir (21) have presented evidence that the radial spokes are involved in the transduction mechanism that converts interdoublet sliding into local bending. Since this would require energy, an ATPase may be situated in the spoke head, and this could be dynein 2. However, the functioning of this system may be dependent upon the functioning of the dynein 1 system, so that inhibition of the latter would obscure the functioning of dynein 2. Another possibility is that dynein 2 is not distributed evenly along the axoneme as dynein 1 is thought to be, and this might explain the slight change in waveform seen in Fig. 2.

Fig. 3 shows that the rate of ATP hydrolysis falls more rapidly than the beat frequency as a function of increasing concentrations of antidynein 1. For example, 10  $\mu$ l of antiserum, which caused only about a 25% decrease in beat frequency, resulted in a 60% decrease in total ATPase activity and an 80% decrease in movement-coupled ATPase activity. This result is quite unlike that obtained in studies by Brokaw in which sperm were placed in media of increased viscosity and the movement-coupled ATPase activity decreased

approximately in proportion to the decrease in beat frequency (4). Since the work performed against the viscous resistance of the medium is proportional to the square of the beat frequency, the present data suggest that the efficiency of mechanochemical energy conversion is higher in sperm which have been slowed down by treatment with a small amount of antiserum. This conclusion is almost certainly true if the movement-coupled ATPase activity represents a true measure of the chemical energy available for conversion to mechanical energy. It may well also be true even if all the energy from the total sperm ATPase activity were available for conversion, although more accurate calculation taking account of the altered waveform would be necessary in this case.

An increased energy efficiency might have several origins. For instance, it could be an intrinsic property of the cross-bridge energy transducing system to function more efficiently when the rate of sliding between tubules is slower than normal. However, in this case it would be expected that the increased efficiency would be manifest in other circumstances in which the beat frequency was lowered (such as increased viscosity) and this does not seem to occur (4, 5).

The exact origin of the low ATPase activity of homogenized sperm is not yet known. Large amounts of antidynein produce a 50% decrease in  $v_{H}$  to a value of approximately 0.02  $\mu$ mol Pi  $\text{min}^{-1}$   $\text{mg}^{-1}$ . This final level may represent activity due to dynein 2 and is consistent with the contribution of dynein 2 being about 15% of the total ATPase activity of the axoneme. The ATPase activity of homogenized sperm which is inhibited by antidynein 1 may correspond either to movement-uncoupled ATPase activity as discussed above, or to short-range oscillatory movements of the tubules that are ineffective in producing bends because of the disruption of other components of the system and so are not visible by light microscopy.

The inhibitory action of antidynein serum on motility might involve the blocking of any of the individual steps in the mechanochemical cycle of the dynein arms such as, for example, the binding and hydrolysis of ATP or the formation of arm-tubule cross-bridges. Evidence counter to the second possibility is provided by the results of the treatment of reactivated and rigor sperm with antidynein 1. Since the tubule-binding sites of the dynein arms in rigor sperm are involved in fixed association with the tubules (7, 11), the antidynein presumably cannot also bind to these sites. How-

ever, antidynein 1 does bind to rigor sperm in such a manner as to reduce the beat frequency upon subsequent reactivation, so that some at least of its binding sites must remain accessible in the rigor state. Moreover, for moderate to low concentrations of antiserum, the rates of reaction with motile and with rigor sperm are almost identical. Several alternative interpretations for this result are possible. One is that only a small fraction of the total number of dynein arms is involved as cross-bridges in rigor sperm, and that the antidynein just reacts with the free arms not involved as cross-bridges. A second possibility is that the tubule-binding and ATPase sites occur in two different regions of the dynein molecule, and that these sites are sufficiently far apart to permit simultaneous binding of tubules and of antidynein, and the latter binding at or near the ATPase site.

Gibbons has presented a detailed discussion of the perturbing effects of various agents on flagellar beating (10). He noted that the majority of them fell into three groups. One group has a strong influence on the flagellar beat frequency but little effect on the waveform, the second group produces substantial changes in waveform but has little effect on the beat frequency, while the third group has only a weak effect on both the waveform and beat frequency. As a perturbing agent, antidynein 1 clearly falls in the category of the first group which chiefly affects beat frequency. In this regard its action resembles the effect of partial removal of the dynein arms by KCl extraction (7). With antiserum-treated sperm, the change in movement presumably results from the inhibition of the dynein arms by their reaction with antibody. Both procedures indicate that preventing the action of the dynein arms on the doublet tubules causes a decrease in beat frequency with relatively little effect upon the flagellar waveform.

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