RECOVERY OF SLIDING ABILITY IN ARM-DEPLETED FLAGELLAR AXONEMES
AFTER RECOMBINATION WITH EXTRACTED DYNEIN I

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SUMMARY
We compared sliding velocity between outer doublet tubules in demembranated axonemes of sea-urchin (Pseudocentrotus depressus) sperm flagella with that of arm-depleted axonemes recombined with extracted dynein I.

The outer arm-depleted axonemes after extraction with 0.5 M NaCl had a velocity of 6.9 ± 1.0 μm/s, while the intact axonemes had a velocity of 14.3 ± 1.5 μm/s in the presence of 1 mM ATP and 2 μg/ml trypsin at 25 °C. The sliding velocity was closely related to the number of remaining outer arms following the NaCl-extraction process. When the outer arm-depleted axonemes were recombined with dynein I, the sliding velocity increased to 11.3 ± 1.3 μm/s. Electron microscopy confirmed the recovery of 94% of outer arms in the axonemes.

After extraction with Tris-EDTA solution for 10 min, the axonemes lost their sliding ability completely, even in the presence of ATP and trypsin. Such axonemes lacked most of both inner and outer arms, although sometimes the basal segment of the arms appeared to remain. When the exogenous dynein I fraction extracted from other axonemes was added, the axonemes could extrude tubules, and both types of arms reappeared clearly and distinctly in the axonemes. The recombined axonemes with one-fold stoichiometric excess of dynein I recovered 58% of the total number of arms and had a velocity of 7.4 ± 1.6 μm/s. Those with 2-fold stoichiometric excess had a velocity of 11.0 ± 1.5 μm/s, up to 82% of the arms in these axonemes being restored.

These results indicated that the exogenous dynein I fraction derived from the outer arms restored sliding ability to arm-depleted axonemes, recombining with the outer doublet tubules as inner and outer arms, and that the sliding velocity had a close relationship to the total number of arms in the axonemes, irrespective of their being inner or outer arms.

INTRODUCTION
Flagellar axonemes are cylindrical and consist of 9 doublet microtubules surrounding a central pair of singlet microtubules. Each doublet tubule has 2 parallel rows of projections called outer and inner arms. Since Summers & Gibbons (1971) demonstrated that ATP induced active sliding movement between outer doublet tubules in trypsin-treated axonemes of sea-urchin sperm flagella, many people now believe that the normal propagation of bending waves in flagellar movement is the result of localized sliding movement between adjacent outer doublet microtubules in the axonemes (Satir, 1968; Brokaw, 1971; Brokaw & Gibbons, 1973; Warner & Satir, 1974; Shingyoji, Murakami & Takahashi, 1977). Experimental evidence (Brokaw & Benedict,
Y. Yano and T. Miki-Noumura

1968; Gibbons & Gibbons, 1972) suggests that the sliding movement may be induced by an ATP-driven mechanochemical cycle involving interaction of dynein arms with adjacent outer doublet tubules.

Brief extraction of demembranated sea-urchin sperm with 0.5 M KCl or NaCl removes the outer dynein arms from the doublet tubules, leaving the inner arms and other axonemal structure apparently intact (Gibbons & Fronk, 1972). The flagellar beat frequency of the KCl-extracted sperm is only about half that of control sperm, although the form of the bending waves is not significantly altered (Gibbons & Gibbons, 1973). We found that the axonemes depleted of outer arms could extrude outer doublet tubules in the presence of ATP and trypsin. The sliding speed was about 60% of that of control axonemes (Hata et al. 1980). These observations indicated that the inner arms have a function equivalent to that of the outer arms in 'walking along' or 'sliding on' the adjacent doublet tubules.

Gibbons & Gibbons (1976, 1979) showed that 21 S latent ATPase activity form of dynein 1 increased the beat frequency of KCl-extracted flagella of sea-urchin sperm from 14 to 25 Hz and restored up to 90% of the outer arms on the doublet tubules.

We report here recovery of the sliding ability of either outer doublet tubules without outer arms or ones without both types of arms after recombination with extracted dynein 1. Using the method for sliding velocity measurement with precision (Yano & Miki-Noumura, 1980), we attempt also to find a relationship between the number of the arms and the sliding velocity of the doublet tubules from the axonemes.

MATERIAL AND METHODS

Material

Sperm of sea urchin, Pseudocentrotus depressus, were obtained by injecting 0.5 M KCl into the body cavity. Sperm were collected by centrifugation at 4000 g for 5 min, kept at 0 °C and used within 2 days.

Isolation of demembranated axonemes

The sperm were suspended in 20 vol. of an axoneme isolation medium (0.15 M KCl, 2 mM MgSO4, 2 mM CaCl2, 0.5 mM EDTA, 1 mM dithiothreitol (DTT), 0.25% Triton X-100, 10 mM Tris-HCl buffer, pH 8.3) and gently stirred for 20 min at room temperature to de-membranate and separate tails from heads. After discarding sperm heads collected by centrifugation, the supernatant was further centrifuged at 10000 g for 10 min to collect axonemes. The axonemes were washed once with the axoneme isolation medium, from which Triton was omitted. Since Triton X-100 inhibited recombination of the axonemes with dynein 1, it was necessary to wash out Triton X-100 thoroughly from the demembranated axonemes.

Preparation of NaCl-extracted axonemes and dynein 1 fraction

The demembranated axonemes were suspended in a NaCl extracting solution (0.5 M NaCl, 4 mM MgSO4, 1 mM CaCl2, 1 mM DTT, 10 mM Tris-HCl buffer, pH 8.0) at 2-4 mg/ml protein for 10 min at 0 °C, unless otherwise specified. Extraction was terminated by diluting the NaCl solution with 100 vol. of an ATP-free reactivation medium (0.15 M KCl, 2.2 mM MgSO4, 0.5 mM CaCl2, 2 mM EGTA, 1 mM DTT, 10 mM Tris-HCl buffer, pH 8.4), or by centrifuging at 15000 g for 10 min. The extracted axonemes were designated as 'NaCl-extracted axonemes'. The supernatant obtained by centrifugation was used as 'dynein 1 fraction'.
Recovery of sliding ability 225

Preparation of Tris-EDTA-extracted axonemes

The demembranated axonemes were suspended in a Tris-EDTA extracting solution (0.5 mM EDTA, 1 mM DTT, 10 mM Tris-HCl buffer, pH 8.0) at 2–4 mg/ml protein for 10 min at 0 °C. Extraction was terminated by transferring the extracted axonemes to 100 vol. of the ATP-free reactivation medium or by centrifuging at 15,000 g for 10 min. The extracted axonemes were designated as 'Tris-EDTA-extracted axonemes'.

Recombination of dynein 1 with the extracted axonemes

To recombine NaCl-extracted axonemes with the endogenous dynein 1, the demembranated axonemes were suspended in the NaCl extracting solution, and after 10 min the extraction was stopped by diluting the 0.5 M NaCl to 0.15 M NaCl with 7/3 vol. of a buffer solution (4 mM MgSO₄, 1 mM CaCl₂, 1 mM DTT, 10 mM Tris-HCl buffer, pH 8.0). The diluted suspension was then incubated for 30 min at 0 °C.

The exogenous dynein 1 fraction prepared from other axonemes was added to the suspension of NaCl-extracted axonemes to recombine them with excess dynein 1. The suspension was incubated for 30 min at 0 °C after reducing the NaCl concentration to 0.15 M.

Tris-EDTA-extracted axonemes were also recombined with the exogenous dynein 1 fraction prepared from other axonemes by incubating them in 0.15 M NaCl solution containing 4 mM MgSO₄, 1 mM CaCl₂, 1 mM DTT, 10 mM Tris-HCl buffer, pH 8.0, for 30 min at 0 °C.

After incubation, each axoneme suspension was transferred to 100 vol. of the ATP-free reactivation medium, or centrifuged at 15,000 g for 10 min and then suspended in the reactivation medium.

Observation by dark-field microscopy and measurement of sliding velocity

Prior to observation under a dark-field microscope, a small portion of the axoneme suspension was diluted with the ATP-free reactivation medium containing 2% polyethylene glycol (PEG) to give a protein concentration of about 20 μg/ml. Sliding movement of the axonemes was induced on the slide by adding the reactivation medium containing 1 mM ATP and 2 μg/ml trypsin. The sliding process was recorded with a CTC-9000 Night Vision Camera system including a video tape recorder (Ikegami Tsushinki Co. Ltd, Tokyo). The length of the extruded tubules from the axoneme and the elapsed time were measured directly on the image of the picture monitor. The sliding velocity of about 20 axonemes in the same axoneme sample was estimated. Sliding percentage was expressed as a ratio of the number of axonemes showing sliding movement to those of all axonemes. Details were described in a previous paper (Yano & Miki-Noumura, 1980).

Assay for ATPase activity and determination of protein concentration

Axoneme samples were suspended in the reactivation medium containing no ATP and PEG. Measurement of ATP hydrolysis by the axonemes (~0.1 mg/ml) was carried out in the PEG-free reactivation medium containing 1 mM ATP at 25 °C. Inorganic phosphate liberated in the early stage of the hydrolysis was measured by the method of Murphy & Riley (1962) with some modifications (Hayashi, 1976). Protein concentration was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), using bovine serum albumin as the standard sample.

SDS-polyacrylamide gel electrophoresis

Sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis was performed according to the method of Weber & Osborn (1966). Electrophoresis with 4% gel was carried out at 8 mA per column for 3 h 10 min, and then the gel was stained with 0.2% Coomassie brilliant blue.

Observation by electron microscopy

Axoneme samples were collected by centrifugation at 15,000 g for 10 min at 4 °C, and fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.5. The pellet was then postfixed with 1% OsO₄ for 2 h at 0 °C, dehydrated with an ethanol series, and embedded in Epon 812.
Thin-sectioned specimens were double-stained with uranyl acetate and lead citrate. Electron micrographs were taken with a JEM 100CX electron microscope at 80 kV at 50,000 magnification. In order to reconfirm our counts of the number of arms present in axonemal cross-sections, the number of arms was counted by an observer who did not know the identity of the micrographs being counted.

RESULTS

Decrease of sliding velocity of the doublet tubules during extraction with 0.5 M NaCl

We reported previously that NaCl-extracted axonemes having only inner arms had the ability to extrude tubules in a manner similar to the intact axonemes having both types of arms (Hata et al. 1980). Sliding velocity of the doublet tubules in NaCl-extracted axonemes was measured by adding 1 mM ATP and 2 μg/ml trypsin at 25 °C. As shown in Table 1, the sliding velocity of the doublet tubules in the NaCl-extracted axonemes was about 7 μm/s, which was about half of the sliding velocity in the intact axonemes. No significant difference was found between the sliding velocity of the NaCl-extracted axonemes, NaCl-extraction of which was stopped by centrifugation or by lowering the salt concentration.

Table 1. Sliding velocity of the outer doublet tubules in intact axonemes having both types of arms and in NaCl-extracted axonemes lacking outer arms

<table>
<thead>
<tr>
<th></th>
<th>μm/s</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact axonemes</td>
<td>14.3 ± 1.5</td>
<td>100</td>
</tr>
<tr>
<td>NaCl-extracted axonemes (by centrifugation)</td>
<td>6.9 ± 1.0</td>
<td>48.3</td>
</tr>
<tr>
<td>NaCl-extracted axonemes (by dilution)</td>
<td>6.7 ± 1.0</td>
<td>46.9</td>
</tr>
</tbody>
</table>

The extraction time of the axonemes could be determined exactly by the dilution method. Using the axonemes extracted for different periods ranging from 1 to 10 min, sliding velocity and number of remaining arms in sectional images of the axonemes in electron micrographs were examined. Fig. 1 shows that both the sliding velocity and the number of remaining arms decreased to about half of the original level after 5 min extraction, and remained almost unchanged thereafter up to 10 min. This means that almost all the outer arms had been extracted by this time, leaving the inner arms intact. The decrease of sliding velocity was accompanied by a substantial decrease in the number of remaining arms. The result indicates that there is a close correlation between the number of arms remaining in the axoneme and the sliding velocity.

To determine exactly the relationship between the number of outer arms and the sliding velocity, we observed carefully the transverse images of sectioned axonemes in the electron micrographs. First, the position of the remaining outer arms in the NaCl-extracted axonemes was examined, as it is possible to determine the position of each outer doublet in relation to the pair of central tubules. As shown in Table 2, no
Recovery of sliding ability

Fig. 1. Time course of decrease in sliding velocity and the number of remaining arms in axonemes during NaCl extraction. Axonemes were extracted with 0.5 M NaCl and were diluted with 100 vol. of the reactivation medium to terminate the extraction. Sliding velocity (○) and the number of remaining arms (●) are represented by ratio (%) to the values in intact axonemes. 50% of the number of remaining arms represents the total number of inner arms.

Table 2. Distribution of remaining outer arms among 9 outer doublets in an axoneme

<table>
<thead>
<tr>
<th>NaCl-extraction time, min</th>
<th>Count of remaining outer arms</th>
<th>Total no. of axonemes examined</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>132 132 134 133 133 133 131 132 132 134</td>
<td>134</td>
</tr>
<tr>
<td>1</td>
<td>83  82  79  81  61  77  86  87  84  157</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>36  33  35  39  37  44  43  42  38  126</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>27  29  24  31  34  30  38  35  26  135</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>9   11  14  13  12  9   16  7   111</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>5   4   6   4   2   3   5   6   3   152</td>
<td></td>
</tr>
</tbody>
</table>

* Position of nine outer doublets in an axoneme.

significant positional difference in the remaining outer arms was detected among all nine doublet tubules. Next, we scored the transverse images on the basis of the number of remaining outer arms in each axonomal image, regardless of their position. Although the number of axonomal images having 0 or 9 outer arms was a little larger than those having 2 to 8 outer arms during 1 and 2 min extraction times, the mean number of the remaining arms per one axonomal image was from 2 to 5, as shown in Fig. 2. Based on the mean number, the probability distribution of the number of outer arms was calculated as shown in Fig. 2. The distribution histogram of the calculated probability did not agree with that of the counted ones. The disagreement
means that the outer arms were not extracted at random. These results suggest that the outer arms were extracted with NaCl in groups from doublet tubules next to each other; that is, as soon as one outer arm was extracted, the adjacent arms would also become easily extracted. The remaining outer arms in groups were often observed in axonemal images.

Fig. 2. Distribution profiles of the number of remaining outer arms after each extraction time, A-F, 0, 1, 2, 3, 5, and 10 min, respectively. Abscissa: number of remaining outer arms in one axonemal image; ordinate: frequency (%) of axonemal images which retained from 0 to 9 outer arms. Solid outline: distribution profiles of remaining outer arms counted from the electron micrographs; vertical broken line: mean number of remaining outer arms; dotted outline: probability distribution profiles of outer arms calculated from the mean number of remaining outer arms.

Increase of the sliding velocity in NaCl-extracted axonemes after recombination with endogenous or exogenous dynein 1

We reported in a previous paper (Hata et al. 1980) that ATPase activity of the axonemes having no outer arms was about half of that of intact axonemes.

When the axoneme suspension was extracted with 0·5 M NaCl for 10 min and then
Recovery of sliding ability

Incubated in 0.15 M NaCl solution for 30 min at 0 °C, the A-band polypeptide of the extracted dynein 1 was found again in the dynein region of the incubated axoneme fraction in SDS-4% polyacrylamide gel, as shown in Fig. 3.

Fig. 3. Electrophoretic band patterns of axonemal proteins in SDS-4% polyacrylamide gel. a, intact axonemes (52.3 μg); b, soluble fraction of axonemes after 0.15 M NaCl extraction for 10 min (dynein 1) (7.5 μg); c, NaCl-extracted axonemes (41.0 μg); d, NaCl-extracted axonemes recombined with dynein 1 (45.2 μg).

An increase in ATPase activity was found in the recombined axonemes. Using ATPase activity as a criterion of the degree of recombination, we determined the optimal conditions for recombination. Because protein concentration of the axoneme and dynein 1 fractions might have an effect on the recombination process, the ATPase activity of the axonemes recombined in various protein concentrations was surveyed. To obtain recombination higher than 80%, an axonemal protein concentration of more than 1 mg/ml and a dynein 1 fraction of around 0.1 mg/ml were used.

In order to examine the effect of divalent cations on the recombination of dynein 1, axoneme suspensions were incubated in 0.15 M NaCl solution containing MgSO₄ or CaCl₂ from 1 to 10 mM. As shown in Table 3, dynein 1 hardly recombined with the axonemes in the absence of divalent cations. Although Ca²⁺ was a little more effective than Mg²⁺, the combined presence of both cations was much more effective.

When the pH of the recombination medium was lowered from 8.0 to 6.0, an increase of ATPase activity of the axonemes was found. However, the dynein molecules had a tendency to aggregate at lower pH. As shown in Table 3, we used a concentration of more than 1 mg/ml of protein and 10 mM Tris-HCl buffer, pH 8.0, containing 4 mM MgSO₄ and 1 mM CaCl₂ to achieve the optimal recombination of dynein 1 with the NaCl-extracted axonemes.

Excess dynein 1 fraction prepared from other axonemes was added to the NaCl-extracted axonemes to get a higher recombination percentage (ratio of ATPase activity of the axonemes recombined with dynein 1 to that of the intact axonemes). The ratio
of endo- or exogenous dynein 1 fraction added to the extracted axonemes was varied from 1:1 to 4:1. The equivalency ratio (1:1) was based upon the relative quantities of these components obtained from intact axonemes. As shown in Table 4, ATPase activity of the recombined axonemes did not reach the level of that of the intact axonemes, although ATPase activity increased in accordance with increasing amount of dynein 1 fraction.

Table 3. Effect of Mg\(^{2+}\) and Ca\(^{2+}\) on recombination of dynein 1 with the NaCl-extracted axonemes

<table>
<thead>
<tr>
<th>Divalent cations in recombination process</th>
<th>ATPase activity (nmol P(_i)/ml per min)</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact axonemes</td>
<td>7.29</td>
<td>100</td>
</tr>
<tr>
<td>NaCl-extracted axonemes</td>
<td>4.11</td>
<td>56.4</td>
</tr>
<tr>
<td>Axonemes recombined with dynein 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>5.17</td>
<td>70.1</td>
</tr>
<tr>
<td>1 mM Mg(^{2+})</td>
<td>5.75</td>
<td>78.9</td>
</tr>
<tr>
<td>4 mM Mg(^{2+})</td>
<td>6.15</td>
<td>84.4</td>
</tr>
<tr>
<td>10 mM Mg(^{2+})</td>
<td>6.31</td>
<td>86.5</td>
</tr>
<tr>
<td>1 mM Ca(^{2+})</td>
<td>6.25</td>
<td>85.7</td>
</tr>
<tr>
<td>4 mM Ca(^{2+})</td>
<td>6.37</td>
<td>87.4</td>
</tr>
<tr>
<td>10 mM Ca(^{2+})</td>
<td>6.46</td>
<td>88.6</td>
</tr>
<tr>
<td>4 mM Mg(^{2+}) + 1 mM Ca(^{2+})</td>
<td>6.80</td>
<td>93.3</td>
</tr>
</tbody>
</table>

When axonemes recombined with dynein 1 were treated again with 0.5 M NaCl, ATPase activity was found in the soluble fraction. After reducing the NaCl concentration to 0.15 M, increased ATPase activity reappeared in the axoneme fraction. Therefore, dynein 1 was reversibly extracted and recombined by changing NaCl concentration. Addition of 1 mM ATP did not dissociate the recombined axonemes into axoneme and dynein 1.

Axonemes recombined with dynein 1 were examined by electron microscopy. As shown in Fig. 4, while the NaCl-extracted axonemes lost their outer arms (Fig. 4b) the outer arms reappeared at almost regular positions on the axonemes recombined with dynein 1 (Fig. 4c). In some cases, 5–6 bridges also reappeared. Even when excess exogenous dynein 1 fraction was added the arms or arm-like structures were not observed at irregular positions on the doublet tubules (Fig. 4d). The number of outer arms was counted in sections of each axoneme fraction and then the ratios of the mean number of outer arms to the original 9 outer arms were calculated. Recombination of about 100% was obtained at 2:1 or 3:1 ratio of dynein 1:axonemes as shown in Table 5.

Recombined axonemes were suspended in reactivation medium and studied by dark-field microscopy. At first they seemed to become less rigid; then, after a twitching movement, the axonemes extruded their tubules successively. The pattern of sliding movement of these axonemes was almost the same as that of the NaCl-extracted
Recovery of sliding ability

Table 4. Recovery of ATPase activity and sliding velocity of the NaCl-extracted axonemes after recombination with dynein 1

<table>
<thead>
<tr>
<th>ATPase activity, nmol of P_i/ml per min</th>
<th>Sliding velocity, μm/s</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact axonemes</td>
<td>7.43 ± 0.3</td>
</tr>
<tr>
<td>NaCl-extracted axonemes</td>
<td>4.32 ± 0.8</td>
</tr>
<tr>
<td>Axonemes recombined with dynein 1</td>
<td>6.46 ± 0.9</td>
</tr>
<tr>
<td>Axonemes recombined with dynein 1</td>
<td>6.71 ± 1.3</td>
</tr>
<tr>
<td>Axonemes recombined with dynein 1</td>
<td>7.13 ± 1.6</td>
</tr>
<tr>
<td>Axonemes recombined with dynein 1</td>
<td>7.18 ± 1.9</td>
</tr>
</tbody>
</table>

axonemes and of the intact axonemes, which was described in a previous paper (Yano & Miki-Noumura, 1980). Table 4 shows that the sliding velocity in the axonemes recombined with dynein 1 was about 80% of that of the intact axonemes, while that of the NaCl-extracted axonemes was about 50%. Even though excess dynein 1

![Fig. 4.](image)

Table 5. The number of outer arms in NaCl-extracted axonemes after recombination with dynein 1

<table>
<thead>
<tr>
<th>Intact axonemes</th>
<th>NaCl-extracted axonemes</th>
<th>Axonemes recombined with dynein 1 (dynein 1:axonemes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>b</td>
<td>c</td>
</tr>
<tr>
<td>47</td>
<td>421</td>
<td>8.96</td>
</tr>
<tr>
<td>60</td>
<td>75</td>
<td>1.25</td>
</tr>
<tr>
<td>1:1</td>
<td>47</td>
<td>399</td>
</tr>
<tr>
<td>2:1</td>
<td>45</td>
<td>401</td>
</tr>
<tr>
<td>3:1</td>
<td>55</td>
<td>488</td>
</tr>
</tbody>
</table>

a, Total no. of counted axonemal images.
b, Total no. of observed outer arms.
c, The mean no. of outer arms per axoneme, represented by b/a.
d, Ratio (%) of the mean no. of outer arms to the original number, represented by 100c/a.
Y. Yano and T. Miki-Noumura

fraction was added to the axonemes, a sliding velocity higher than 80% was not observed.

Recovery of sliding ability in Tris-EDTA-extracted axonemes after recombination with exogenous dynein 1

Dialysis of the axonemes against Tris-EDTA solution for a longer period solubilized paired central tubules, both types of arms, nexin links and spokes (Gibbons, 1965). Extraction of axonemes with Tris-EDTA solution for a shorter period brought about a gradual loss of sliding ability in the axonemes. After extraction for 1 min, the sliding velocity decreased to $5.2 \pm 0.7 \mu m/s$, which corresponds to 35% of that of the intact axonemes. After 2 min, the sliding percentage (ratio of number of axonemes showing sliding movement to the total number of axonemes) was around 10%, the axonemes extruding only some of their tubules and doing so very slowly. After more than 3 min, the axonemes disintegrated into bundles of outer doublet tubules on addition of ATP

Fig. 5. The process of disintegration and sliding movement in axonemes under a dark-field microscope. 1 mM ATP and 2 $\mu g/ml$ trypsin were added to the axonemes. A, disintegration process in the Tris-EDTA-extracted axoneme; B, sliding movement in the Tris-EDTA-extracted axoneme recombined with dynein 1. Arrows indicate the sliding edge of the doublet microtubules on adjacent doublets. Scale bar, 10 $\mu m$.

and trypsin (Fig. 5A), in a similar manner to the disintegration (peeling) of intact axonemes caused by trypsin in the absence of ATP, as described previously (Yano & Miki-Noumura, 1980). The Tris-EDTA-extracted axonemes disintegrated and divided into bundles did not show any bending motion and then separated from each
Recovery of sliding ability

233

other longitudinally. Thus, the Tris-EDTA-extracted axonemes lost their sliding ability completely.

After extraction for 10 min, axonemes were suspended in 0.15 M NaCl solution containing 4 mM MgSO₄, 1 mM CaCl₂, 1 mM DTT and 10 mM Tris-HCl buffer, pH 8.0, and incubated for 30 min at 0 °C to recombine them with the dissociated endogenous dynein. Sliding movement could not be restored in these axonemes by adding ATP and trypsin. However, when the Tris-EDTA-extracted axonemes were incubated with exogenous dynein 1 fraction for 30 min at 0 °C, the axonemes recovered their sliding ability, as shown in Fig. 5B. When the ratio of added dynein 1 fraction to the Tris-EDTA-extracted axonemes was 1:1 (equivalent to the relative quantities of these components in the intact axonemes), the sliding percentage of the axonemes was about 50%. When the ratio was raised to 2:1, the percentage increased to about 80%. The sliding process of these axonemes, however, was somewhat different from that of the intact axonemes. The recombined axonemes did not show the twitching movement which was observed in intact axonemes before tubule extrusion, while ATP diffused into the axonemes. In addition, while the intact axonemes extruded doublet tubules successively, the sliding movement in the recombined axonemes occurred only between two or three groups of doublet tubules.

Table 6. Recovery of ATPase activity and sliding velocity of Tris-EDTA-extracted axonemes after recombination with dynein 1

<table>
<thead>
<tr>
<th>ATPase activity</th>
<th>Sliding velocity</th>
</tr>
</thead>
<tbody>
<tr>
<td>nmol of P₆/ml per min</td>
<td>μm/s</td>
</tr>
<tr>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>Intact axonemes</td>
<td>2.87</td>
</tr>
<tr>
<td>Tris-EDTA-extracted axonemes</td>
<td>1.06</td>
</tr>
<tr>
<td>Axonemes incubated in 0.15 M NaCl without exogenous dynein 1</td>
<td>1.31</td>
</tr>
<tr>
<td>Axonemes recombined with dynein 1 (dynein 1:axonemes)</td>
<td>1:1</td>
</tr>
<tr>
<td>2:1</td>
<td>2.42</td>
</tr>
<tr>
<td>3:1</td>
<td>2.46</td>
</tr>
</tbody>
</table>

As shown in Table 6, when the ratio of added exogenous dynein 1 fraction to the Tris-EDTA-extracted axonemes was 1:1, the sliding velocity was found to be about 50% of that of the intact axonemes, and increased to about 75% when the ratio was 2:1.

We compared the ATPase activity of the dynein 1-recombined axonemes with that of the Tris-EDTA-extracted axonemes. When the exogenous dynein 1 fraction was added to the axonemes, the axonemes had higher ATPase activity than the axonemes incubated with the endogenous dynein fraction. Increasing the amount of added dynein 1 fraction brought about a gradual increase in ATPase activity as shown in
Table 6, although the activity of the axonemes recombined at the ratio of 2:1 and 3:1 remained at a level of about 85% of that of the intact ones.

These axoneme samples were run in SDS-4% polyacrylamide gel, and the resulting electrophoretic patterns are shown in Fig. 6. The soluble fraction extracted from the axonemes with Tris-EDTA solution for 10 min (Fig. 6b) consisted mainly of A-band in the dynein region and some faint bands in the region of lower molecular weight. The similarity of the band pattern of the soluble fraction (Fig. 6b) to that of the soluble fraction after NaCl extraction (Fig. 3b) suggests that at least part of the inner arm consisted of A-band polypeptide. The recombined axonemes in the ratio of 2:1 (Fig. 6e) had an intensity in each band of the dynein region similar to that of the intact axonemes (Fig. 6a).

Cross-sections of these axonemes were examined by electron microscopy as shown in Fig. 7. In the Tris-EDTA-extracted axonemes, almost all the outer arms disappeared (Fig. 7A), although small segments sometimes remained in the basal area of outer arms. Most of the spoke heads and central microtubules were partially solubilized. Because of this damage, the arrangement of the outer doublet tubules became disordered in many axonemes. Most of the inner arms appeared to have been extracted, as shown in Fig. 7A. Due to overlapping of the nexin links and the inner arms, it is difficult to ascertain the absence of inner arms. However, when we carefully observed the intact axonemes having both types of arms, the distal part of the inner arms was always seen to be located a little inside the nexin links which connected adjacent doublet tubules. Since the density of inner arms inside the nexin links was considerably lower in the
Recovery of sliding ability

Tris-EDTA-extracted axonemes the inner arms appeared to have been extracted in these axonemes.

The axonemes with exogenous dynein 1 fraction added recovered their outer arms in the correct positions on the doublet tubules. Furthermore, the inner arms became clearly visible again a little inside the nexin links and could be counted (Fig. 7B-D). This result suggests that the extracted dynein 1 which was derived from the outer arms might have recombined with the outer doublet tubules as the inner arms. Increasing the amount of dynein 1 added to the axonemes resulted in an increase in

Fig. 7. Electron micrographs of: A, Tris-EDTA-extracted axoneme; B, C, D, Tris-EDTA-extracted axonemes recombined with dynein 1. The equivalency ratios (exogenous dynein 1 added to the Tris-EDTA-extracted axonemes) were 1:1 (B), 2:1 (C) and 3:1 (D). X 100,000.

Table 7. Number of outer and inner arms of Tris-EDTA-extracted axonemes after recombination with dynein 1

<table>
<thead>
<tr>
<th></th>
<th>a</th>
<th>b</th>
<th>c</th>
<th>d(%)</th>
<th>e</th>
<th>f</th>
<th>g(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact axonemes</td>
<td>56</td>
<td>497</td>
<td>8:88</td>
<td>98:6</td>
<td>493</td>
<td>8:80</td>
<td>97:8</td>
</tr>
<tr>
<td>Tris-EDTA-extracted axonemes</td>
<td>66</td>
<td>2</td>
<td>0:03</td>
<td>0:3</td>
<td>141</td>
<td>2:14</td>
<td>23:7</td>
</tr>
<tr>
<td>Axonemes incubated in 0:15 M NaCl without exogenous dynein 1</td>
<td>46</td>
<td>26</td>
<td>0:57</td>
<td>6:3</td>
<td>100</td>
<td>2:14</td>
<td>24:2</td>
</tr>
<tr>
<td>Axonemes recombined with dynein 1 (dynein 1: axonemes)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:1</td>
<td>49</td>
<td>261</td>
<td>5:33</td>
<td>59:2</td>
<td>251</td>
<td>5:12</td>
<td>56:9</td>
</tr>
<tr>
<td>2:1</td>
<td>61</td>
<td>479</td>
<td>7:85</td>
<td>87:2</td>
<td>425</td>
<td>6:97</td>
<td>77:4</td>
</tr>
<tr>
<td>3:1</td>
<td>54</td>
<td>452</td>
<td>8:37</td>
<td>93:0</td>
<td>420</td>
<td>7:70</td>
<td>86:4</td>
</tr>
</tbody>
</table>

a, Total no. of counted axonemal images.
b, Total no. of observed outer arms.
c, The mean no. of outer arms per axoneme, represented by b/a.
d, Ratio (%) of the mean no. of outer arms to the original no., represented by 100c/9.
e, Total no. of observed inner arms.
f, The mean no. of inner arms per axoneme, represented by e/a.
g, Ratio (%) of the mean no. of inner arms to the original no., represented by 100f/9.

number of both types of rebound arms, although the number of rebound outer arms was larger than that of the inner arms, as shown in Table 7. Arm-like structures rebound at irregular positions in the axonemes were not observed.
In order to verify whether the exogenous dynein 1 recombined with the Tris-EDTA-extracted axonemes as the inner arms, we tried to recombine dynein 1 with axonemes which had been dialysed against Tris-EDTA solution for 18 h. The axonemes extracted for a longer period lost both types of arms, including the basal parts, and lost their sliding ability completely. After incubating the extracted axonemes and the exogenous dynein 1 in 0.15 M NaCl solution, we examined them by electron microscopy, and found that the axonemes recovered the outer arms, but not the inner arms. The addition of ATP and trypsin could not induce active sliding movement between the doublet tubules in these axonemes, in spite of the presence of the rebound arms. Dialysing the axonemes for a longer period might have caused some damage of the axonemal structure, such as the recombination site of the inner arms on the A-tubules or the attachment site of the outer arms on the B-tubules.

DISCUSSION

Sliding velocity and the number of outer arms in the axonemes

In our previous work (Hata et al. 1980), the sliding velocity of axonemes having only inner arms was 5.3–5.7 μm/s, which was around 60% of the sliding velocity in the axonemes having both outer and inner arms (8.3 μm/s). However, as the method for measuring sliding velocity has been improved (Yano & Miki-Noumura, 1980), the sliding velocities of these axonemes were re-examined. Differences of the sliding velocities reported here and those reported previously might be due to the difference in the axoneme isolation method, ATP concentration and temperature.

As shown in Fig. 1, decrease in sliding velocity was accompanied by a decrease in the number of the total remaining arms, which also agreed with the decrease of ATPase activity of the axonemes previously reported (Hata et al. 1980). After 10 min of NaCl-extraction, the velocity and the number of remaining arms were about 50% of these of the intact axonemes. We might have overestimated the number of remaining outer arms, because the thickness of cross-sectioned specimens contained several periods of arms and absence of only one outer arm could not be detected, as was already pointed out by Gibbons & Gibbons (1973). However, the outer arms were extracted from the axonemes in groups, as shown in Fig. 2, which suggests that the overestimate might not be great. Further study of this point awaits more precise observation of the transverse and longitudinal image of serially-sectioned axonemes during NaCl-extraction.

Recovery of sliding ability in NaCl-extracted axonemes after recombination with dynein 1

Gibbons & Gibbons (1976) reported that the beat frequency of the KCl-extracted sperm of Colobocentrotus atratus was increased by recombining it with dynein 1 prepared from sperm of Tripneustes gratilla. In this study, we succeeded in restoring the sliding velocity of the NaCl-extracted axonemes by recombining them with dynein 1, which was prepared from the same species of sea urchin, Pseudocentrotus depressus.

Even though excess dynein 1 fraction prepared from other axonemes was added to the NaCl-extracted axonemes, the sliding velocity of the axonemes did not reach more than 80% of that of intact axonemes. The upper limit of this functional recovery
Recovery of sliding ability

The recovery of sliding ability corresponds to that of the recovery of beat frequency reported by Gibbons & Gibbons (1979). In their report, a 5- to 500-fold excess of LAD-1 (latent ATPase form of dynein 1) was needed for maximal recovery in beat frequency. In this study, the recovery of sliding velocity was achieved by using a quantity of dynein 1 fraction equivalent to that removed during extraction. The higher efficiency in our recombination experiment might be due to the axoneme and dynein 1 samples being prepared from the same species of sea urchin, or to longer incubation time. In the report of Gibbons & Gibbons (1979), the beat frequency of a KCl-extracted sperm model decreased from 31 to 14 Hz, and increased to 25 Hz after recombination with dynein 1. The sliding velocity of the NaCl-extracted axonemes decreased from 14 to 7 μm/s and increased again to 11 μm/s after recombination with dynein 1. Beat frequency and sliding velocity decreased to 50% of original values after extraction of outer arms and increased to 80% of original values after recombination with outer arms. These results suggest that beat frequency might be directly dependent on the sliding velocity between doublet tubules.

The degree of recovery of the number of outer arms, ATPase activity of the axonemes, and sliding velocity were 98, 90 and 80% of those of intact axonemes, respectively. Some discrepancy among these recovery percentages might be due to several factors: the overestimation of the number of arms in counting as pointed out by Gibbons, or recombination of denatured arms, etc.

Recovery of sliding ability in the Tris-EDTA-extracted axonemes after recombination with dynein 1

Brief extraction of axonemes with Tris-EDTA solution brought about complete loss of sliding ability of the axonemes. However, about 35% of ATPase activity and at most less than 30% of inner arms were retained by these axonemes. Although we do not know at present whether the remaining inner arms were functional or not, they could not generate sliding movement between the adjacent doublet tubules.

After extraction of axonemes with Tris-EDTA solution for 10 min, small segments sometimes remained on the site where outer arms were located. These segments were the proximal parts of intact outer arms and corresponded to basal segments observed by Baccetti, Burrini, Dallai & Pallini (1979) in flag midge sperm. Only the distal part of the outer arm seems to have been extracted. The dynein molecules in the fraction solubilized with Tris-EDTA did not recombine with the Tris-EDTA-extracted axonemes, and did not restore sliding ability of the axonemes. Gibbons & Fronk (1979) reported that 21S latent ATPase form of dynein 1 extracted with 0.6 M NaCl was converted to a 10S form by dialysis against imidazol-EDTA. The dynein molecule in Tris-EDTA solution might have also changed into small particles, and could not rebind to the Tris-EDTA-extracted axonemes. In Tetrahymena cilia, 30S dynein extracted with Tris-EDTA solution recombined with doublet microtubules in the presence of Mg2+ (Gibbons, 1965; Shimizu, 1975; Takahashi & Tonomura, 1978).

Sliding percentage of the recombined axonemes (ratio of the number of axonemes showing sliding movement to the total number of axonemes) was about 80%, and the sliding movement was observed only between two or three of the adjacent doublet.
tubules in the axoneme. Therefore, some rebound arms might be unable to interact with the adjacent B-tubules due to the disordered arrangement of doublet tubules and the greater distance between the adjacent doublet tubules in the axonemes, which were sometimes observed by electron microscopy.

Sliding velocity of the axonemes with dynein 1 fraction added at the ratio of 1:1 (dynein 1:axonemes) was 8 μm/s and that of the axonemes with dynein 1 fraction added at the ratio of 2:1 was 11 μm/s. Recovery of ATPase activity and the number of arms in the recombined axonemes was also larger when the ratio was 2:1 than 1:1. The axonemes with an equivalent quantity of dynein 1 fraction added had a value of sliding velocity similar to that of the NaCl-extracted axonemes. The NaCl-extracted axonemes had about 50% of the total number of arms as the inner arms. When the Tris-EDTA-extracted axonemes were recombined with dynein 1 at a 1:1 ratio, the axonemes also restored 55-60% of the total number of arms, as outer or inner arms. These results suggest that sliding velocity maintained a close relationship with the total number of arms in the axonemes, irrespective of whether they were outer or inner arms. However, we have no information about the exact localization of arms in the longitudinal sectional image of the recombined axonemes or of the NaCl-extracted axonemes during the extracting process, as mentioned above. At present, it would be premature to conclude that the sliding velocity may be determined strictly by simple summation of the force generated by each arm without interaction with other arms, simply on the basis of a close relationship between the sliding velocity and the 'apparent total number' of arms in the axonemes.

Takahashi & Tonomura (1978) reported that 30S dynein of *Tetrahymena* cilia recombined with both A- and B-tubules in the presence of Mg^{2+}, and that the arms recombined with B-tubules were dissociated in the presence of ATP. The axonemes of *Tetrahymena* cilia recombined with excess 30S dynein revealed 'fluffy material' (Shimizu, 1975). As shown in this study, when the dynein 1 recombined with the extracted axonemes of sea-urchin sperm, the recombined arms always reappeared at the regular positions on A-tubules and arm-like structures were not observed on B-tubules or at irregular positions. These results agreed well with those of Gibbons & Fronk (1979). The recombination of dynein with the doublet tubules seems to be more specific in sea-urchin sperm flagellar axonemes than in ciliary axonemes of *Tetrahymena*.

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Recovery of sliding ability

REFERENCES


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