Effect of Adenosine Triphosphate on *Perca fluviatilis* Sperm Tails Isolated in Buffered Sucrose

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1. Sperm tails were isolated from mechanically disrupted spermatozoa of *Perca fluviatilis* and suspended in buffered sucrose solution. When freshly prepared suspensions were treated with ATP and magnesium chloride and centrifugal pellets obtained these weighed up to about 40% heavier than controls. 2. The effect is closely linked to the enzymic splitting of ATP and under conditions where this was inhibited no increase in weight was observed. Pellets from suspensions aged overnight at 2° and then treated with ATP and magnesium chloride were no heavier than the controls although the suspensions had undiminished enzymic activity. Sperm tails from *Salmo trutta* did not show the effect. 3. Analyses showed that the weight increases were the result of uptake of sucrose solution and that sucrose completely penetrated the pellet water. Polyethylene glycol (mol. wt. 4000) did not penetrate the water of the pellets completely and an amount approximately equal to 1.9 times the flagellar dry weight was inaccessible. 4. Electron-microscopic studies indicated that the effect is the result of tail disintegration.

During the hydrolysis of added ATP by a tail fraction from a homogenate of *Perca fluviatilis* spermatozoa a decrease in turbidity occurred (Tibbs, 1962a). The possibility that this effect might be the result of swelling in the tail and tail fragments was strengthened by the observation that centrifugal pellets obtained from these preparations after treatment with ATP under conditions activating dephosphorylation were heavier than the controls (Tibbs, 1962b, 1963). This paper reports this work in more detail and also describes attempts to determine how the intraflagellar water as distinct from the total pellet water is changed during the reaction with ATP. The results of these experiments were difficult to reconcile with the idea of a simple swelling process and an electron-microscopic examination of some of the preparations used provided a modified explanation for many of the observations.

EXPERIMENTAL

Materials. Regular supplies of live male perch (*Perca fluviatilis*) were obtained from the Freshwater Biological Station, Ambleside, Westmorland, during the spawning season from late April to early June. These fish were kept in running water and milt was obtained from them as and when required by applying pressure along the line of the testes. Suitable precautions were taken to exclude water and faecal material. Before use the milt was tested by observing under the microscope the effects of mixing a little with water. In all preparations used this caused the sperm to become motile.

A few experiments were conducted on sperm from trout (*Salmo trutta*). For this purpose, live male trout were purchased from a local hatchery during November, the month in which they were spawning.

All the nucleotides used in the present work were products of the Sigma Chemical Co., St Louis, Mo., U.S.A.

Preparation of sperm-tail suspensions. Precautions were taken at all stages to keep the temperature of the preparations as near 0° as possible. Fresh milt was diluted approximately tenfold with ice-cold 0.5M-sucrose in 0.05M-tris buffer, pH 7.6 (hereafter called 'buffered sucrose'), containing 2.5mm-EDTA. The suspension was homogenized for 20sec. in a high-speed, top-drive homogenizer (Measuring and Scientific Equipment Ltd.) to remove the tails from the heads. The vast majority of the heads were then removed by spinning for 8min. at 2500g_{av.}. Sometimes a second spinning was necessary to reduce the number of heads in the supernatant to insignificant proportions. The fraction obtained from the supernatant by sedimentation at 15000g_{av.} for 8min. was dispersed in buffered sucrose solution to give the final suspension of sperm tails and no attempt was made to wash the preparations. Plate 1(a) is an electron micrograph showing that such a preparation contained sperm-tail fragments of varying lengths. Suspensions were of the order of five times the volume of original milt and usually contained about 3–4mg. dry wt. of sperm tails/ml. Such a concentration was reasonably economical in material and provided unambiguous experimental results, but sometimes when ample material was available less suspending medium was used to produce more concentrated suspensions (up to 10mg. dry wt. of sperm tails/ml.).

Measurement of pellet weights. All additives to the tail suspensions were made up in buffered sucrose. Portions (2ml.) of suspension were measured into weighed poly-

propylene centrifuge tubes of approx. 7 ml. capacity. This was followed by 2 ml. of a solution of any additional additive required by the particular experiment and 0.8 ml. of MgCl_2 . The concentration of the latter was usually 20 mM but this was varied in some of the experiments described. The reaction was started by adding 1.6 ml. of 8 mM-ATP and stirring. Controls were tubes in which either MgCl_2 or ATP or both were absent. After standing for 10 min. at room temperature the tubes were centrifuged for 10 min. at 18000 $g_{av.}$. After removal of the supernatants the insides of the tubes were dried with rolled filter paper and the tubes weighed.

Measurement of pellet composition. For the estimation of the amount of water present the pellet, of known wet weight, was freeze-dried in the centrifuge tube and kept *in vacuo* overnight over P_2O_5 . The tube and dried pellet were then weighed and the water originally present was estimated by difference.

Sucrose was determined by extracting the pellet with 5 ml. of 0.34 M-perchloric acid for 10 min. at room temperature. To the extract (1 ml.) was added 0.2 ml. of 60% (w/w) perchloric acid and the solution heated at 70° for 2 min. to hydrolyse the sucrose. The solution was diluted to 5 ml. and the glucose in 0.2 ml. determined by the glucose oxidase-peroxidase method as described by Bergmeyer & Bernt (1963).

Nucleotide in the pellet resulting from the addition of ATP to the suspension was estimated as adenylic acid by measuring the extinction at 260 $m\mu$ of the same 0.34 M-perchloric acid extract as was used for the sucrose estimation. A correction was made for ultraviolet-absorbing material normally present in the sperm tails and a molar extinction coefficient of 18.2×10^3 was used. This was the value, found in this Laboratory, for a preparation of Sigma adenylic acid.

Pellet inorganic phosphate was estimated by applying the Gomori (1942) method to the perchloric acid extract.

The micro-Kjeldahl method was used to measure the nitrogen in the pellets after perchloric acid extraction. The small amount of nitrogen lost from the pellets during this extraction was readily accountable for on the basis of the removal of tris buffer (nucleotide nitrogen was negligible). A value of 10.54% for the nitrogen content of tails prepared in buffered sucrose was used to calculate the dry wt. of sperm tails present. The figure 10.54 ± 0.09 was the mean of six nitrogen estimations carried out on sperm-tail preparations prepared in buffered sucrose, exhaustively dialysed and then freeze-dried.

Estimation of rate of enzymic dephosphorylation of ATP. For the sake of brevity the enzymic dephosphorylation of ATP is referred to below as adenosine-triphosphatase activity. ADP and ITP were the only compounds, other than ATP, which were dephosphorylated at an appreciable rate by perch sperm tails in buffered sucrose (Tibbs, 1959, 1962a). The general method for the determination of adenosine-triphosphatase activity has been described (Tibbs, 1959). In the present experiments sperm tails were incubated in buffered sucrose with substrate and activator in the concentrations required by the particular experiment for 5 min. at 37°.

Estimation of phosphate released during determinations of pellet weight. It was occasionally necessary to measure the actual amount of phosphate released in a particular tube during a determination of pellet weight. The Gomori (1942)

method of estimation was applied to samples from the supernatant from the final centrifugation.

Penetration of pellet water by sucrose, nucleotide and polyethylene glycol. The extent to which a solute penetrates a centrifugal pellet can easily be calculated if the water and solute content of the pellet and solute concentration in the supernatant are known (Conway & Downey, 1950). These calculations were made for the sucrose of the suspending medium and for the added ATP (or dephosphorylated ATP). They were also made for polyethylene glycol (mol. wt. 4000), when added to the extent of about 1.5% to the suspending medium from which the pellets were obtained.

Pellet water, sucrose and nucleotide and the concentrations of sucrose and nucleotide in the supernatants were estimated as already described. Polyethylene glycol was measured in the pellets and supernatants by the turbidimetric method of Hydén (1956) as modified by Smith (1959).

For the purposes of these calculations the specific gravity of the sucrose buffer was taken as 1.067, of which 174 mg./ml. was sucrose. These were the values obtained by direct experiment.

Electron microscopy. This examination was kindly undertaken by Professor R. E. Coupland of the Department of Anatomy, Queen's College, Dundee. Specimens were fixed in buffered osmium tetroxide (pH 7.3) and washed before dispersing and drying on copper grids and examining in an EM6 electron microscope.

RESULTS

Choice of suspending medium for use during isolation of tail fractions

Sucrose was included in the suspending medium in the first instance with the idea of reducing the possibility of osmotic disruption. In view of the fact that the sperm-tail fragments in the preparations presumably had open ends (Plate 1a) it is doubtful if sucrose offered any osmotic protection. It certainly exerted a protective influence of some kind, however, for when tail preparations were obtained without the use of sucrose these, after treatment with ATP under conditions favouring ATP breakdown, did not provide pellets any heavier than the controls.

Since the whole object of the work was to detect physical changes consequent upon ATP hydrolysis it was considered necessary to guard against the breakdown of endogenous ATP during the preparative procedures by inhibiting reversibly the tail adenosine-triphosphatase activity. This was carried out by including 2.5 mM-EDTA in the suspending medium and the inhibition caused by this compound could be overcome by magnesium chloride (Tibbs, 1962b).

Effect on pellet weight of treating suspensions with adenosine triphosphate and magnesium chloride

Freshly prepared sperm-tail suspensions in buffered sucrose were treated with 2 mM-ATP and

Table 1. *Percentage increases in wet weight observed in centrifugal pellets obtained from perch sperm-tail suspensions after treatment with adenosine triphosphate and magnesium chloride*

Preparative methods were as described in the text. Pellets which were compared with each other were always obtained during the same centrifugation. Nitrogen contents of pellets from ATP-treated suspensions were slightly higher than those from untreated controls (Table 2) and the wet weights of pellets from treated suspensions were corrected to control pellet nitrogen. Results describe the behaviour of 49 different preparations.

Percentage increase in weight in pellets from ATP-treated tails	No. of preparations showing this increase in weight
0.0-4.9	5
5.0-9.9	4
10.0-14.9	6
15.0-19.9	5
20.0-24.9	8
25.0-29.9	8
30.0-34.9	4
35.0-39.9	4
40.0-44.9	4
45.0 upwards	1

2.5mm-magnesium chloride, the centrifugal pellets obtained and the weights compared with those of control pellets from the same preparations devoid of ATP or magnesium chloride or both. The nitrogen contents of the pellets were determined after extraction with 0.34M-perchloric acid and, although these figures for pellets from ATP-treated suspensions appeared to be slightly higher than those from controls (Table 2), on a constant nitrogen basis pellets from treated suspensions were usually considerably heavier than the controls. Table 1 shows the weight increases which were observed with 49 different preparations investigated during four spawning seasons. Wet pellet weights from different preparations normally ranged from 50 to 100mg, and the increase in weight was an obvious and clear phenomenon. It was even possible to see visually that the pellets treated with ATP and magnesium chloride were bulkier than the controls. There was no pH change during the treatment. The percentage nitrogen of the wet pellets from treated suspensions varied between 0.80 and 1.15, the corresponding range for untreated suspensions being from 1.10 to 1.55.

When aged preparations (stored overnight at 2°) were used the phenomenon was usually no longer observable although the adenosine-triphosphatase activity of such preparations had hardly decreased.

Specificity of effect. When ATP was replaced by 2mm-ADP, -AMP, -ITP, -sodium pyrophosphate or 4mm-sodium phosphate in the presence of 2.5mm-

magnesium chloride only the ADP-treated pellets showed any increase in weight over the controls. Neither calcium nor barium could replace the magnesium.

Proportion of ATP split, and effect of adding more ATP. An estimate of the amount of hydrolysis taking place during the 10min. treatment with ATP was made for six different preparations varying in pellet wet wt. from 50 to 151mg. From 1.36 to 1.82 phosphate groups had been liberated per molecule of ATP and a longer period of standing in no case increased this figure beyond 1.82. To see whether the available ATP was a factor limiting the weight increase more ATP and magnesium chloride were added at the end of 10min. but this did not result in a further increase in pellet weight.

Analysis of pellets. Table 2 shows the results of analyses carried out on pellets from ATP-treated material and controls. It can be seen that the increases in weight were accounted for as sucrose solution. The weight contribution due to added nucleotide or phosphate was negligible.

The weight of this buffer plus magnesium chloride in the pellet was obtained by calculation, their concentration in the pellet water being assumed to be the same as in the supernatant. Magnesium was not estimated, the total amount of magnesium chloride in the incubated mixture only being 1.5mg. Table 2 also shows that slightly more tail material was actually sedimented from the suspension after ATP treatment. The reason for this is unknown.

Apparent absence of effect with trout sperm tails. Tails from the sperm of the brown trout (*Salmo trutta*) were isolated in the same way as perch sperm tails and treated with ATP and magnesium chloride. No increase in pellet weight was observed. This was repeated six times with the same result.

Use of inhibitors to show dependence of increases in pellet weight on hydrolysis of adenosine triphosphate

EDTA and Mg²⁺ ions. When the ratio of EDTA to Mg²⁺ ion concentration was high enough to cause enzyme inactivation the pellets from ATP-treated suspensions were no heavier than the controls. Under conditions of enzyme activation, however, the usual increases in pellet weight could be observed (Tibbs, 1962b).

Sodium fluoride, cyanide and chloride. At a concentration of 0.01M only sodium fluoride of these three salts caused appreciable enzyme inhibition (about 50%), and only this salt prevented that change which results in increased pellet weights. All salts in 0.1M concentration inhibited the water uptake although the chloride had little if any inhibiting effect on the adenosine triphosphatase.

Iodoacetate. This compound inhibited the adenosine triphosphatase only slightly in a concentration

Table 2. *Analyses of centrifugal pellets obtained from sperm-tail suspensions, after treatment with adenosine triphosphate and magnesium chloride, compared with controls*

Preparative and analytical methods were as described in the text. Pellets which were compared with each other were always obtained during the same centrifugation. In each case except for preparation 7 (64) the wet pellet weights were the average values obtained from four pellets obtained in an identical manner. The wet pellet weight of 7 (64) was the average of two determinations. Agreement was such that all observations of wet weight fell within $\pm 2\%$ of the values indicated. Other determinations were the average of duplicate analyses; with the dry weight of tails obtained from nitrogen values no individual observation differed by more than $\pm 2\%$ from the mean; in all other cases the corresponding figure was $\pm 3\%$.

Preparation no.	Pellet wet wt. (mg.)	Water found (mg.)	Sucrose found (mg.)	Dry wt. of tails found from N (mg.)	Tris buffer + MgCl ₂ calculated (mg.)	Wet pellet material accounted for (mg.)	Nucleotide found (expressed as $\mu\text{g. of AMP}$)	Inorganic phosphate found (expressed as $\mu\text{g. of P}$)
Pellets from suspensions not treated with ATP								
2 (64)	84.4	60.2	12.4	11.3	0.4	84.3	19.1	
3 (64)	122.8	88.5	14.9	16.9	0.6	120.9	29.8	1.2
4 (64)	97.9	70.2	13.5	13.1	0.5	97.3	20.7	
6 (64)	60.7	43.5	8.5	7.6	0.3	59.9	11.7	3.4
7 (64)	59.7	43.7	8.3	7.4	0.3	59.7	15.4	
Pellets from suspensions treated with ATP								
2 (64)	102.8	75.7	13.8	12.0	0.5	102.0	45.5	
3 (64)	148.8	110.8	17.5	17.3	0.8	146.4	72.8	12.0
4 (64)	108.7	79.8	15.1	13.4	0.6	108.9	49.5	
6 (64)	72.4	53.2	10.5	8.0	0.4	72.1	30.9	9.1
7 (64)	79.9	59.8	11.5	7.6	0.4	79.3	39.7	

of 2mm, to the extent of about 50% in 5mm and 80% in 10mm. Similarly the weight increases observed in the absence of inhibitor were reduced by about 50% in 2mm-iodoacetate and abolished in 10mm.

Pyrophosphate. Pyrophosphate in a concentration higher than that of the magnesium inhibits the adenosine triphosphatase. Under such conditions an increase in pellet weight cannot be observed. 5mm-Pyrophosphate proved to be a suitable concentration in which to demonstrate this, for in concentrations appreciably less than this pyrophosphate was ineffective as an enzyme inhibitor and in higher concentrations the amount of magnesium required to overcome the inhibition resulted in the precipitation of magnesium pyrophosphate.

In 1.25mm-magnesium chloride, 5mm-pyrophosphate reduced the enzyme activity and the increases in pellet weight observed in a preparation by about 60% and 100% respectively. In 2.5mm-magnesium chloride the corresponding figures were 30% and 50%, whereas in 5mm-magnesium chloride no inhibition of either property was recorded.

Reversibility

A number of attempts were made to reverse the effect. Thus EDTA in buffered sucrose was added to sperm-tail suspensions which had been treated with ATP and magnesium chloride for 10min. The EDTA concentrations used were such as would

result in inhibition of adenosine triphosphatase. When the pellet weights from these suspensions were compared with those from the appropriate controls it was found that the increases in pellet weights were normal and no reversal had taken place.

Time required in ATP and magnesium chloride for increase in pellet weight to occur

In the presence of suitable concentrations of EDTA increases in pellet weight do not take place and, since the addition of EDTA and ATP to a suspension of tails already treated with ATP and magnesium chloride did not reduce the weight increases, this suggested a method of finding the rate at which the phenomenon normally occurred. Fig. 1 shows the weight increases which resulted when the treatment of two sperm-tail preparations with ATP and magnesium chloride was stopped after various time-intervals by the addition of EDTA and ATP. A considerable proportion of the total effect took place within the first minute or so.

Penetration of pellet water by solutes

Penetration by sucrose. Appropriate analyses were carried out on ATP-treated and control pellets from five different preparations and the results are summarized in Table 3. These results show that all of the pellet water in both ATP-treated material and in the controls was available to sucrose.

Penetration by ATP. No attempt was made to distinguish between ATP and dephosphorylated ATP in these analyses. Control pellets contained a small amount of ultraviolet-absorbing material extractable by dilute perchloric acid. The absorption curve of this material corresponded closely to

Fig. 1. Effect on the increases in pellet weight of the time of treatment with 2mm-ATP and 2.5mm-MgCl₂. Uptake of water was stopped at specified intervals by the addition of more 2mm-ATP and 2.5mm-MgCl₂ containing sufficient EDTA to give a final concentration of 5mm. ● and ○ refer to two different preparations.

an adenine nucleotide and calculated as such a value of 4.8 ± 0.4 μ moles of nucleotide bound/g. dry wt. of sperm tails was obtained from seven different preparations. This bound material was still present in the ATP-treated pellets after added ATP had been removed by washing. When calculating the amount of added nucleotide which was present in the ATP-treated pellets allowance was therefore made for the endogenous contribution. The results in Table 3 indicate that under the conditions described a proportion of the pellet water, averaging about 2.1 times the flagellar dry weight, is inaccessible to nucleotide.

Penetration by polyethylene glycol (mol.wt. 4000). The extent to which a high-molecular-weight solute penetrated the centrifugal pellets was investigated in an attempt to distinguish between intraflagellar and extraflagellar water. Because of the ease of estimation polyethylene glycol was used. The results of this work, which was started before the electron-microscopic evidence became available, were rather surprising when viewed against a background of the latter (see the Discussion section).

Table 4 summarizes the results obtained with ATP-treated pellets and controls when polyethylene glycol was used as the penetrating solute. These results show quite clearly that not all of the pellet water was available to this solute, and that the increase in pellet water which takes place when the suspension is treated with ATP is accounted for entirely by an increase in the water accessible to polyethylene glycol. The water inaccessible to this compound, about 1.9 times the flagellar dry weight, has not changed.

Table 3. *Accessibility to sucrose and adenosine triphosphate of water in pellets from perch sperm-tail suspensions treated with adenosine triphosphate and to sucrose in pellets from control suspensions*

Preparative and analytical methods were as described in the text. Pellets which were compared with each other were always obtained during the same centrifugation. Nitrogen contents of pellets from ATP-treated suspensions were slightly higher than those from untreated controls (Table 2), and analytical results for the former were corrected to control pellet nitrogen. Determinations were the average of duplicate analyses and direct estimations (as opposed to those obtained by difference or addition) did not differ from the mean by more than the amounts indicated in Table 2.

Preparation no.	Sperm-tail dry wt. (mg.)	Total water in pellet from ATP-treated tails (mg.)	Total water in control pellet (mg.)	Water in pellet from ATP-treated tails accessible to sucrose (mg.)	Water in control pellet accessible to sucrose (mg.)	Water in pellet from ATP-treated tails accessible to ATP (mg.)	Water in pellet from ATP-treated tails inaccessible to ATP (mg.)	Ratio of pellet water inaccessible to ATP: sperm-tail dry wt.
2 (64)	11.3	71.2	60.2	66.0	63.1	43.7	27.5	2.4
3 (64)	16.9	108.4	88.5	104.0	90.9	73.9	34.5	2.0
4 (64)	13.1	77.9	70.2	76.6	71.9	49.2	28.7	2.2
6 (64)	7.6	50.1	43.5	51.6	40.1	31.4	18.7	2.5
7 (64)	7.4	57.6	43.6	57.1	42.0	40.7	16.9	2.3
8 (64)	5.1	42.8	29.8	—	—	34.4	8.4	1.6
9 (64)	24.2	141.1	102.8	—	—	105.0	36.1	1.5

Plate 1. Sperm-tail preparations of *Perca fluviatilis*, isolated in buffered sucrose and treated with 2 mM-ATP and 2.5 mM-MgCl₂ for varying times at room temperature before fixing with osmium tetroxide. (a) Fixative added before ATP and MgCl₂. (b) Fixative added after 1 min. (c) Fixative added after 4 min. (d) Fixative added after 60 min. The sample (a) used as control was prepared from some untreated suspension about the same time as fixative was added to (d). The preparation had already been standing at room temperature for several hours and the disintegration seen in (d) was not spontaneous. Magnification $\times 5600$.

Table 4. *Accessibility to polyethylene glycol (mol.wt. 4000) of water in pellets from adenosine triphosphate-treated and control perch sperm-tail suspensions*

Preparative and analytical methods were as described in the text. Pellets which were compared with each other were always obtained during the same centrifugation. Nitrogen contents of pellets from ATP-treated suspensions were slightly higher than those from untreated controls (Table 2) and analytical results for the former were corrected to control pellet nitrogen. Determinations were the average of duplicate analyses and direct estimations (as opposed to those obtained by difference or addition) did not differ from the mean by more than the amounts indicated in Table 2. The figure for the polyethylene glycol (PEG) analyses was $\pm 3\%$. Pellets from preparations 8 (64) and 9 (64) analysed in Table 3 were not obtained at the same time as those discussed here, nor were the preparations used in the same concentration.

Preparation no.	Sperm-tail dry wt. (mg.)	Total water in pellet from ATP-treated tails (mg.)	Water in pellet from ATP-treated tails accessible to PEG (mg.)	Water in pellet from ATP-treated tails inaccessible to PEG (mg.)	Total water in control pellet (mg.)	Water in control pellet accessible to PEG (mg.)	Water in control pellet inaccessible to PEG (mg.)	Ratio of water in pellet from ATP-treated tails	Ratio of water in control pellet
								inaccessible to PEG: sperm-tail dry wt.	
7 (64)	7.4	57.6	40.8	16.8	43.6	25.1	18.5	2.3	2.5
8 (64)	6.5	41.7	31.9	9.8	34.1	23.0	11.1	1.5	1.7
9 (64)	14.0	78.6	48.8	29.8	67.1	37.9	29.2	2.1	2.1
10 (64)	10.5	66.3	43.6	22.7	56.0	33.5	22.5	2.2	2.1
13 (64)	7.2	41.5	31.7	9.8	38.4	29.8	8.6	1.4	1.2
14 (64)	12.9	73.7	50.4	23.3	66.7	45.8	20.9	1.8	1.6

Electron microscopy

Plate 1(a) shows a suspension of sperm tails untreated with ATP. Although many of the tails appear to be fragmented laterally the individual filaments cohere longitudinally. The consequences of treating this preparation for varying lengths of time with ATP and magnesium chloride are shown in Plates 1(b)–(d). The sperm tails appear to swell and then to split into constituent filaments and groups of filaments. Plate 1(d) shows that, with few exceptions, the sperm tails have become highly swollen or disintegrated.

DISCUSSION

The observation of uptake of sucrose solution by the centrifugal pellets only under conditions of ATP splitting leaves little doubt that the phenomenon is associated with the splitting and not just the presence of ATP. Only ADP appears able to substitute and this is probably the result of a myokinase-like transmutation to AMP and ATP (Brokaw, 1961). A high rate of ATP splitting seems to be more important than the total amount actually split, for in some experiments in which the enzyme was partially inhibited and little weight increase could be observed the duration of the experiment was long enough for all the ATP present to be hydrolysed. ATP splitting is not invariably accompanied by water uptake. The adenosine-triphosphatase activity of suspensions stored overnight at 2° was hardly

diminished but such suspensions, on treatment with ATP and magnesium chloride, did not usually provide pellets any heavier than the controls. This suggests that the weight-increase phenomenon is not an artifact resulting from ATP splitting.

The fact that no water uptake by trout sperm tails could be observed is not unduly disturbing. Freshly prepared suspensions from perch did not always show the effect and all preparations became inactive after storage for quite short periods. The ability to take up additional water seems to be one which is easily lost.

The electron micrographs indicate that the increased pellet weights are due to the sperm tails swelling and finally splitting into individual filaments and groups of filaments. It is easy to understand how a pellet with a structure such as shown in Plate 1(d) is able to hold more solution than one of the type seen in Plate 1(a). Moreover the effect could hardly be expected to be reversible.

At the same time as material was being processed for electron microscopy, parallel experiments were being performed in an attempt to distinguish between intraflagellar and extraflagellar water and to determine how these components of the total pellet water altered as the result of ATP treatment. The electron micrographs indicate, however, that a distinction between these two kinds of water may not be valid. Even the untreated sperm tails appear to have open ends, which should allow all solutes to permeate the structure without difficulty. The con-

cept of two kinds of water is even more difficult to maintain in the structure shown in Plate 1(d). In the light of these observations, the results which were obtained with nucleotide and polyethylene glycol are interesting and their correct interpretation may be significant.

It was found that in neither the untreated nor the ATP-treated pellets was the whole of the water available to polyethylene glycol. In fact, an amount of water approximately equal to double the sperm-tail dry weight was unavailable to this solute and this water did not alter even when the structure was disrupted, as shown in Plate 1(d). One interpretation of this is that the concept of intraflagellar water may mean little at the flagellar level of structure but may have some validity at the level of the constituent filaments.

The results also indicated that although the total pellet water was invariably available to sucrose this was not so for nucleotide. The amount of pellet water unavailable to ATP was similar to that unavailable to polyethylene glycol. Here again it is not easy to suggest an explanation for this, and at this stage, as far as nucleotide is concerned, it may not be fruitful to speculate. The nucleotides are negatively charged, and, at the relatively low ionic strengths used, the Gibbs-Donnan effect might explain, in part at least, the exclusion of nucleotide from any water which is closely associated with flagellar protein.

Thus it appears that *in vitro* an effect on flagellar structure closely correlated with ATP hydrolysis has been observed. The swelling and subsequent disintegration of the tails could well be a secondary

effect, resulting from the breakage of bonds within the structure. The relevance or otherwise of these observations, made on fragmented sperm tails, to the problems of normal motility must await further work. If the function of the tail enzyme *in vivo* is to liberate energy for movement an understanding of the forces responsible for the disruption of the preparations described in this paper might help considerably towards an understanding of the mechanism of sperm motility.

The biochemical techniques used here have been useful in detecting the effect and defining the conditions under which it takes place, and have provided a semi-quantitative method of measuring and comparing the reaction towards ATP of different preparations.

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