Mechanochemical Coupling in Flagella

II. *Effects of viscosity and thiourea on metabolism and motility of Ciona spermatozoa*

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ABSTRACT The relation between oxygen consumption and motility of *Ciona* spermatozoa has been measured by using pH stats to measure the acid production of spermatozoa swimming in dilute suspensions where their motility can be analyzed accurately, and calibrating the acid production by measuring it simultaneously with measurements of oxygen consumption, using more concentrated sperm suspensions. When the motility of the spermatozoa is inhibited by thiourea or by increased viscosity, their oxygen consumption decreases in proportion to the decrease in beat frequency. 80–85% of their oxygen consumption appears to be tightly coupled to motility. The amount of movement-coupled oxidative metabolism per beat remains nearly constant, even when there are significant changes in the energy required per beat for movement against the viscous resistance of the medium. This implies that under these conditions, where the radius of curvature of flagellar bending remains constant, the amount of ATP used is determined by a stoichiometric relation to bending rather than by the energy requirement. The movement-coupled oxidative metabolism appears to be sufficient to generate approximately two molecules of ATP per beat for each molecule of the flagellar ATPase, dynein.

Studies of the relation between ATP dephosphorylation and motility of glycerinated sea urchin spermatozoa have indicated that the use of ATP for movement is proportional to the frequency of flagellar beating, and that there is an average use of approximately one ATP molecule per molecule of the ciliary ATPase, dynein, during each beat cycle (Brokaw, 1967; Brokaw and Benedict, 1968). However, the interpretation of these experiments was limited because it was not possible to measure accurately the relatively low and possibly variable percentage motility obtained with glycerinated spermatozoa. Similar experiments can be carried out with live spermatozoa under conditions where nearly 100% of the spermatozoa are motile, but it is then necessary to

measure the over-all metabolic rate of the spermatozoa, instead of the dephosphorylation of ATP. Although numerous experiments have been carried out with metabolic inhibitors, demonstrating that the inhibition of metabolism leads to inhibition of motility (e.g., Rikmenspoel, 1965), this does not necessarily require that respiration and motility will be so tightly coupled that the independent inhibition of motility by factors such as the viscosity of the medium will lead to an inhibition of metabolism. However, Rothschild's report that the heat production of bull spermatozoa was decreased when they

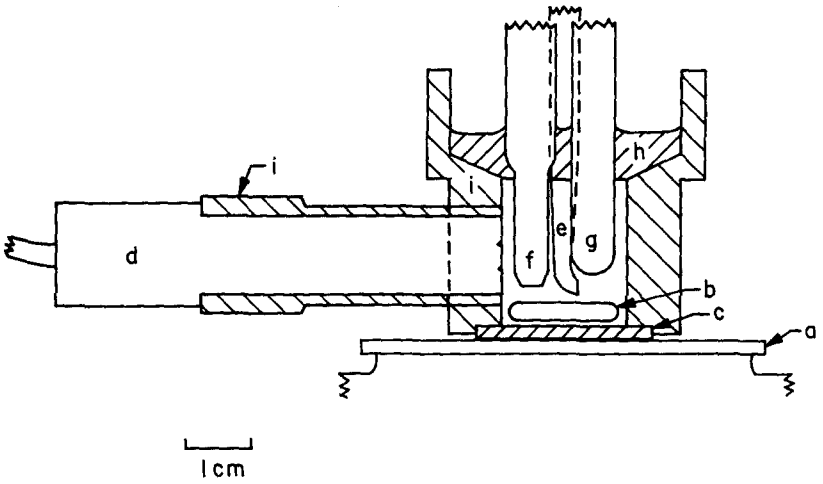

FIGURE 1. Diagram of the assay vessel and probe arrangement used for simultaneous measurement of acid production and oxygen consumption by a sperm suspension. *a*, thermoelectric plate of pH stat; *b*, magnetic stirring bar; *c*, ground glass bottom sealed into vessel with epoxy cement; *d*, polarographic oxygen sensor (Beckman No. 39065); *e*, tip of pH stat delivery burette; *f*, reference pH electrode (Beckman No. 41239); *g*, glass pH electrode (Beckman No. 41252); *h*, mineral oil seal; *i*, Plexiglas vessel. (A thermistor probe, not shown, is also inserted into the assay vessel).

swam in more viscous solutions (Rothschild, 1961) suggests that metabolism and motility may be tightly coupled in spermatozoa.

Sperm motility can only be accurately measured in relatively dilute suspensions, where individual spermatozoa can be observed microscopically and where hydrodynamic interactions between spermatozoa, of the type discussed by Rothschild (1961) and by Reynolds and Rothschild (1963), are negligible. At sperm dilutions suitable for microscopic observation or photography of individual spermatozoa, sperm metabolism will cause only a very slow decrease in the oxygen partial pressure in the sperm suspension, so that polarographic measurements of oxygen uptake are not very satisfactory. We have found that the acid production associated with aerobic metabolism—presumably due primarily to CO_2 production—can be successfully measured in

dilute sperm suspensions by using a recording pH stat. A similar technique was used by Nevo et al. (1963) to measure the anaerobic lactic acid production of fowl spermatozoa over long periods of time. The metabolic rate measured by the pH stat under conditions suitable for the measurement of motility can then be equated to an oxygen uptake rate on the basis of comparisons between oxygen uptake and acid production in more concentrated sperm suspensions.

This paper describes the results obtained from pH stat measurements of the metabolism of *Ciona* spermatozoa when their motility was varied by varying the viscosity of the medium in which they were swimming or by an inhibitor, thiourea. The effects of viscosity on the parameters of movement of these spermatozoa were described in detail in a previous study (Brokaw, 1966), so that for the purposes of the present study it was only necessary to measure the beat frequencies of the spermatozoa in order to characterize their movements at various viscosities. As in the earlier work with glycerinated spermatozoa (Brokaw and Benedict, 1968), the measurements of metabolic activity have been directly compared with beat frequencies, rather than with measured values of the viscosity, in order to eliminate one source of experimental variability and some of the variability between sperm preparations. The effects of thiourea on motility were described briefly in earlier work (Brokaw, 1965, 1966); some supplementary results are presented in this paper.

MATERIALS AND METHODS

We collected spermatozoa from the sperm duct of the tunicate *Ciona intestinalis*, after dissecting the animal and opening and rinsing out the egg duct, to prevent contamination with eggs. Occasional animals yielded 100 μl or more of concentrated spermatozoa, depending on the size and condition of the animals, but almost all the experiments described in this paper were performed with spermatozoa pooled from 10–50 animals. These concentrated samples consistently contained 4–6 $\times$ 10^{10} spermatozoa per ml. The pooled spermatozoa were stored at 16°C for several hours during a period of experimentation, either undiluted or diluted 1:1 with 0.5 M NaCl in order to obtain a less viscous suspension to facilitate reproducible sampling. There was often a decrease of about 5% between the beat frequencies of samples of spermatozoa removed from the concentrated suspension at the beginning and at the end of a sequence of experiments (4–6 hr), but this did not influence the results significantly, since both metabolism and beat frequency decreased by about the same amount.

All experiments were carried out at pH 8.1 and 16°C in solutions containing 0.5 mM EDTA, 2 mM histidine, and 0.5% polyvinylpyrrolidinone in a mixture of 60% filtered seawater and 40% 0.5 M NaCl. Solutions containing methyl cellulose were prepared by replacing some of the 0.5 M NaCl solution with the proper amount of a methyl cellulose (4000 cP; Fisher Scientific Co., Pittsburgh, Pa., No. M-281) stock solution made up in 0.5 M NaCl, usually containing 1.5% by weight of methyl cellulose.

Acid production by sperm suspensions was measured with recording pH stats (E. H. Sargent & Co., Chicago, Ill.). The sperm suspension was covered with a layer of mineral oil, to prevent the uptake of atmospheric CO_2 and/or the loss of respiratory CO_2. This mineral oil seal effectively stops the significant CO_2 absorption which is measured if the assay vessel is open to the atmosphere. Spermatozoa were added to the solution in the assay vessel at the beginning of an experiment by a micropipette inserted through the mineral oil layer, and samples of the sperm suspension were

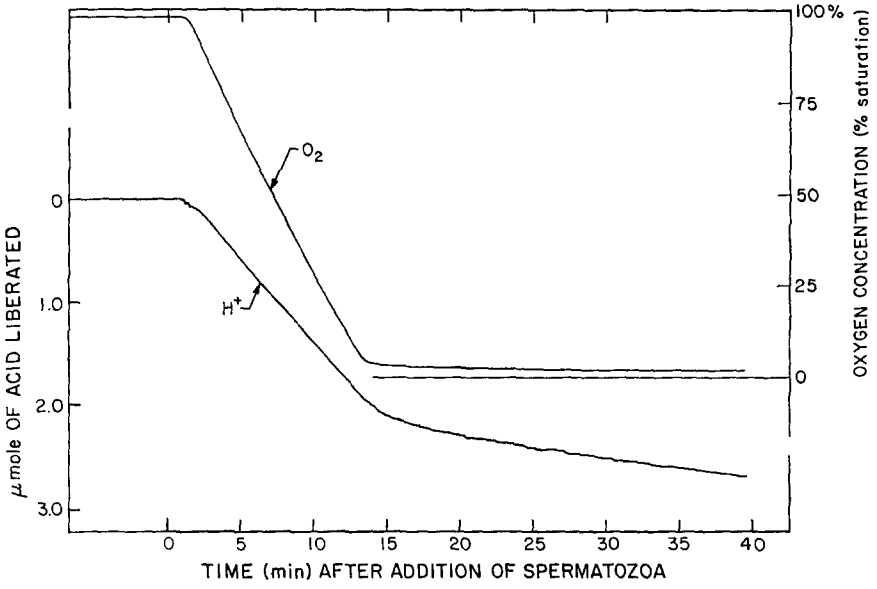

FIGURE 2. Tracings of records from the oxygen sensor and the pH stat burette during an experiment in which 50 μl of sperm suspension were added to the solution in the assay vessel at $t = 0$ min.

easily withdrawn through the oil layer for microscopic examination. In order to maintain conditions similar to those in the assay vessel during microscopic observations, sperm samples were removed and observed under mineral oil, without exposure to the air.

For comparison of acid production with oxygen consumption, a special pH stat assay vessel was constructed of Plexiglas so that a polarographic oxygen sensor could be inserted at the side of the vessel, as shown in Fig. 1. 6.0 ml of solution were added to the chamber, and after the proper temperature and pH were established, the solution was stirred vigorously for 10–20 min, in order to approach atmospheric equilibrium, and the oxygen recorder was then adjusted to read full scale. From the rate of change of the oxygen reading during this equilibration period, we estimated that the oxygen partial pressure in the solution was approaching a limiting value not more than 1–2% greater than the value which was set to correspond to a full scale record. After equilibration, the mineral oil layer, approximately 0.5 cm thick, was added, the stirring rate being reduced so that the oil layer was stable. The decrease in stirring rate caused the reading of the oxygen sensor to drop to 98–99% full scale;

it was not readjusted to 100%, in order to introduce a compensation for the possibility that the solution might not have been fully saturated.

The oxygen content of the solution at complete equilibration with air was assumed to be equal to that of seawater at 16°C, or about 5.6 μl/ml (Harvey, 1963). The 6.0 ml initial volume of solution should therefore have contained 1.50 μM of O_2 if fully equilibrated with air, so that each chart division measuring 1% saturation was equivalent to 0.0150 μM of O_2. A small additional correction is required for the dissolved oxygen which was probably added in the pH stat titrant, so that the calibration constant for the oxygen measurements was 0.0154–0.0156 μM per chart division in various experiments, depending on the normality of the pH stat titrant. For these measurements, approximately 0.01 N KOH solutions were used as the pH stat titrant. The titrant and delivery system were standardized by measuring the rate of KOH delivery required to maintain constant pH when dilute HCl was added at a known rate with a syringe infusion pump.

For the comparison of acid production with motility, the pH stat measurements were carried out with 13 ml of solution in a standard glass assay vessel. An approximately 0.002 N KOH solution was used as the pH stat titrant, and standardization was carried out as described above. Small samples of the sperm suspension were removed from the assay vessel during the measurement of acid production and observed microscopically, using stroboscopic illumination (Brokaw, 1963, 1965). Beat frequency measurements were made on 20 spermatozoa in each sample, using a digital counter and print-out device integrated with the stroboscope. Sperm numbers were determined by microscopic counts on a diluted sperm sample, as described previously (Brokaw, 1967).

Multiple-exposure dark-field photomicrographs of spermatozoa swimming in the presence of various concentrations of thiourea were obtained for measurement of the parameters of the bending waves, by techniques similar to those used in previous studies (Brokaw, 1963, 1965, 1966). Three or four flashes, at 50 or 100 flashes per second, were used to obtain exposures at a magnification of 160× on Kodak 2485 high speed recording film, and measurements were made on prints at a final magnification of 2000×. The sperm suspension was observed with stroboscopic illumination at a frequency corresponding to the average beat frequency, in order to select for photography spermatozoa which were beating at approximately the same frequency. The sperm suspension occupied a thin layer between the bottom of a cover glass and mineral oil contained in a glass well slide. Under these conditions, the beat frequencies were much more consistent than when the suspension was confined between a slide and cover glass in the conventional manner, and there was very little tendency to beat at half or twice the normal frequency, as described previously (Brokaw, 1966). The reason for this difference has not been discovered.

RESULTS

Comparison of Acid Production with Oxygen Consumption

Records from a typical experiment, using a 50 μl sperm sample, are reproduced in Fig. 2. Acid production and oxygen consumption began to be detect-

able about 1 min after addition of spermatozoa to the solution; we have not attempted to determine whether this is a biological or an instrumental lag. Both rates reached near maximum levels within 2–3 min, and continued with little change until the oxygen concentration fell to about 5% of its saturation value. Most records are at least as straight as those shown in Fig. 2. The metab-

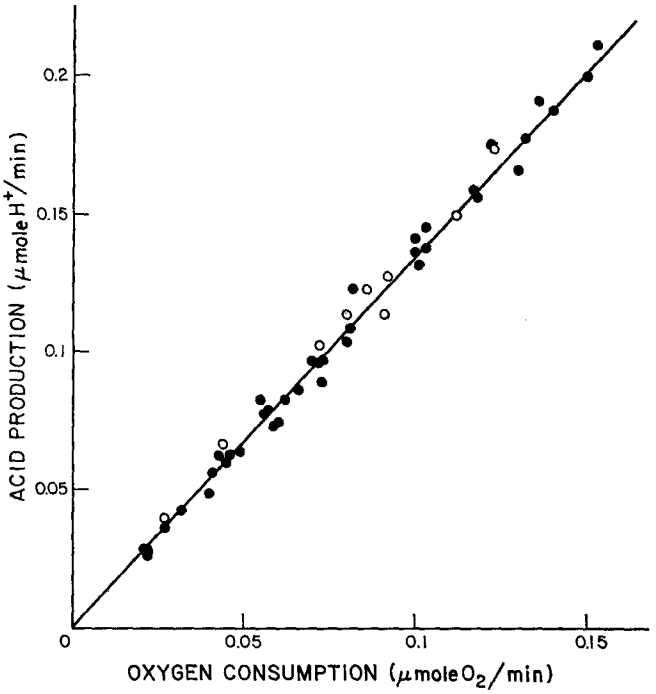

FIGURE 3. Results from experiments in which the oxygen consumption and acid production of *Ciona* sperm suspensions were measured simultaneously. The solid circles represent results from experiments without methyl cellulose. The average molar ratio of acid production to oxygen consumption is 1.34 and is represented by the line drawn through the origin. The open circles represent results from experiments in which the solutions contained 0.1–0.4% methyl cellulose.

olism of *Ciona* spermatozoa appears to be nearly independent of oxygen partial pressure in this range, so that there is no difficulty in obtaining representative average rates of acid production and oxygen consumption.

Below 5% oxygen saturation, the rate of oxygen consumption fell abruptly. The oxygen reading fell gradually to about 2% saturation after another 10–15 min, and to about 1.5% saturation over the next hour. The rate of acid production decreased somewhat less abruptly to a level between 10 and 15% of its aerobic rate; this rate decreased only slightly during the next hour. During this anaerobic (?) phase, samples of spermatozoa removed and examined under mineral oil still showed vigorous motility. Accurate measurement of

motility in such a concentrated sperm suspension is not possible, but there appeared to be very little change in the movement parameters of the motile spermatozoa; the number of nonmotile spermatozoa increased gradually to about 50% after 1–2 hr.

The results from 43 experiments of this type are summarized in Fig. 3. In most of the experiments only the rates during the aerobic phase, or its first 10–15 min, were measured. These experiments were carried out with seven different pooled sperm collections, on different days, using samples of 10–50 μl. The mean molar ratio of H^+ production to O_2 consumption was 1.34, with 90% confidence limits of approximately 1.32–1.36; the line has been drawn through the origin with a slope corresponding to this ratio. The ratio

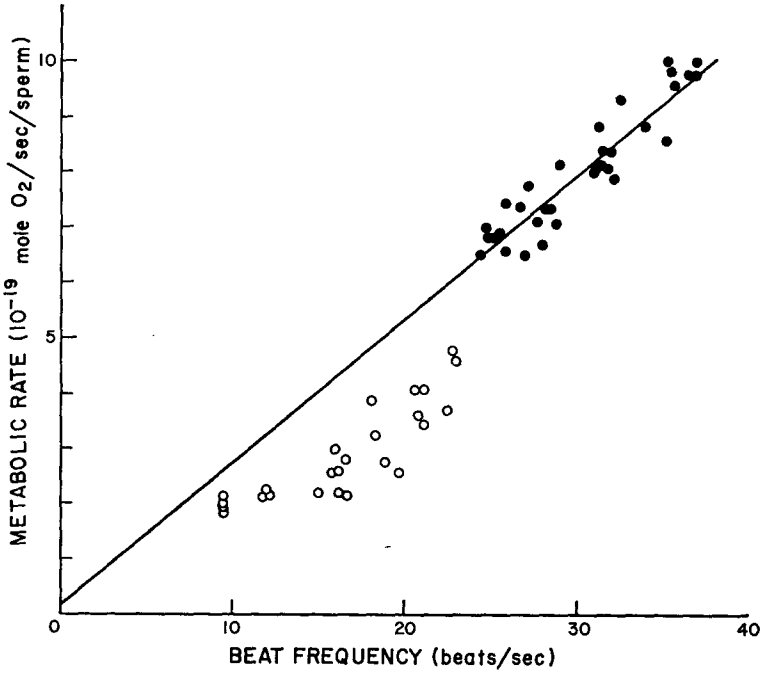

FIGURE 4. Measurements of metabolic rate, converted to rate of oxygen uptake per spermatozoon, and beat frequency of *Ciona* spermatozoa at different viscosities. The solid circles represent results from two experiments (Nos. 3 and 4 in Table II) with methyl cellulose concentrations ranging from 0 to 0.24%. The line through these points has been derived by the method of least squares and has a slope of 0.265, ±0.03 (90% confidence interval), $\times 10^{-19}$ mole O_2 per beat per sperm.

The open circles represent results from other experiments with methyl cellulose concentrations ranging from 0.45 to 1.0%. Each of these experiments also contained some measurements without methyl cellulose. The points from measurements in the presence of methyl cellulose were plotted after adjusting the metabolic rates by a factor which places the average of the measurements obtained in the absence of methyl cellulose with a particular sperm preparation on the regression line.

appears to be independent of sperm dilution, so that it can be used to convert the results of measurements of acid production at low sperm densities suitable for the measurement of motility to equivalent rates of oxygen consumption. Fig. 3 also contains a few points measured in solutions containing 0.1–0.4% methyl cellulose; these do not appear to differ appreciably from the others.

A similar series of measurements was carried out, using 50 μl sperm samples, in solutions containing 0, 0.1, 0.2, or 0.3 M thiourea. The molar ratio between acid production and oxygen uptake was not changed significantly in the

TABLE I
MEASUREMENTS OF THE METABOLISM OF BROKEN *CIONA* SPERMATOZOA

Experiment No.	13-11	15-11	16-11	17-11
Metabolic rate ($\times 10^{-19}$ mole O_2 per sec per sperm)				
Unbroken spermatozoa; 10 μl samples; average of 4 measurements				
Without methyl cellulose	9.3	10.2	9.9	9.8
Broken spermatozoa; 50 μl samples; average of 2 measurements				
Without methyl cellulose	0.52	0.87	0.54	0.65
With 0.2% methyl cellulose	0.57	0.86		
With 0.4% methyl cellulose	0.51	0.79		
With 0.6% methyl cellulose	0.61	0.83		

presence of 0.1 M thiourea. With 0.2 M thiourea this ratio increased to about 1.42, and with 0.3 M thiourea it increased to about 1.60. These results were used in converting measurements of acid production at lower sperm densities in the presence of thiourea to equivalent rates of oxygen uptake, as shown in Fig. 5.

Effect of Viscosity on Metabolism and Motility

Results of measurements of the acid production and beat frequencies of *Ciona* spermatozoa in the presence of various concentrations of methyl cellulose are illustrated in Fig. 4. The points indicated by solid circles are from two experiments using 20 μl samples of 1 : 1 diluted spermatozoa at relatively low concentrations (0–0.24%) of methyl cellulose, corresponding to increases in viscosity up to approximately three times normal. At increased viscosities within this range, *Ciona* spermatozoa continue to propagate bending waves along nearly the full length of their flagella, with reduced amplitude and velocity, but with no apparent change in the radius of curvature of the bent regions of the flagellum (Brokaw, 1966). At somewhat higher viscosities, the movements become very erratic and unstable, so that no representative measurements of beat frequency could be obtained at methyl cellulose concentrations between 0.25 and 0.45%.

The movements of the spermatozoa stabilize again at high viscosities, and they swim with bending waves of small amplitude, which are usually restricted to the proximal region of the flagellum. The open circles in Fig. 4 represent measurements made in experiments with methyl cellulose concentrations from 0.45 to 1.0%. Larger sperm samples were used for measurements at 0.8 and

TABLE II

SUMMARY OF RESULTS FROM EXPERIMENTS COMPARING THE METABOLISM AND MOTILITY OF *CIONA* SPERMATOZOA AT LOW CONCENTRATIONS OF METHYL CELLULOSE

Experiment No.	1	2	3	4	5	6	7
Methyl cellulose concentration range	0–0.25%	0–0.25%	0–0.24%	0–0.2%	0–0.2%	0	0
No. of measurements	14	14	17	16	16	10	11
Estimated % motility	95–100%	80%	90–95%	90–95%	80–90%	80–90%	90–95%
Parameters obtained by linear regression of metabolism, measured by acid production, against beat frequency							
Intercept at 0 beat frequency ($\times 10^{-19}$ mole O_2 per sec per sperm)	0.4	1.7	−0.1	0.3	1.1		
90% confidence interval for intercept	±1.7	±1.7	±0.8	±1.5	±1.9		
Slope ($\times 10^{-19}$ mole O_2 per beat per sperm)	0.28	0.15	0.27	0.25	0.27		
90% confidence interval for slope	±0.06	±0.06	±0.025	±0.05	±0.06		
Slope obtained by assuming 0 intercept at 0 beat frequency	0.29	0.21	0.265	0.265	0.30	0.27	0.30
Slope obtained from measurements in the absence of methyl cellulose, by assuming intercept at 0 beat frequency = 1.5 moles O_2 per sec per sperm	0.25	0.16	0.23	0.22	0.26	0.23	0.26

1.0% methyl cellulose, in order to get a more accurate measure of the reduced rate of acid production. In solutions containing 0.8% methyl cellulose, most of the spermatozoa swim with bending waves of very small amplitude visible in about the proximal one-fourth to one-third of the flagellum, and with beat frequencies of 10–12 per sec. In solutions containing 1.0% methyl cellulose, most of the spermatozoa appeared to be motionless, but some could be seen to be swimming slowly with barely visible bending waves and beat frequencies of 8–11 per sec. Because of the variation in the length of the flagellum on which bending waves are visible in this range of viscosities, the relation between metabolism and beat frequency is not particularly meaningful. However, as the movement is inhibited by increasing viscosity, the metabolism appears to

approach a limiting value in the vicinity of 1.5×10^{-19} mole of O_2 per sec per sperm, suggesting either that only about 85% of the metabolism of normal spermatozoa is coupled to movement, or that at high viscosities, mechanochemical coupling is not absolutely tight.

Control experiments were carried out using 50 μl samples of undiluted *Ciona* spermatozoa which had been rendered nonmotile by vigorously shaking the concentrated sperm suspension for 10 sec in a Vortex mixer, to break apart the heads and flagella of the spermatozoa. Results of some typical experiments are summarized in Table I. Methyl cellulose has no effect on the measured metabolism of broken spermatozoa, in agreement with the assumption that its effect on unbroken spermatozoa is a consequence of the inhibition of motility. The metabolic activity of broken spermatozoa appears to be about 6–7% of that of unbroken spermatozoa. This is probably not the best method for estimating the amount of metabolism which is coupled to movement, since breakage of the spermatozoa may inhibit metabolism not only by separating the site of oxidative metabolism from the flagellar enzymes which dephosphorylate ATP, but also by directly damaging the metabolic components of the spermatzoa.

Table II summarizes the results obtained in experiments at low concentrations of methyl cellulose, in terms of the parameters obtained by linear regression of metabolism against beat frequency, using the method of least squares. The results from experiment 2, in which there was an unusually large percentage of nonmotile spermatozoa, are somewhat different from the remainder, and have not been included in averaging the results.

Extrapolation of the regression lines indicates that at 0 beat frequency there will still be a metabolic rate of about 0.4×10^{-19} mole of O_2 per sec per sperm, suggesting that about 96% of the metabolism of these spermatozoa under normal conditions is coupled to movement. However, the precision of this extrapolation to 0 beat frequency is not sufficient to distinguish the intercept from 0 or from the residual metabolism obtained with broken spermatozoa. A more conservative conclusion would be that 90–100% of the metabolism of *Ciona* spermatozoa under normal conditions appears to be coupled to movement. This appears to be a significantly higher coupling than is indicated by the measurements at high viscosities shown in Fig. 4. The slopes of the calculated regression lines may be made steeper if there is a slight decrease in the extent of bend propagation along the flagellum as the viscosity is increased. This effect becomes obvious at the higher viscosities, where the metabolic rate falls well below the regression line obtained for the measurements at low viscosities (Fig. 4).

The average slope for the regression of oxygen uptake on beat frequency is about 0.27×10^{-19} mole of O_2 per beat per sperm, or 0.28×10^{-19} mole of O_2 per beat per sperm if it is assumed that all the metabolic activity is coupled

to movement so that the regression lines should pass through the origin. The results at low viscosities suggest that metabolism is directly proportional to beat frequency, if all the spermatozoa are propagating bending waves all along their flagella. However, if the slope of the regression lines is influenced by second-order effects such as a slight decrease in the activity of the distal regions of the flagellum, the best estimate of the amount of metabolism per beat may be obtained by using the results obtained in the absence of methyl cellulose and the limiting value obtained at very high viscosities, rather than

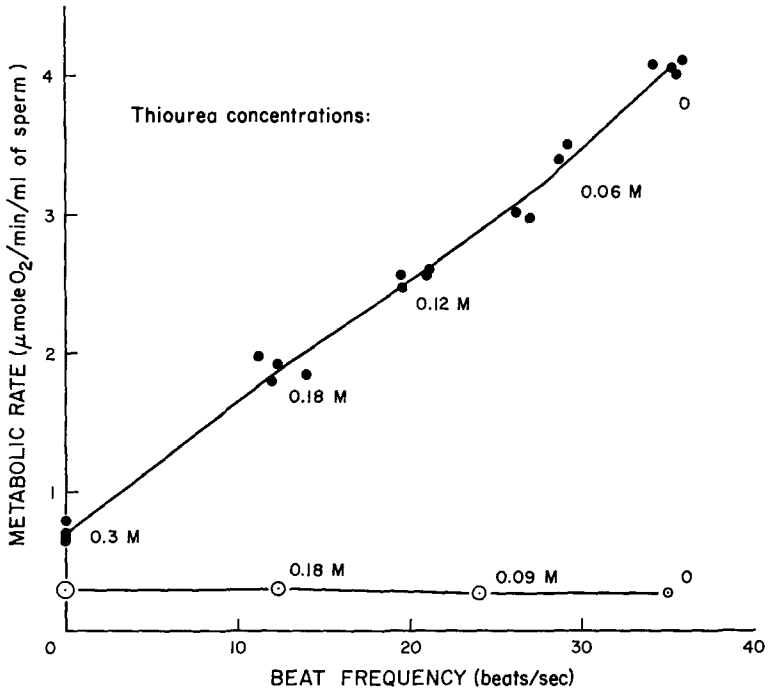

FIGURE 5. The solid circles represent results from two typical experiments in which the metabolic rates and beat frequencies of *Ciona* spermatozoa were measured in the presence of various concentrations of thiourea. Each point represents the average of duplicate measurements with the same sperm preparation and the same pH stat, and a beat frequency based on measurement of 40 spermatozoa. The location of the points for 0.3 M thiourea at 0 beat frequency is somewhat arbitrary, since these suspensions usually contained some motile spermatozoa beating at up to 2 beats per sec, although extrapolation of the results obtained by measuring beat frequencies at lower concentrations of thiourea indicates that the beat frequency should reach 0 at a thiourea concentration of 0.28 M.

Each of the open circles represents the mean of 9 to 12 measurements of the metabolic rate of broken spermatozoa at various thiourea concentrations; the diameter of each circle represents the 90% confidence interval for the mean. They have been located along the abscissa to approximately match the thiourea concentrations used for the measurements with unbroken spermatozoa.

the slopes of the regression lines. In this case, the metabolism per beat will be approximately 0.24×10^{-19} mole of O_2 per beat per sperm.

All the sperm samples examined in these experiments contained a few nonmotile spermatozoa and a few spermatozoa which were only weakly motile. Their numbers were difficult to estimate accurately, but probably averaged between 5 and 15% of the total. Taking into account both this uncertainty and

TABLE III
EFFECT OF THIOUREA ON PARAMETERS OF THE BENDING WAVES OF *CIONA* SPERMATOZOA

Thiourea concentration, *moles/liter*	0	0.12	0.18
Beat frequency (f) obtained from stroboscopic measurements (Fig. 5), *beats/sec*	35.3±0.6*	20.3±1.1	12.4±1.1
No. of sperm photographs measured	33	30	20
Beat frequency (f) estimated from photographs, *beats/sec*	34.9±1.2	21.7±0.8	11.9±0.4
Radius of curvature (ρ) of bent regions, μ	5.5±0.1	5.25±0.1	5.3±0.2
Wavelength (L) measured along the flagellum, μ	32.3±0.5	32.4±0.5	33.3±1.3
$1 - (\lambda V_e/LV_s)$‡	0.56±0.02	0.63±0.01	0.59±0.02
Bending moment required to overcome the viscous resistance of the medium,§ 10^{-10} *dyne cm*	4.2	2.7	1.6
Energy expenditure per beat,§ 10^{-8} *erg*	1.3	0.9	0.5

* Variance ranges indicated in this table are 90% confidence intervals for the mean value, determined by the t test.

‡ This quantity appears in the formulae for calculating bending moments and energy expenditures, and is characteristic of the shape of the bending waves. λ and V are respectively the wavelength and the phase velocity relative to the medium, measured along the axis of progression. L and V_s are respectively the wavelength and the phase velocity relative to the flagellum, measured along the flagellum (Brokaw, 1965).

§ Bending moments and energy expenditures were calculated by formulae given in Brokaw (1965) using the mean values for the parameters. The bending moment is proportional to $f\rho L^2[1 - (\lambda V_e/LV_s)]$ and the energy expenditure is proportional to $fL^2[1 - (\lambda V_e/LV_s)]$.

the uncertainty in the correct slope, the metabolism of a motile spermatozoon is probably between 0.25 and 0.32×10^{-19} mole of O_2 per beat per sperm, with a best value of about 0.27×10^{-19} mole of O_2 per beat per sperm.

Effect of Thiourea on Metabolism and Motility

Thiourea is an inhibitor of sperm motility (Brokaw, 1965) which appears to act directly on the mechanochemical processes in the flagellum, rather than on the generation and supply of ATP, since it has similar inhibitory effects on live spermatozoa (Brokaw, 1965, 1966) and on ATP-reactivated, glycerinated spermatozoa (Brokaw and Benedict, 1968). As shown by the results in Fig. 5, thiourea concentrations sufficient to completely inhibit motility cause about the same degree of inhibition of metabolism that is obtained when movement is inhibited by high viscosities, and thiourea has no significant effect on the

metabolic rate of broken, nonmotile spermatozoa. Both thiourea and increased viscosity therefore appear to specifically inhibit the same movement-coupled fraction of sperm metabolism, corresponding to 80–85% of the metabolism of normal spermatozoa.

The results presented in Fig. 5 also show that there is a nearly linear relation between metabolism and beat frequency when the motility is inhibited by thiourea, so that the amount of movement-coupled oxidative metabolism per beat remains nearly constant as the beat frequency decreases. In this respect, the inhibitory effect of thiourea resembles that of low concentrations of methyl cellulose. However, the effects of thiourea and of increased viscosity on the wave pattern of the sperm flagellum are very different. Although in both cases there is no noticeable change in the radius of curvature of the bent regions of the flagellum, increased viscosities cause major decreases in the amplitude of the bending waves, whereas thiourea causes no noticeable change in any parameters of the bending waves, other than the beat frequency. Although this observation was reported in earlier work (Brokaw, 1966) and illustrated by a few representative photographs, the effect of thiourea on the parameters of the bending waves was not studied quantitatively to provide data for calculation of the energy expenditure for movement in various concentrations of thiourea.

This analysis has now been carried out, on photographs of spermatozoa from the same suspensions used to obtain the results presented in Fig. 5, and the results are presented in Table III. These results confirm the conclusion that thiourea has almost no effect on the size and shape parameters of the bending waves, so that both the active bending moment required to overcome the viscous resistance of the medium, and the energy expended per beat against the viscous resistance of the medium, decrease nearly in proportion to the decrease in beat frequency. The inhibition of active bending moment by thiourea is very similar to its inhibition of the generation of tension by muscle (Rüegg et al., 1963).

DISCUSSION

The work done against external viscous resistances by *Ciona* spermatozoa swimming at various viscosities was calculated from photographic measurements of wave parameters in a previous paper (Brokaw, 1966). These data are plotted in Fig. 6. The energy expenditure per beat appears to be relatively constant as the frequency is changed by increasing the viscosity. These calculations did not take into account the restriction of bending to the proximal part of the flagellum which occurs at high viscosities; if this factor is included, a relationship more nearly like that shown in Fig. 4 is obtained. The maintenance of a constant energy expenditure per beat and a constant radius of curvature by *Ciona* spermatozoa swimming at moderate viscosities implies

that the local bending and unbending events are the same at various viscosities, and that only their timing is altered to adjust the movement of the flagellum to increased viscous resistances. The observation that the oxygen uptake per beat is independent of changes in frequency caused by increased viscosity implies that the efficiency with which metabolism is coupled to movement is also constant.

If the parameters describing the size and shape of the flagellar bending waves remain constant, the energy expenditure for flagellar movement will be

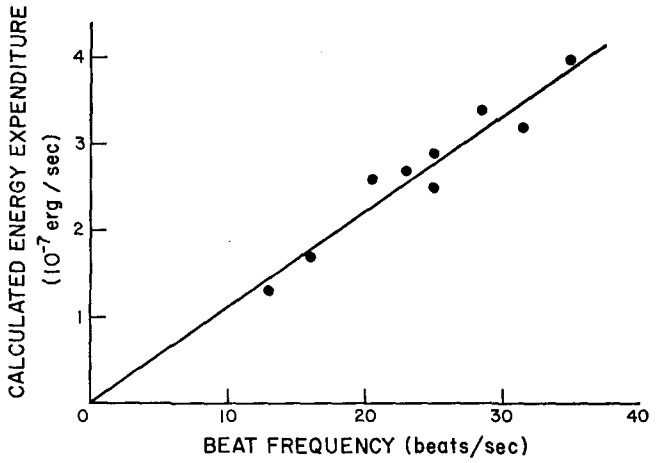

FIGURE 6. Calculated values of the energy expended by *Ciona* spermatozoa to overcome the viscous resistance of the medium, at various beat frequencies obtained by the addition of methyl cellulose. Data from Table II of Brokaw (1966). The line has been drawn through the origin with a slope equal to the average ratio between energy expenditure and beat frequency.

proportional to the square of the beat frequency (Brokaw, 1965), and the energy expenditure per beat will be proportional to the beat frequency. When *Ciona* spermatozoa are inhibited by thiourea, the energy expenditure per beat decreases almost in proportion to the decrease in beat frequency, as shown by the results in Table III, but the movement-coupled oxidative metabolism per beat remains nearly constant, as shown by the results in Fig. 5. Under these conditions, where the radius of curvature remains constant, the stoichiometry of mechanochemical coupling appears to remain constant, while the energy efficiency is variable. Measurements of the rates of ATP dephosphorylation by ATP-reactivated, glycerinated sea urchin spermatozoa under conditions where the radius of curvature of the bending waves was expected to vary with changes in viscosity have suggested that under these conditions also, the stoichiometry of mechanochemical coupling remains constant, while the energy efficiency varies (Brokaw and Benedict, 1968). However, this conclusion needs

further examination, since under certain conditions, such as the movement of *Chaetopterus* spermatozoa at high viscosities, the necessary increase in energy expenditure appears to be incompatible with constant stoichiometry (Brokaw, 1968).

In a study of the aerobic metabolism of bull spermatozoa at 35°C, Rikmenspoel (1965) found that the ratio between the energy expended by the spermatozoa in swimming against the viscous resistance of the medium, and their total oxygen consumption, was equivalent to 8.8 kcals per mole of O_2. Our results give a value for this ratio of 8.8–9.8 kcals per mole of O_2, depending on the estimate of the percentage of spermatozoa which were nonmotile. The similarity between these two results is interesting, because bull spermatozoa have a considerably more complex flagellar ultrastructure, a more complex three-dimensional pattern of flagellar movement, and a normal oxygen consumption per spermatozoon approximately 6 times that of *Ciona* spermatozoa.

Oxidative metabolism can generate 6⅓ moles of ATP per mole of oxygen (O_2) from glucose (Cantarow and Schepartz, 1967) or 5⅔ moles of ATP per mole of oxygen from saturated fatty acids (Stumpf and Barber, 1960). Although the endogenous substrate and the metabolic pathways of *Ciona* spermatozoa have not been determined, it seems reasonable to expect that they could generate about 6 moles of ATP per mole of oxygen, so that the movement-coupled metabolism of *Ciona* spermatozoa should be able to make available for movement 1.6×10^{-19} mole of ATP per beat per sperm. This does not take into consideration the possibility that other reactions coupled to metabolism might be involved in the generation or hydrolysis of ATP. The metabolism of *Ciona* spermatozoa does not appear to have been studied previously and there is no indication as to whether or not it is valid to compare their metabolism with that of sea urchin spermatozoa, which has been studied by many investigators. Sea urchin spermatozoa apparently have a respiratory quotient of 0.7, consistent with the oxidative metabolism of endogenous phospholipid (Mohri and Horiuchi, 1961). If this is also the case with *Ciona* spermatozoa, the molar ratio of 1.34 measured between acid production and oxygen consumption is too great to be explained by the evolution of CO_2, which will appear as $H^+ + HCO_3^-$ at this pH. Further study of the metabolism responsible for movement during the anaerobic phase of experiments such as that summarized by the records in Fig. 2 is also required. The rate of acid production during this anaerobic phase appears to be much lower than would be required to generate energy for the observed movement if these spermatozoa obtained energy under anaerobic conditions by glycolysis leading to the release of lactic acid.

The average length of *Ciona* sperm flagella, determined by measurements on some of the photographs used to obtain the results in Table III, is 47.5 $\mu \pm$ 0.4 (90% confidence limits; $n = 56$) μ. With the same assumptions used

previously for the slightly shorter flagella of sea urchin spermatozoa (Brokaw, 1967, 1968), each *Ciona* spermatozoon should contain 0.67–1.0 $\times$ 10^{-19} mole of the enzyme, dynein, which is believed to utilize ATP for flagellar movement. The movement-coupled oxidative metabolism of *Ciona* spermatozoa therefore could generate 1.6–2.5 molecules of ATP per dynein molecule per beat.

A minimum ATP requirement per beat can be estimated on the basis of the amount required to supply energy for movement against the viscous resistance of the medium, which amounts to 1.3 $\times$ 10^{-8} erg per beat, or 3.1 $\times$ 10^{-19} kcal per beat (Table III). Although under physiological conditions the free energy potentially available from the hydrolysis of ATP will probably be somewhat greater than the standard $-\Delta F^{\circ}$ of 7.0 kcal per mole (Huennekens and Whiteley, 1960), Carlson et al. (1963) found that in a muscle the work output per mole of creatine phosphate hydrolyzed was only 5.9 kcal, so that it seems unlikely that flagella would be able to utilize more than 6–7 kcal per mole of ATP. This would require them to use approximately 0.5 $\times$ 10^{-19} mole of ATP per beat per sperm, or about 0.5–0.7 molecule of ATP per beat per dynein molecule.

From the above arguments it appears that *Ciona* spermatozoa use somewhere between 0.5 and 2.5 molecules of ATP per dynein molecule per beat. Previous work involving the direct measurement of the rate of ATP dephosphorylation by glycerinated sea urchin spermatozoa suggested that the use of ATP amounted to 0.3–2.5 molecules of ATP per dynein molecule per beat. These estimates do not differ significantly. The uncertainty in the estimate obtained from glycerinated spermatozoa was largely the result of the uncertainty about the number of motile spermatozoa in the suspensions. This uncertainty has been reduced to a negligible level in the present study with live *Ciona* spermatozoa, but an additional source of uncertainty has been introduced by measuring metabolism, rather than the dephosphorylation of ATP. Nevertheless, since most of the uncertainty caused by variation in the number of motile spermatozoa has been eliminated, the measurements of *Ciona* sperm metabolism provide a more rigorous indication that there is a stoichiometric relation between ATP dephosphorylation and beat frequency as long as the radius of curvature of the bending waves remains constant, even if the energy expenditure per beat varies. There also appears to be a constant degree of coupling between oxygen uptake and ATP generation, but in these experiments we have not been able to obtain independent measures of stoichiometric constants for metabolism and for mechanochemical coupling in the flagella, in order to evaluate the hypothesis that mechanochemical coupling in flagella might involve the use of 1 ATP molecule per dynein molecule per beat.

If this hypothesis is correct, the metabolism of *Ciona* spermatozoa must

produce significantly less than the expected 6 moles of ATP per mole of O_2 consumed. If the metabolism of these spermatozoa does lead to the phosphorylation of approximately 6 moles of ATP per mole of O_2, and if an integral number of ATP molecules is used per dynein molecule per beat, this number must be 2, rather than 1.

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