Energy Conversion in Bull Sperm Flagella

ROBERT RIKMENSPOEL, SANDRA SINTON,
and JOHN J. JANICK

From the Department of Biological Sciences, State University of New York, Albany, New York 12203

ABSTRACT With the use of a specially developed incubation chamber the rates of motility, respiration, and fructolysis were measured simultaneously on semen samples. By inhibiting the respiration with antimycin A, and/or the fructolysis with 2-deoxyglucose, the rates of each of the two ATP-producing pathways could be reduced independently. In this way the ratio of the amount of free energy produced by respiration and by fructolysis could be varied at will from 1 to 0. In uninhibited preparations approximately 75% of the free energy derives from respiration, and 25% from fructolysis. By the use of the absolute rates of respiration, fructolysis, and motility, the efficiency of the conversion of free energy into hydrodynamic work was calculated. After correction for the decay of the preparation during the experiment, this conversion efficiency was found to be 30–45% lower for free energy from respiration than for free energy from fructolysis. The difference in distribution of the enzymes for fructolysis and respiration over the flagellum was ruled out as the cause of the efficiency difference. The respiration could be 70% inhibited by oligomycin. It is concluded that approximately one-third of the free energy from respiration is used for maintenance of the mitochondria.

INTRODUCTION

The problem of the energy involved in flagellar motion has received considerable attention. Spermatozoa have generally been used as experimental material, because it can be assumed that the energy produced by metabolism is used for the motile process only. RNA is found in sperm in minute (but variable) quantities (Leuchtenberger et al., 1956; Bhargava et al., 1959). Only traces of ribose have been reported to be present in mammalian sperm (Kubicek and Santavy, 1958). Synthesis of ribose, via the hexose monophosphate shunt, was found not to occur in ejaculated sperm (Wu et al., 1959). In view of these observations it is generally assumed that no RNA or protein synthesis takes place in spermatozoa.

The energy for sperm motility is produced via the pathways of oxidative phosphorylation and of fructolysis. The reserves of ATP in sperm have been found to be sufficient to maintain motility for a period of only approximately

30 sec (Rikmenspoel, 1965 *a*). This shows that the rate of motility is dependent on the instantaneous rate of ATP synthesis.

Many measurements of the rate of respiration of bull sperm have been made, resulting in an average value of 21 μl O_2 per hr consumed by 10^8 sperm (Mann, 1964). This is equivalent to 1.7×10^6 molecules of O_2 per sec per sperm. Rikmenspoel (1965 *a*) has compared the respiratory rate, R, directly to the number of *moving* sperm in the preparation: $R = 3.6 \times 10^6$ molecules O_2 per sec per sperm. Since diluted bull sperm usually contains 50–60% moving sperm, the two figures are in good agreement. Mohri et al. (1965) have reported tight coupling of oxidative phosphorylation in fragmented bull sperm tails. This leads to the conclusion that respiration yields approximately 2×10^7 molecules ATP per sec to a moving sperm.

The rate of fructolysis of bull sperm is about 2 mg fructose per hr for 10^9 sperm, equivalent to 1.8×10^6 molecules of fructose per sec per sperm (cf. the review by Mann, 1964). Rikmenspoel and Caputo (1966) found the saturated rate of fructolysis to be 3×10^6 molecules per sec per moving sperm. Since the breakdown of fructose to lactic acid yields 2 ATP, fructolysis yields 6×10^6 molecules ATP per sec per moving sperm.

It has been claimed (Mann, 1964; Nevo[1]) that respiration alone, as well as fructolysis alone, is able to drive the full motility of bull sperm. Pasteur effects in bull sperm are known to be quite small (Lardy and Phillips, 1941; Schmidt et al., 1959). The figures for ATP turnover in sperm which are driven by respiration or by fructolysis indicate a difference in efficiency of the conversion of ATP hydrolysis energy into motility for the two pathways.

The present paper describes experiments aimed at elucidating this apparent contradiction. The design of the experiment was to perform simultaneous measurements of motility, respiration, and rate of fructolysis on the same sperm preparation. By selective inhibition of either of the two metabolic pathways, the preparation could be studied when the motility of the sperm was driven by either respiration or fructolysis, or by both.

In addition the coupling of the oxidative phosphorylation was investigated by means of inhibition with oligomycin. The waveform of the sperm flagella in which either the fructolysis or the respiration was inhibited, was measured from cinemicrographs.

A preliminary communication of the results has been made recently (Rikmenspoel, 1969 *a*).

EXPERIMENTAL METHODS

Instrumentation

During experiments the diluted semen samples were incubated in a specially designed microchamber. The chamber, shown in Fig. 1, was a modification of the one described

[1] A. C. Nevo. Unpublished data.

previously (Rikmenspoel, 1964 *a*). A cuvette of 1 × 2 × 1 cm^3 holds the bulk sample of sperm. Through a slot in one sidewall the cuvette is connected to a slide, in which a channel is ground 50 μ deep and 8 mm wide. The motility of the sperm is observed in this slide. The rate of fructolysis is measured from the change in pH of the bulk sample due to lactic acid production. The respiration is measured by monitoring the oxygen tension with an oxygen electrode. The two electrodes are held in place in a nylon block which covers the top of the cuvette.

The pH electrode used was a combined electrode (GK2641C) of Radiometer Copenhagen, Denmark. The ground-glass, tapered part of the electrode fits directly in a tapered hole in the nylon cover of the cuvette.

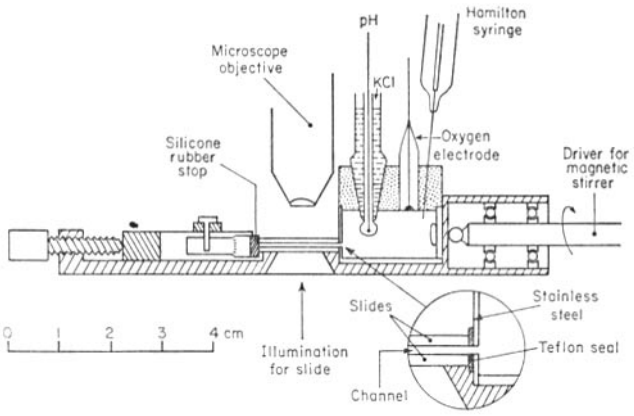

FIGURE 1. Incubation chamber used for simultaneous measurement of respiration, rate of fructolysis, and motility. The rubber stop at the left side of the slide can be opened to flush the slide and to draw a fresh sample from the cuvette.

Recording of the pH changes is made via the electrometer amplifier A4311 of Radiometer Copenhagen. Fig. 2 shows the circuit diagram. By increasing the RC time of the detector circuit of the A4311, the indication time was raised to 2.5 sec. This decreases the noise to acceptable levels.

This high ohmic recording system is very sensitive to interference. With appropriate grounding technique (see Fig. 2) the rms noise is equivalent to approximately 5 × 10^{-4} pH units. 1 hr after switching on the apparatus and the temperature control, the drift is less than a few times 10^{-4} pH per min.

The medium in which the sperm were suspended had a buffer capacity of 1 mM phosphate plus the capacity of the protein (see below), in all equivalent to approximately 2 mM phosphate. In these circumstances 1 μg of lactic acid (LA) per ml causes a pH drop of approximately 1 × 10^{-2} pH. By making a series of LA additions it was established that the recorded deflection was linear with LA addition in the range up to 50 μg LA per ml (starting pH 7.2, final pH 6.7). In the sperm samples in actual experiments, the total LA production is of the order of 40 μg LA in 40 min (1 μg LA per min). Sensitivity and range of the system are thus satisfactory (compare Figs. 4 and 7).

After every experiment the pH measurement system was calibrated by the addition of a known amount of LA. This is necessary as the sperm contain an unknown and variable amount of buffer capacity.

In a sperm sample which shows respiration, lactic acid disappears from the sample into the Krebs cycle. In order to avoid interference of this effect with the pH change recording 2 mM sodium lactate was always added as a carrier in the medium. This

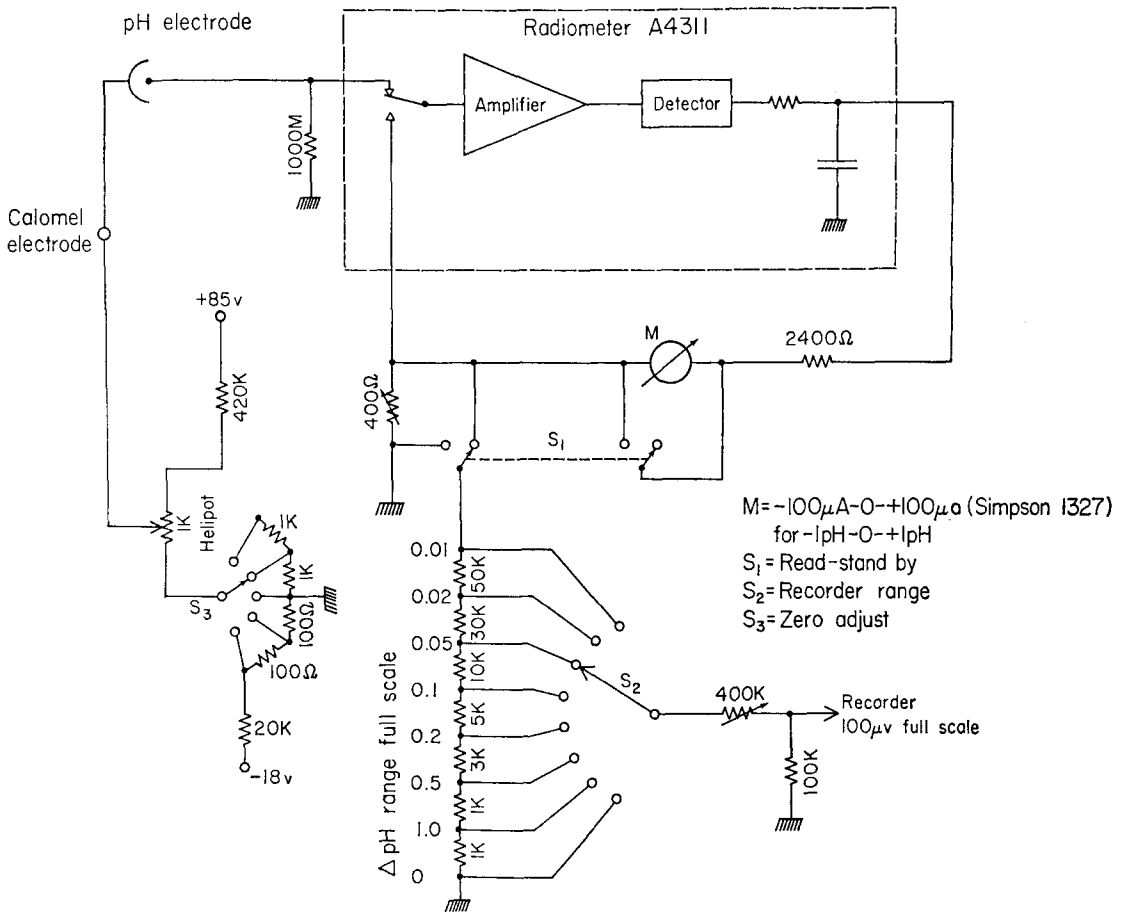

FIGURE 2. Wiring diagram of the pH recording apparatus. The whole circuit is grounded through the KCl reference electrode.

largely suffices to separate the pH change measurement completely from respiratory effects.

During respiration, one CO_2 is produced for every O_2 consumed. During all experiments the respiration was measured (see below). After calibration of the buffer capacity of the medium, the part due to CO_2 production was subtracted from the total pH change, to arrive at the LA production. This correction was usually of the order of 10% of the pH change.

For oxygen measurement a small oxygen electrode was constructed, as shown in Fig. 3. The electrode is a scaled-down version of the electrode described by Clark (see Davies, 1962). The total outside diameter is 6 mm. The electrode is operated in the usual way at −0.6 v. Noise and drift of the electrode are slightly better than those reported with the Clark electrode (Rikmenspoel, 1964 *a*) due to its present location closer to the magnetic stirrer.

Motility Measurements

Motility of the sperm was measured with the photoelectric-electronic apparatus previously described (Rikmenspoel, 1964 *b*). In this device a photomultiplier observes the intensity of the light originating from a small area (15 μ diameter) in the slide (see Fig. 1). Whenever a sperm passes this area a light signal occurs. The number of

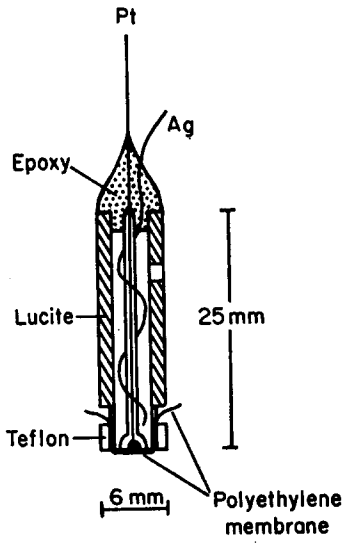

FIGURE 3. Cross-section of the microoxygen electrode for use with the chamber shown in Fig. 1.

sperm, N, which pass over the area per second is equal to $N = c.V.q.d.$, where c is the concentration of moving sperm, V the average velocity of the moving sperm, d is the depth of the slide (5×10^{-3} cm), and q is a calibration constant ($q = 2.8 \times 10^{-3}$ cm).

Each of the light signals initiated by a passing sperm is fed into an electronic analyzer, which determines the velocity of the sperm from the shape of the signal. During a measuring time of e.g. 3 min, signals from many sperm are received. Averaging these signals then yields the average velocity of the sperm in the sample. The apparatus works well in the range 50 μ per sec $< V <$ 150 μ per sec. In those cases in which $V <$ 50 μ per sec, no reliable measurement of V can be obtained. The details of the method have been extensively discussed previously (Rikmenspoel, 1957, 1964 *a*).

Thus the primary information obtained with the device is the "amount of motility" $cV = N/qd$. For samples with $V >$ 50 μ per sec, values for the average velocity, V, and also for the concentration, c, of moving sperm are obtained separately.

For each motility measurement a fresh sample was always drawn into the slide, to insure that the conditions in the slide corresponded to those in the bulk sample.

Kinetic measurements of sperm motility upon the addition of inhibitor were done with the photographic technique described previously (Rikmenspoel, 1957, 1965 *a*).

Cinemicrographs at a speed of 200 frames per sec were made at the Research Institute for Animal Husbandry at Zeist, Holland. The apparatus, described previously in detail (Eykhout and Rikmenspoel, 1960), was made available through the kindness of Mr. Ed Blokhuis.

Sperm Preparations

Semen from Holstein bulls was generously provided by the Eastern Artificial Insemination Cooperative in Ithaca, New York.

The semen was diluted and washed in an egg yolk containing diluent as described previously (Rikmenspoel, 1965 *a*). The only modification of the diluent was the use of 1 mM phosphate buffer instead of 10 mM, and the addition of 250 μg fructose per ml and of 2 mM Na lactate.

For experiments the spermatozoa were diluted to a final concentration of approximately 3×10^7 sperm per ml.

All experiments were performed at a temperature of 37°C.

Inhibitors

Fructolysis was inhibited with 2 deoxyglucose, obtained from Calbiochem in Los Angeles, Calif. Respiration was inhibited by means of antimycin A, generously provided by Ayerst Laboratories in Rouses Point, New York. Oligomycin (containing 15% oligomycin A and 85% B) was obtained from Sigma Chemical Co. in St. Louis, Mo. The antimycin A and the oligomycin used for experiments were dissolved in ethanol. Total addition of ethanol to the preparations never exceeded 5 μl per ml.

RESULTS

Control Experiments

A number of sperm samples were incubated at 37°C, as described above and the rates of fructolysis and respiration were recorded for approximately 40 min. At intervals of approximately 10 min a fresh sample was drawn into the slide, and a motility measurement was made. Fig. 4 shows the recordings and motility figures for a typical experiment.

Fig. 5 summarizes the results of six experiments. The values for respiration, rate of fructolysis, and concentration of moving cells are plotted in Fig. 5 relative to the value for these quantities at the moment ($t = 0$) the incubation started. These values for $t = 0$ were obtained by extrapolating the data back to $t = 0$ as described previously (Rikmenspoel, 1965 *a*).

The average velocity, V, of the sperm samples is plotted as the absolute value in Fig. 5.

Table I shows the values of the rate of respiration and of fructolysis per

sperm, during the first 10 min in incubation averaged over the six experiments. The rate of fructolysis per moving sperm is equal to 2.7×10^6 molecules per sec. This agrees very well with the figure obtained from direct chemical analysis (2.9×10^6 molecules per sec [Rikmenspoel and Caputo, 1966]) and confirms that the pH change recording gives an accurate measure of the fructose utilization.

Also shown in Fig. 5 is the change in the efficiency of the sperm motility,

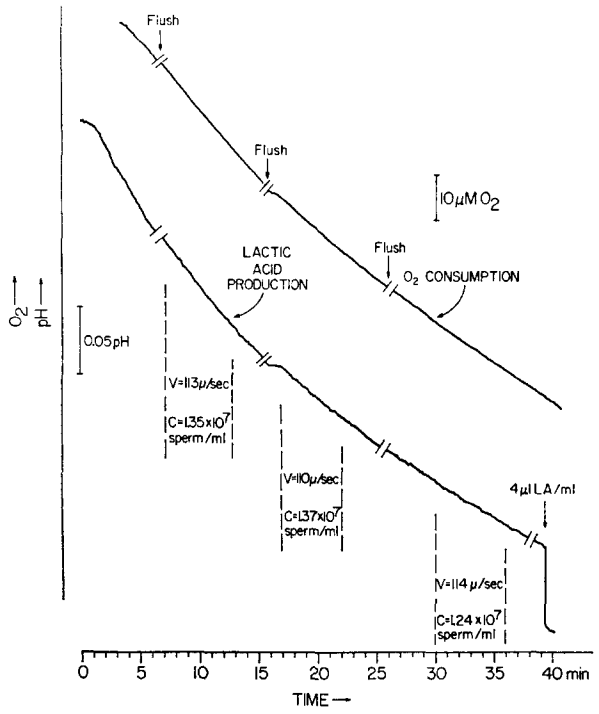

FIGURE 4. Tracings of the oxygen electrode and pH electrode recordings during a control experiment. The values for three consecutive motility measurements are shown in the figure. At the end of the experiment the buffer capacity of the preparation is calibrated by means of a small addition of lactic acid.

as a function of the incubation time of the sample. As is shown under the heading "Efficiency measurements" in this section and in the Discussion, the amount of work performed by the sperm during motility is probably proportional to the forward velocity of the sperm. For this reason the efficiency is expressed as the forward velocity per free energy of the reaction driving the motility. The free energy is expressed as the number of high energy bonds ($\sim$) being produced by the respiration plus the fructolysis. It is assumed that 6 $\sim$ are produced per molecule of O_2 and 2 $\sim$ per molecule of fructose consumed.

Fig. 5 shows that the respiration, the rate of fructolysis, and the number of moving sperm decrease during incubation by 1–2% per min. This has been observed previously (Rikmenspoel, 1965; Freund et al., 1957, 1959). The average velocity and the efficiency of the motility increase during incubation, however.

The most likely interpretation of these effects is that the sperm with the lower initial velocities die most rapidly.

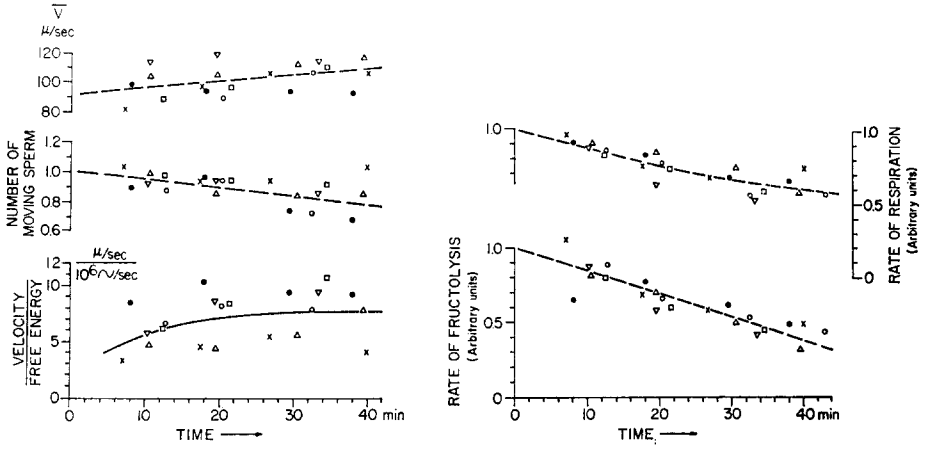

FIGURE 5. Change in respiration, fructolysis, and motility during 40 min incubation of six sperm samples. The lower left part of the figure shows the change in "efficiency" of conversion of free energy into motility by the sperm, during the incubation.

TABLE I

AVERAGE AND STANDARD DEVIATION OF RESPIRATION AND FRUCTOSE UTILIZATION PER MOVING SPERM DURING THE INITIAL 10 MIN OF INCUBATION

Number of experiments, six. Respiration provides 72%, fructolysis, 28% of the free energy made available to the sperm.

	Respiration		Fructolysis	
Average velocity	Molecules O_2/sec	No. of high energy bonds/sec	Molecules fructose/sec	No. of high energy bonds/sec
95±15 μ/sec	2.3±1.0×10⁶	13.8±6.0×10⁶	2.7±1.5×10⁶	5.4±3.0×10⁶

Shortly after the start of the incubation the efficiency of the sperm motility is equal to 4.4 μ per sec per 10^6 ~ per sec. This is in excellent agreement with the value of 4.6 μ per sec per 10^6 ~ per sec reported in an earlier paper (Rikmenspoel, 1965 *a*). After some 20 min the efficiency reaches a constant value of 7.8 μ per sec per 10^6 ~ per sec. The experiments in the section on Efficiency measurements were corrected for this change during incubation.

Action of Individual Inhibitors

ANTIMYCIN A When antimycin A is added to a sperm preparation the respiration is inhibited after a delay of several minutes. The membrane of the sperm obviously forms a penetration barrier for this antibiotic. This finding conflicts with the report of Gonse (1962) that the addition of antimycin A "immediately" inhibited motility and respiration. We cannot offer an explanation for our different observations.

The final percentage of inhibition is dependent on the amount of antimycin A added, however. At a concentration of antimycin A of 1.2×10^{-7} M, respiration is inhibited approximately 50%; at a concentration of $> 6 \times 10^{-7}$ M, 95 ± 5% inhibition occurs.

The time course for the effect of antimycin A inhibition upon the motility of the sperm was measured by means of the photographic technique. The fructolysis of the sperm in these experiments had been cut off completely by

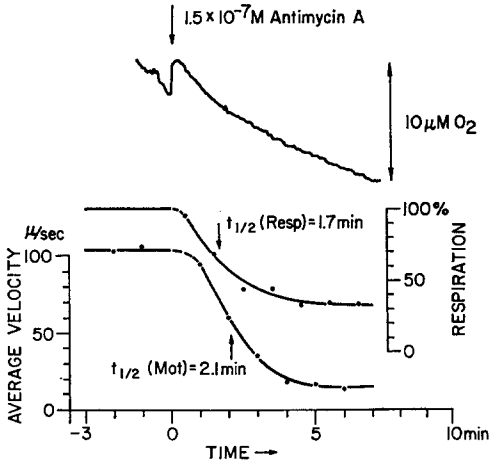

FIGURE 6. Kinetics of respiration and average velocity of a sperm preparation following addition of antimycin A.

2 mM deoxyglucose. After the sperm respiration had reached a steady state, 1.5×10^{-7} M antimycin A was added. The bulk sample was stirred for 20 sec, and a fresh sample was drawn in the slide. Track photographs were made of the sperm starting 1 min after the addition of antimycin A. The results of a typical experiment are shown in Fig. 6; the delay of motility lags behind the respiration delay.

Table II summarizes the results of four experiments. The average value of the time at which the inhibition is 50% of its final value ($t_{1/2}$) is 0.7 min longer for the motility than for the respiration. This indicates that the ATP reserve in the sperm is sufficient to maintain motility for approximately 40 sec, when respiration is the source of energy. This value is in good agreement with the figure of 30 sec reported previously for inhibition by means of amytal (Rikmenspoel, 1965 *a*).

DEOXYGLUCOSE Sperm samples were incubated as usual, and respiration was cut off completely by the addition of 10^{-6} M antimycin A. Upon addition of deoxyglucose (DOG) an inhibition of the rate of fructolysis is indeed observed. The relation between the amount of inhibition and the DOG concentration shows the inhibition to be competitive. At the fructose concentration used, 250 μg per ml (= 1.4 mM), half-inhibition is reached

TABLE II

KINETICS OF ANTIMYCIN A INHIBITION OF RESPIRATION AND MOTILITY

Fructolysis was abolished in these preparations by the addition of 2 mM DOG. Antimycin A concentration, 1.5×10^{-7} M.

Experiment No.	Average velocity		Respiration		Delay time	
	V Initial	V Inhibited	Initial	Inhibited	$t_{1/2}$ for respiration	$t_{1/2}$ for velocity
	μ/sec	μ/sec	μM O_2/min	μM O_2/min	min	min
1	99	25	3.0	0.7	2.7	4.8
2	96	30	3.4	1.1	2.6	3.1
3	102	14	3.0	1.1	1.7	2.1
4	86	40	2.4	1.0	2.0	1.9
Average					2.3±0.4	3.0±0.5

TABLE III

KINETICS OF DEOXYGLUCOSE (DOG) INHIBITION OF FRUCTOLYSIS AND MOTILITY

Respiration of all samples was inhibited by the addition of 10^{-6} M antimycin A. The characteristic times shown are the times, after the addition of 2 mM DOG, at which 50% of the final effect was obtained.

Rate of fructolysis		Motility	
No. of experiments	$t_{1/2}$ (Average ± SD)	No. of experiments	$t_{1/2}$ (Average ± SD)
	sec		sec
4	17±7	6	19±6

with 0.2 mM DOG. This value is much lower than the one reported by Salisbury and Lodge (1962).

The K_M for fructose in bull sperm is 1.04 mM (Rikmenspoel and Caputo, 1966). In our present experiments the rate of fructolysis was therefore approximately 75% of the saturated rate.

The kinetics of the inhibition of the motility when driven by fructolysis was measured as described above for antimycin A inhibition. The kinetics of inhibition of the fructolysis by DOG could not be measured in this way. When a fresh sperm sample is drawn into the slide upon DOG inhibition,

the pH electrode becomes unstable, with a recovery period of approximately 10 sec. The DOG kinetics was therefore measured separately. The pH electrode was mounted in a glass cuvette described previously (Rikmenspoel, 1969 *b*), which contained the sperm suspension.

Table III summarizes the results of four experiments for measuring the kinetics of the fructolysis, and six experiments for that of the motility. The motility decays in phase with the fructolysis upon DOG addition, and the ATP reserve is obviously small. The sperm motility when driven by fructolysis only, is, therefore, energy-limited.

OLIGOMYCIN The action of oligomycin on sperm motility and respiration was measured in the same way as was the effect of antimycin A. Fructolysis of the preparations was always blocked by means of 2 mM DOG.

TABLE IV

EFFECT OF OLIGOMYCIN ON RESPIRATION AND MOTILITY OF BULL SPERM

Fructolysis in all preparations was abolished by inhibition with 2 mM DOG. The delay times shown are the times at which 50% of the final inhibitory effect was obtained.

Oligomycin concentration	No. of experiments	$t_{1/2}$ (Average ± SD)	Final respiration (Average ± SD)	Final average velocity
M		*min*	%	*μ/sec*
10^{-7}	6	10.1±2.2	30±10	
3×10^{-7}	6	3.2±0.4	28±13	
10^{-6}	4	2.1±0.5	33±8	
3×10^{-7}	4		32±9	0*

* Judged by visual observation.

If oligomycin is added to a concentration of 1×10^{-7} M, the steady-state level of inhibition is reached only after approximately 15 min. Therefore, oligomycin penetrates the sperm more slowly than antimycin. Raising the oligomycin concentration reduces the delay time, but the percentage inhibition is not increased. It is, therefore, not possible to obtain submaximal inhibition by means of this antibiotic. Table IV shows the delay times at which the inhibition has reached its half-value, for three different oligomycin concentrations.

For all the experiments shown in Table IV the average value of the respiration after oligomycin inhibition was 30.5 ± 8.5% (SD).

In the presence of oligomycin inhibition of the respiration, the motility of the sperm always reached a final value of 0. This shows that motility is powered by ATP only, and not by one of the intermediate compounds of the oxidative phosphorylation. It also shows that ADP phosphorylation is only

partially coupled to respiration in the sperm. This point is further elaborated in the Discussion.

DINITROPHENOL No reproducible results could be obtained with 2,4-dinitrophenol (DNP) in our sperm preparations. A short burst of extra respiration was sometimes detectable after the addition of DNP. Sperm motility was not affected by DNP in DOG-inhibited sperm preparations. We have concluded that DNP is not a practical tool with which to investigate oxidative phosphorylation in our sperm preparations. Since DNP apparently has

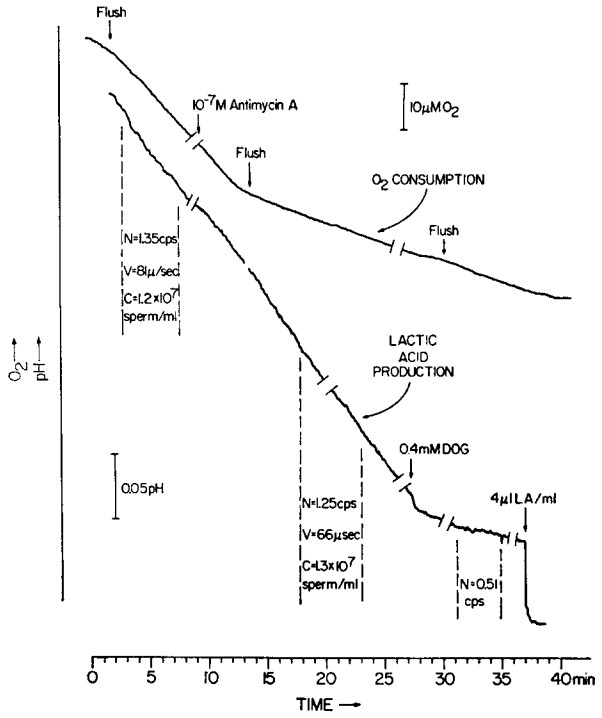

FIGURE 7. Tracing of oxygen electrode and recording of pH shift of a sperm preparation which was inhibited first with antimycin A, then with deoxyglucose. The values for the motility of the sperm are shown in the figure.

several effects other than uncoupling, this finding is not too surprising (Gonse, 1962; Kraayenhof and van Dam, 1969; Wilson and Merz, 1969).

Efficiency Measurements

A large humber of preparations were incubated in the chamber, and the rates of metabolism and motility were measured. Following this, either the respiration was partially inhibited by antimycin A, or the fructolysis by DOG. Varying degrees of inhibition were used in different experiments.

The amount of inhibition effected in a preparation cannot, of course, be

exactly predicted from the amount of inhibitor added. In a number of experiments, the respiration was inhibited by more than 85%. In these cases the remaining respiration is of the same order as the drift effects of the oxygen electrode ($\approx$ 0.15 μM O_2 per min). The respiration was taken to be 0 in these cases.

In uninhibited preparations roughly three-quarters of the free energy for the sperm is suppled by respiration. When respiratory inhibition is applied, the fraction of the free energy which is supplied by respiration becomes less. When fructolysis is inhibited, it becomes more.

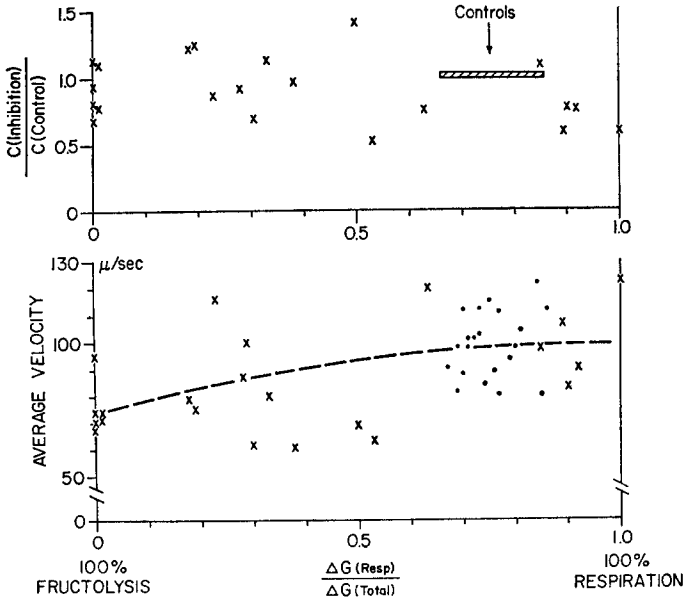

FIGURE 8. Average velocity and number of moving sperm upon partial inhibition of either respiration or fructolysis. Solid circles, uninhibited preparations. After inhibition the ratio of the rates of respiration to fructolysis changes, and the sample becomes displaced along the horizontal axis (×).

Subsequently, the pathway left uninhibited was itself subjected to inhibition. This brings the fraction (respiratory free energy/total free energy) back towards its original value. The rates of both pathways, and of the motility are, of course, greatly reduced after this second inhibition.

Fig. 7 shows tracings of the oxygen electrode and pH recordings during a typical experiment. The results of the motility measurements taken initially, after respiratory inhibition and after subsequent inhibition of the fructolysis are inserted in Fig. 7.

Fig. 8 shows the data obtained for average velocity and number of moving cells of the uninhibited preparation and after the first inhibition had been

applied. The data are plotted as a function of the ratio $\Delta G(\text{resp})/\Delta G(\text{resp} + \text{fruct})$. It can be seen that the velocity of the sperm is lower when fructolysis is the only pathway driving the sperm. This is in agreement with the kinetic data on DOG inhibition, which showed the fructolysis-driven sperm to be energy-limited.

As shown in Fig. 8 the number of moving sperm is not dramatically changed after the first inhibition. This indicates that only the rate of the motility of the sperm is reduced, but that no "damage" is inflicted.

After the second inhibition the velocity of the sperm usually drops well below 50 μ per sec, and consequently the velocity, V, and the concentration, c, of moving sperm cannot be measured separately (see the section on Experimental Methods). The number of cells counted photoelectrically, $N = cVqd$, is, of course, always obtained, even at very reduced motility rates. The rate of respiration, $R = \alpha \times c$, where α is the O_2 consumption per sperm; the rate of fructolysis, $F = \beta \times c$. The total number of high energy bonds ($\sim$) produced in the preparation is $6R + 2F = c\,(6\alpha + 2\beta)$ in which R, F, α, and β are taken to be expressed in molecules per second. The free energy produced per sperm per second, $\Delta G = 6\alpha + 2\beta$, in which ΔG is expressed in number of $\sim$ per second. The efficiency, $V/\Delta G$, can be found as

$$\frac{V}{\Delta G} = \frac{N/cqd}{(6R + 2F)/c} = \frac{N}{6R + 2F} \cdot \frac{1}{qd} \qquad (1)$$

Equation (1) shows that the average velocity of the sperm per unit of free energy can be obtained with our apparatus without an actual measurement of the velocity.

In Fig. 9 the data for $V/\Delta G$ are plotted against the ratio of respiration to total free energy available to the sperm. It can be seen that the quotient, $V/\Delta G$, is larger when fructolysis provides the greater fraction of the free energy. The value for $V/\Delta G$ is independent of the previous history of the preparation, however. This is shown by the fact that the points for the second inhibition follow the same trend as the points for the controls and the first inhibition. The insert in Fig. 9 shows the average and standard error of $V/\Delta G$ for all points, divided into five groups.

The control (uninhibited) values in Fig. 9 were obtained after approximately 8 min incubation of the samples. The value for the average of $V/\Delta G$ for the controls in the insert of Fig. 9 was corrected for the increase in this quantity during incubation, as shown in Fig. 5. This makes this average comparable to the averages in the other groups of points which were generally taken from 20 to 35 min after the start of the incubation. During this period $V/\Delta G$ has reached a constant value (see Fig. 5) and no correction is necessary.

It should be noted that the rates for the triangular points in Fig. 9 are from 8 to 15 times lower than the rates for the uninhibited preparations. The ratio, $V/\Delta G$, is thus constant over a wide range of velocity, and it is dependent only on the ratio of fructolysis/respiration.

Cinemicrographic Measurements

Cinemicrographs were made of sperm samples in which either the respira-

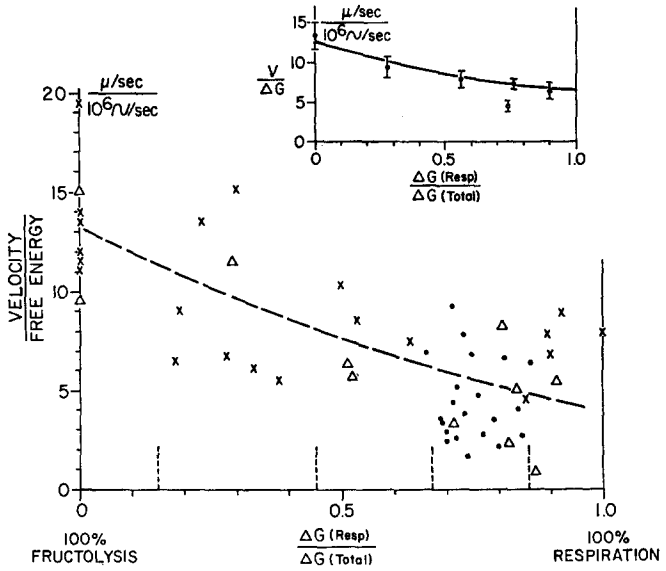

FIGURE 9. Average velocity of the sperm per 10^6 high energy bonds produced per second. The horizontal axis gives the fraction of the total number of high energy bonds which are produced by the respiration. Solid circles, control values, for uninhibited preparations. ×, values after first inhibition. Open triangles, values after the second inhibition (of the complementary pathway). The insert shows the averages of the points grouped around the values for ΔG(resp)/ΔG(total) of 0, 0.28, 0.56, 0.76, and 0.90 respectively (the limits of these groups are indicated in the main figure). The average value of $V/\Delta G$ for the uninhibited preparations in the insert at ΔG(resp)/ΔG(total) = 0.76 has been corrected for the change in the preparations during incubation, the open and solid circles representing the uncorrected and corrected values, respectively. The vertical bars in the insert are standard errors. The curves drawn in the main figure and in the insert are for heuristic purpose only.

tion was inhibited with 2×10^{-6} M antimycin A, or in which the fructolysis was abolished by 5 mM DOG. At the laboratory in Holland no facilities were available for measuring metabolic rates. The amount of inhibitor used was, therefore, chosen on the generous side. The effectiveness of the inhibition was assured from the fact that when the remaining pathway was inhibited, the motility of the sperm fell to zero.

Respiration takes place in the mitochondria of the sperm, close to the head. Fructolysis is divided over the entire tail length (Mohri et al., 1965). The contractile elements are distributed along the flagellum (Nelson, 1962; Rikmenspoel, 1965 *b*). The obvious question arises whether respiratory ATP can reach the more distal part of the tail.

If respiratory ATP could not diffuse or be transported into the distal tail parts, the flagellar wave would be reduced in amplitude as it travels distally. It has been shown that a free running, passive wave will dampen out long before it reaches the end of the flagellum (Machin, 1958; Rikmenspoel, 1965 *b*).

Fig. 10 shows the amplitude of the tail wave as a function of the distance along the tail. The data are the averages for 10 cells driven by respiration and 7 cells driven by fructolysis. No difference is apparent between the two conditions.

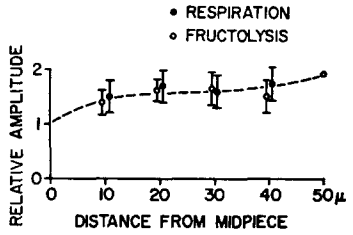

FIGURE 10. Amplitude of the tail wave of sperm driven by either respiration or by fructolysis, as a function of the distance along the flagellum. The amplitudes are plotted relative to the amplitude at the midpiece-tail junction. The data are averages of 10 cells driven by respiration and 7 cells driven by fructolysis.

DISCUSSION

The amount of hydrodynamic work, W_{hyd}, performed by a moving sperm has been calculated by Taylor (1952):

$$W_{hyd} = \frac{4\pi^3 \eta L f^2 b^2}{0.62 - \ln\left(\frac{2\pi\rho}{\lambda}\right)} \qquad (2)$$

where η is the viscosity of the medium (0.01 poise [Rikmenspoel, 1957]), L is the length of the flagellum ($L = 60\ \mu = 6 \times 10^{-3}$ cm), ρ is the radius of the cross-section of the flagellum ($\rho = 0.2\ \mu = 2 \times 10^{-5}$ cm), λ is the wavelength of the tail wave ($\lambda \approx 35\ \mu = 3.5 \times 10^{-3}$ cm), f is the frequency, and b the amplitude of the tail wave. Substituting the values for η, L, ρ, and λ into equation (2) yields:

$$W_{hyd} = 1.9 \times 10^{-3} f^2 b^2 \text{ erg per sec} \qquad (3)$$

The amount of energy needed for the elastic deformation of the flagellum may be taken, according to Machin (1958), as one-third of the hydrodynamic work. The total work, W, performed by a motile sperm then becomes

$$W = 2.6 \times 10^{-3} f^2 b^2 \text{ erg per sec} \quad (4)$$

The forward velocity, V, of a sperm is given by $V = \xi f b^2 / \lambda$, where ξ is a dimensionless constant (Rikmenspoel, 1966); for bull sperm $\xi = 2.1$. With $\lambda = 3.5 \times 10^{-3}$ cm:

$$V = 6 \times 10^2 f b^2 \text{ cm per sec} \quad (5)$$

In equations (4) and (5), f and b are expressed in sec^{-1} and in centimeters, respectively.

When a sperm sample is inhibited, the forward velocity of the sperm decreases. This can be due to the fact that either the amplitude, b, of the tail waves is reduced (while the frequency remains relatively constant), or the frequency is reduced, while b stays the same, or because both f and b decrease.

In the first case f in equations (4) and (5) can be treated as a constant, and W (the total work performed by the sperm) can be taken as proportional to V, the average velocity of the sperm under the various conditions.

In the second case b in equations (4) and (5) can be treated as a constant. W would now be proportional to the square of the average velocity, V, of the sperm.

When both f and b are reduced upon inhibition of the sperm, the effect will be between the two extremes mentioned above, or $W \propto V^p$ where $1 < p < 2$.

Brokaw and Benedict (1968) have reported that in *Ciona* sperm the efficiency remains constant when the sperm motility is reduced by increased viscosity. Motility reduction by metabolic inhibition resembles that by viscosity increase in that both change only the rates of the processes, and do not damage the motile apparatus. It would appear, therefore, that $W \propto V$ and constant efficiency applies in our case.

In the case the motility of *Ciona* sperm was inhibited with thiourea, Brokaw and Benedict (1968) concluded that the efficiency was reduced after inhibition, however.

A final answer to the question can only be given by measurements of the frequencies and amplitudes of the tail wave of the sperm under the various conditions. In view of the very large amount of work involved in these measurements, they have not been undertaken within the scope of this paper.

However, in order to check for the influence of the rate of motility, the data have been replotted in two ways. Fig. 11 shows the value of $V^2/\Delta G$ as

a function of the ratio $\Delta G(\text{resp})/\Delta G(\text{total})$. In this figure only data can be shown for which separate values for V and c (the concentration of moving cells) were obtained. This means in practice that only three points could be plotted which represent values after the second inhibition was applied. However, the trend toward a higher "efficiency" for small values of $\Delta G(\text{resp})/\Delta G(\text{total})$ is clearly present in Fig. 11.

After the various steps in the inhibition, the rate of motility of the sperm samples is, of course, drastically changed. To show the influence of this factor

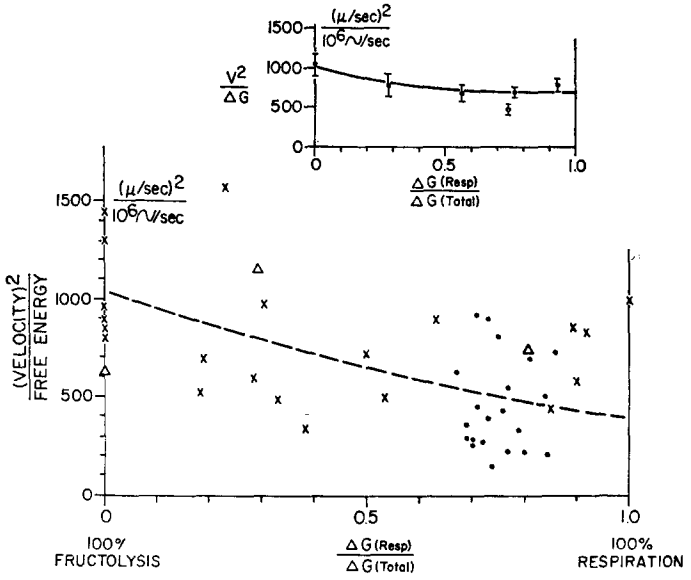

FIGURE 11. Ratio of the square of the velocity of sperm to number of high energy bonds produced. The horizontal axis represents the fraction of the total number of high energy bonds which is produced by respiration. Solid circles, uninhibited preparations. ×, after first inhibition. In only three cases an independent value for V was obtained after the second inhibition (△). The insert is derived by averaging as in Fig. 9.

on the efficiency, the value of $V/\Delta G$ for our samples has been plotted in Fig. 12 as a function of the average velocity, V, of the sperm. Only the data for which a separate measurement of V was made could be accurately plotted in Fig. 12. Generally this applies to the uninhibited state, and to the situation after the first inhibition, when $V > 50$ μ per sec. After the second inhibition, when all rates are low, the velocity of the sperm is usually about 20 μ per sec, as judged by visual observation. For this reason the points in Fig. 12 which represent these samples after the second inhibition have been grouped around $V = 20$ μ per sec.

The group of triangles in Fig. 12 at $V = 20$ μ per sec all represent a situation in which $\Delta G(\text{resp})/\Delta G(\text{total})$ is > 0.5. They are, therefore, comparable

to the uninhibited preparations for which $\Delta G(\text{resp})/\Delta G(\text{total})$ is > 0.65. Fig. 12 shows that the efficiency, $V/\Delta G$, is not much different for these two sets of points, even though the rate of motility of the uninhibited preparations is up to a factor of 10 higher than that for the group of twice inhibited preparations.

Almost all the other points in Fig. 12 refer to a ratio, $\Delta G(\text{resp})/\Delta G(\text{total}) < 0.5$, and reveal the higher efficiency in that situation.

Figs. 9, 11, and 12 show that the difference in efficiency is not related to the rate of motility, but to the ratio of fructolysis to respiration. The conversion of free energy from fructolysis has a higher efficiency than that from respiration.

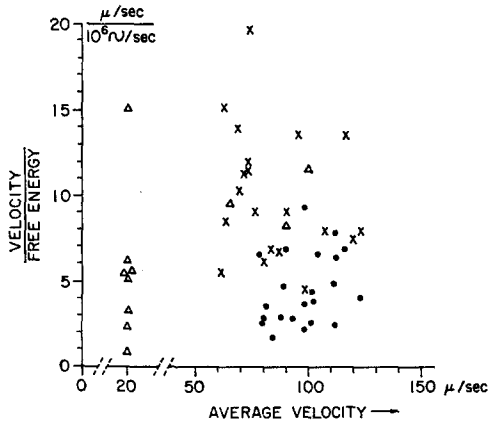

FIGURE 12. Efficiency, $V/\Delta G$, of sperm preparations as a function of the average velocity of the sperm. Solid circles, uninhibited preparations. X, after first inhibition. △ after second inhibition. The velocities of ≈ 20 μ per sec are based on a visual estimate (see text).

If the efficiency for fructolysis is written as E_F, the efficiency E_R for respiration is

$$E_R = (0.53 \text{ to } 0.67) \times E_F \tag{6}$$

dependent on whether $W \propto V$ or $W \propto V^2$. This means that approximately 30–45% of the respiratory free energy is channeled off to energy-requiring pathways other than motility.

In trying to explain this difference in efficiency the first aspect which is apparent, is the difference in localization of the enzymes for respiration and for fructolysis as was explained in the section entitled Cinemicrographic measurements. The results of the film data as shown in Fig. 10 indicate that ATP generated by respiration is indeed able to power the distal contractile elements. However, this does not in itself show that no energy is used for transporting the ATP to the distal part of the flagellum.

A detailed calculation of the diffusion of ATP from the midpiece through the flagellum will be published elsewhere (Nevo and Rikmenspoel, 1969).

In that paper it is shown that ordinary diffusion is amply sufficient to transport ATP to the distal parts of the flagellum.

A rough insight into the importance of diffusion of ATP can be obtained as follows: Katchalsky and Oster[2] have shown that if a substance diffuses through a cylindrical tube, from which it is continuously removed by a chemical reaction (as in our case by the motility ATPase), the concentration of the substance will decrease along the tube with a characteristic length, Λ, and

$$\Lambda^2 = 2Dt_c \tag{7}$$

where D is the diffusion coefficient, and t_c is the relaxation time of the reaction. For ATP in the flagellum $D = 4 \times 10^{-6}$ cm^2 sec^{-1} (Bowen and Martin, 1964; Nevo and Rikmenspoel, 1969). For the relaxation time the time lag between the respiratory cutoff and the motility cutoff as measured by the kinetic experiments should be taken as $t_c = 40$ sec. This yields a value

$$\Lambda = 180\ \mu$$

or three times the length of the flagellum. We may conclude, as was also conclusively shown in the paper by Nevo and Rikmenspoel, that normal, passive diffusion maintains an adequate concentration of ATP into the distal part of the flagellum.

ATP and ADP in respiring sperm are present in approximately equal amounts (Newton and Rothschild, 1962; Bomstein and Streberl, 1959). The diffusion calculation given above shows that the ATP concentration decreases only slowly towards the distal end of the tail. This means that large local variations in ATP concentration (and by analogy also in ADP concentration) along the flagellum can be excluded. The influence of the RT ln {[ATP]/[ADP]} term in the free energy of hydrolysis of ATP is therefore small.

In sperm in which only fructolysis is operative no gradients of ATP and ADP concentration will be present since the enzymes for that pathway are distributed all along the flagellum. The kinetic experiments have shown that in these sperm the ATP content is "low." The free energy of ATP hydrolysis in this situation thus is lower than when respiration is taking place (ATP concentration "high"). A quantitative estimate of the correction for the free energy of ATP when only fructolysis is present is unfortunately not possible, as no values are available for the ATP and ADP concentrations. Since the fructolysis is able to drive almost the full motility, the correction is probably small, however.

The arguments in the two preceding paragraphs show that the difference

[2] Katchalsky, A., and G. Oster. To be published.

in the efficiency is not due to a higher free energy yield per ATP in the case of fructolysis. On the contrary, the available evidence points to a lower free energy for ATP produced by fructolysis, compared to ATP produced by respiration.

In the foregoing it has been shown that the difference between E_F and E_R is not due to the model used for the work calculation, not due to the difference in rate between the sperm driven by fructolysis or respiration and also not due to the difference in location between fructolysis and respiration in the flagellum. The difference between E_F and E_R, therefore, has to be inherent in the difference of the processes for generating the ATP. It is known (Harris et al., 1966) that mitochondria can transport ions (K, Na, Ca) against concentration gradients. This transport is not driven by ATP hydrolysis, but by hydrolysis of an intermediate compound (presumably $I \sim X$ [Chance and Williams, 1956]) of the oxidative phosphorylation. The ion transport is, therefore, not sensitive to inhibition by oligomycin.

Our experiments show that 30% of the respiration of the sperm is oligomycin-insensitive. The conclusion seems, therefore, justified that 30% of the free energy generated by respiration is used in ion transport into the mitochondria, presumably for maintenance. This agrees very well with the observation that 30–45% less of the free energy from respiration has been found to reappear as motility, compared to free energy from fructolysis.

The physiological role of ion transport into mitochondria has been extensively discussed (Harris et al., 1966; Lehninger, 1964). The present experiments indicate that this role is a major one, utilizing approximately one-third of the free energy from respiration. Efficiency measurements which compare a useful output to a driving energy derived from respiratory measurements will be subject to correction for this effect. If an inhibition has been applied (for example of one of the cytochromes), the fraction of the free energy utilized for maintenance may vary dramatically, resulting in large changes in efficiency. The thermodynamic theory of this variation in efficiency has been extensively treated by Kedem and Caplan (1965) and Caplan (1966), and need not be elaborated here.

Finally our data can be used for an estimate of the absolute efficiency of sperm motility. The average velocity of the sperm in an uninhibited preparation is 95 μ per sec (Fig. 8). The insert of Fig. 9 shows that to obtain this velocity $7.5 \times 10^6 = 12.7 \times 10^6$ high energy bonds per sec are required. Of these, 75% or 9.5×10^6 are generated by respiration, and 3.2×10^6 by fructolysis. Since one high energy bond from respiration yields 0.7 ATP, the total ATP hydrolyzed per sperm is 9.9×10^6 ATP per sec. At an ATP hydrolysis-free energy of 10 kcal per mol (equivalent to 0.7×10^{-12} erg per molecule), the free energy input to a sperm $\Delta G_{ATP} = 7 \times 10^{-6}$ ergs per sec.

Detailed measurements of the motility parameters of normal, uninhibited bull sperm have been reported recently by one of us (Rikmenspoel, 1965 *b*). The tail wave of normal sperm is a flattened helix; the amplitudes of the two perpendicular components are 9 μ and 3.5 μ, respectively. The energy dissipated by the two perpendicular components can be taken as the sum of the dissipation of each component (Taylor, 1952; Holwill and Burge, 1963). The average frequency of the tail wave of normal bull sperm, $f = 22$ cps.

The insertion of these values for amplitude and frequency in equation (4) gives:

$$W = 1.3 \times 10^{-6} \text{ erg per sec.}$$

For the efficiency $W/\Delta G$ is thus found: $W/\Delta G = 19\%$. This value appears to be very close to the efficiency of 22% reported in an earlier paper (Rikmenspoel, 1965 *b*). It has to be kept in mind, however, that this closeness is coincidental. Due to the availability of the more recent measurements for the wave parameters, the present value calculated for W is much lower than the one used in the earlier paper. Our present measurements of metabolic rates are more accurate than the earlier ones. Also, the corrections applied for the aging of the preparations during the experiments make the value of ΔG per sperm lower than the earlier reported values. All factors together seem to cancel in the final efficiency value, however.

The value reported here still represents a lower limit for the efficiency. In all sperm preparations a number of abnormally moving cells are present which do not register in our photoelectric apparatus (Rikmenspoel, 1964 *b*). The number of these abnormal cells, from visual estimates, was from 10–25% of the total number of moving cells. The true, absolute efficiency of sperm motility is, therefore, probably closer to 25%.

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