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Quantitative Evaluation of Sperm Motility Control Mechanisms[1]

LEONARD NELSON

Department of Physiology, Medical College of Ohio, Toledo, Ohio 43614 and Marine Biological Laboratory, Woods Hole, Massachusetts 02543

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Propulsion of the spermatozoon depends on the precise coordination of a propagated flagellar wave. True acetylcholinesterase, which is concentrated in the flagellum, may regulate the motility by controlling the intracellular level of acetylcholine. Spermatozoa oriented in a centrifugal field showed a strong biphasic response to added eserine, diisopropylfluorophosphate and ouabain; higher concentrations of those agents (100 to 1000 μM and above) slowed sperm migration while lower concentrations increased sperm motility above the control rate. Strong positively charged quaternary ammonium compounds, tetraethylammonium, neostigmine and acetylcholine had only slight effect on the swim rate, presumably because of their inability to penetrate the sperm plasma membrane. Potassium chloride added to the filtered sea water slowed sperm motility at all concentrations tested, causing 50% inhibition when added in an equivalent amount to that already present in sea water (9 mM). *Arbacia* sperm motility determined by the change in optical density of centrifugally oriented suspensions averaged 187 μm/sec.

The spermatozoa of at least some mammalian and marine invertebrate species exhibit high acetylcholinesterase activity which is concentrated in their flagella (Sekine, 1951; Nelson, 1964, 1966). Ten micromolar eserine inhibited the cholinesterase activity of *Mytilus* sperm cell homogenates while 50 μM eserine increased the rate of flagellation determined stroboscopically (Applegate and Nelson, 1962). The kinetics of eserine action on both *Mytilus* and bull sperm conforms to that of a classical competitive inhibitor and the spermatozoa preferentially hydrolyze acetylcholine over butyryl and benzoylcholine (Applegate and Nelson, 1962; Nelson, 1964). Moreover, disruption of sperm cell integrity by physical or chemical means inactivates the precisely coordinated propagation of the flagellar wave and abolishes propulsive movement, although under appropriate conditions the sperm tail may be induced to resume vibratile activity. For example, treatment with the detergents digitonin or Triton X-100 rapidly halts all motion, but mammalian epididymal spermatozoa may be reactivated to repetitive contractility with ATP and Mg^{2+} (Bishop and Hoffman-Berling, 1959). Bull sperm with membranes disrupted by rapid bombardment with fine glass beads may be similarly reactivated (Morton and Lardy, 1967). Oscillation occurs in noncontiguous regions of the flagellum, and so even though the amplitude of the contractions may equal that of the normal wave, orderly fusion of the twitches and consequently propagation of the flagellar wave fail to occur. In epididymal rat sperm, separation of the conductile process from contraction may commonly be observed. The "impulse" passes through seemingly noncontracting regions to initiate flagellar wave production beyond a block which topographically coincides with the cytoplasmic droplet. Flagellar waves distal to the droplet are capable of propelling the sperm cell (Nelson, 1967). The question to be re-

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solved then is: To what extent does sperm cell motility relate to a functioning acetylcholine–acetylcholinesterase system?

As a working hypothesis, we considered that the acetylcholinesterase may serve to regulate sperm cell motility by controlling the intracellular level of acetylcholine; and that control may be mediated through periodic acetylcholine-initiated reversible alterations in membrane stability. To test the validity of at least parts of this hypothesis, the present work deals with the response, namely, change in swimming speed, of sea urchin spermatozoa, upon exposure to several concentrations of a variety of agents. Among the substances tested were included some which influence membrane transport and transmission in excitable systems. Preliminary observations (Shelley and Nelson, 1969) showed that eserine and diisopropylfluorophosphate could increase starfish sperm motility by 40 to 60% at concentrations which inhibited acetylcholine hydrolysis by about 50%.

MATERIALS AND METHODS

Injection of isotonic (0.53 M) KCl through the oral surface into the perivisceral cavity induces spawning in male *Arbacia punctulata* (Costello *et al.*, 1957). The "dry" sperm collected in Syracuse watch glasses were diluted with filtered sea water immediately prior to the motility rating tests. The sperm cell concentration was adjusted with filtered sea water to give optical density readings in the range between 0.500 and 0.700 in the Bausch and Lomb Spectronic 20 Colorimeter at a wavelength of 540 nm.

Each experimental tube contained 0.01 to 0.5 ml filtered sea water solution of the reagent being tested. The control tube contained an equal volume of filtered sea water and one additional tube contained 0.1% formaldehyde to kill the cells. Five milliliters of the freshly diluted sperm sample was rapidly added to each of the tubes and the contents mixed thoroughly by inverting twice. Then the cells were permitted to incubate in the reagents at room temperature (23–25C) for fixed intervals.

The change in motility was taken as the quantitative response of the sperm cells to the treatments. To expedite the measurement of motility, the spermatozoa were subjected to orientation in a low centrifugal field (Branham, 1966). Under these conditions, the sperm cells tend to swim in a centrifugal direction; when Branham sampled cell density by removing aliquots from near the bottom of the centrifuge tube, he found variations in numbers of cells sedimented depending on age of specimens or exposure to CO_2.

In the present experiments, using a modification of Branham's method, the centrifugations were performed directly in the round colorimeter tubes, permitting periodic monitoring of the rate of change in sperm population density with a minimum of disturbance to the resultant cell distribution. After the initial optical density was measured, the colorimeter tubes were placed in the horizontal rotor of an IEC clinical centrifuge and spun at 120 *g* (940 rpm by Photo Tach Tachometer) at room temperature. Optical density readings were taken after each of three 10-min centrifugations.

The reagents, dissolved in filtered sea water, tested included:

KCl 100 μM to 50 mM
Eserine sulfate 0.5 μM to 1 mM (Physostigmine)
Acetylcholine chloride 10 μM to 10 mM
Neostigmine bromide 50 μM to 1 mM
Tetraethylammonium chloride 5 μM to 5 mM
Ouabain 1 nM to 500 mM.

RESULTS

The optical densities of the *Arbacia* sperm cell suspensions follow Beer's Law, showing a linear relationship over the range 0.100 to 0.700 corresponding to 1×10^6 to 1×10^7 sperm cells/ml (Fig. 1a).

A gravitational field of 120 *g* was arrived at empirically since at low speeds, formalin-killed cells or cells otherwise immobilized tended to sediment out more slowly than the centrifugally-oriented free-swimming spermatozoa. The gravitational asymmetry of the sperm cell derives from the fact that the high molecular weight DNA is situated anteriorly and that the bulk of the head exceeds that of the flagellum.

One may readily determine the relative speed of swimming sperm cells by subtracting the concentration change of the dead cells from the changes in concentration of the control or experimental cells, and expressing the rate of change of the experimental cells as percentages of that of untreated cells. The "absolute" velocity may be calculated from the length of the colorimeter tube and

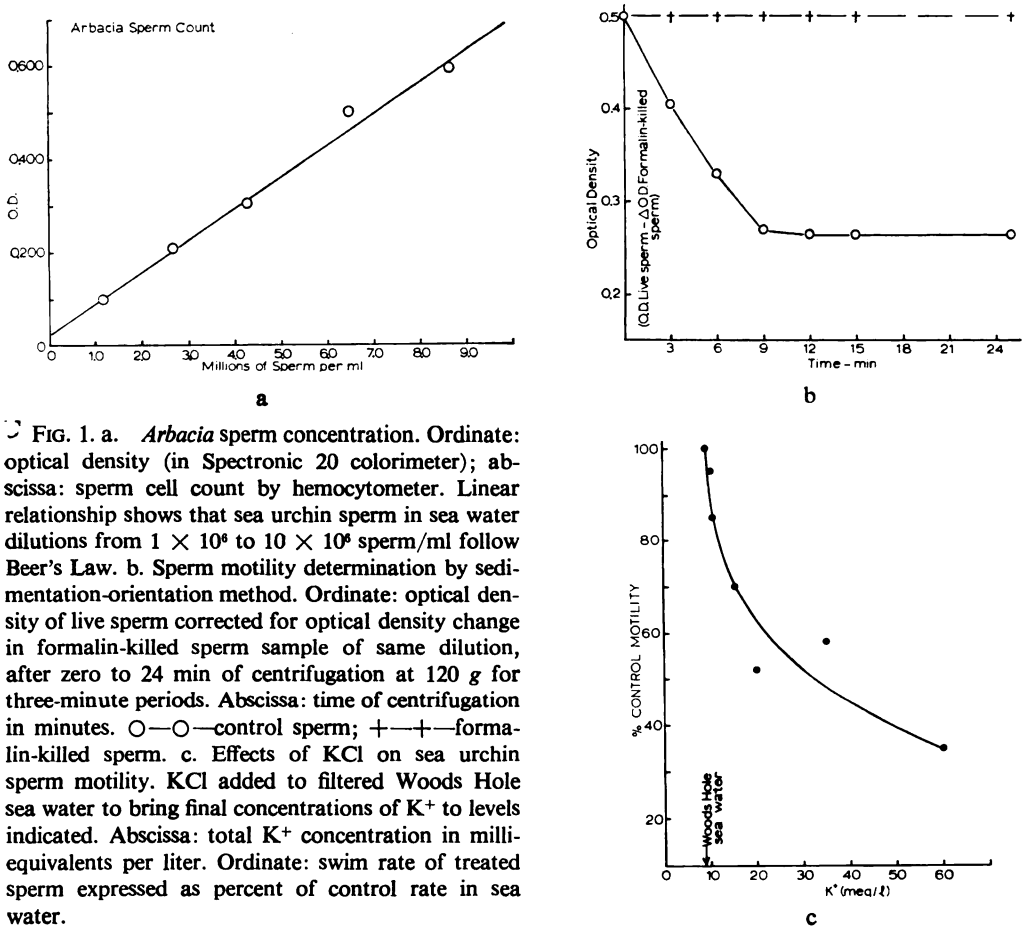
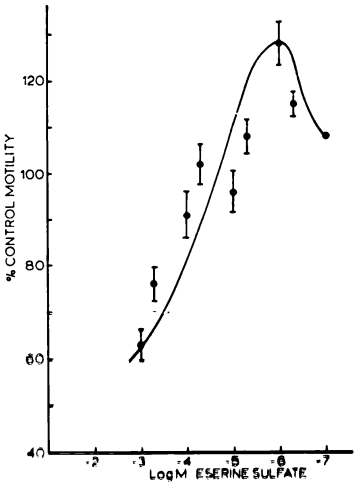


FIG. 1. a. *Arbacia* sperm concentration. Ordinate: optical density (in Spectronic 20 colorimeter); abscissa: sperm cell count by hemocytometer. Linear relationship shows that sea urchin sperm in sea water dilutions from 1×10^6 to 10×10^6 sperm/ml follow Beer's Law. b. Sperm motility determination by sedimentation-orientation method. Ordinate: optical density of live sperm corrected for optical density change in formalin-killed sperm sample of same dilution, after zero to 24 min of centrifugation at 120 *g* for three-minute periods. Abscissa: time of centrifugation in minutes. O—O—control sperm; +—+—formalin-killed sperm. c. Effects of KCl on sea urchin sperm motility. KCl added to filtered Woods Hole sea water to bring final concentrations of K^+ to levels indicated. Abscissa: total K^+ concentration in milliequivalents per liter. Ordinate: swim rate of treated sperm expressed as percent of control rate in sea water.

the rate at which the centrifugally-oriented cells swim beyond the light beam path of the colorimeter. Under the conditions of the experiments, the swimming field was 5 cm long, and each starting suspension appeared to consist of a uniform distribution of several subpopulations of spermatozoa. These proved to be characteristically separable according to differences in their swimming rate (Fig. 1b). First, only about half of the total population swims actively, and their velocity distribution as the sperm cells approach the bottom of the tube appears somewhat asymptotic. The rate of displacement of the rest of the cells does not exceed that of the formalin-killed cells, and so they may be presumed to be nonmotile or dead. In a sperm suspension containing an initial 8×10^6 sperm cells/ml approximately 22% or 1.6×10^6 cells/ml clear the optical path in three minutes, for an average velocity of 280 μm/sec; another 16% or 1.2×10^6 cells/ml cleared the optical path in the next three-minute centrifugation interval for an average velocity of 140 μm/sec, while the final 12% that clear the field take nine minutes altogether, giving them an average speed of 93 μm/sec. The overall average velocity of the swimming sea urchin sperm therefore comes to about 187 μm/sec, which is in remarkable agreement with the average of 191 μm/sec obtained from measurements of individual sea urchin spermatozoa by Gray and Hancock (1955).

Arbacia spermatozoa react intensely to small increases in their environmental potassium. Increasing the potassium from 10 up to 15 meq/liter slows the sperm by 30%; while doubling the initial concentration by the addition of an equimolar amount of KCl to the sea water reduces the motility nearly by half (Fig. 1c).

Most of the other agents tested exerted a biphasic effect on the sperm cell's swimming speed. While higher concentrations had a dampening influence, lower concentrations speeded up the spermatozoa's descent to the bottom of the tube. For instance 1 mM eserine sulfate, a competitive inhibitor of acetylcholinesterase, depressed the motility by about 40%, while 1 μM eserine accelerated the sperm cells in some cases by nearly 40% above the control rate and 100 nM eserine caused only about a 10% increase (Fig. 2a).



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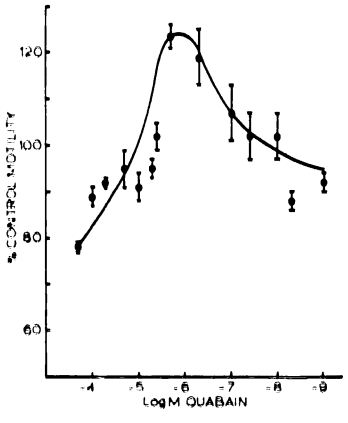

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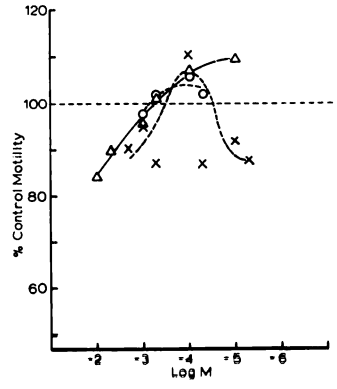

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FIG. 2. a. Effect of eserine on sea urchin sperm motility. Abscissa: log molarity of eserine sulfate in sea water/sperm suspensions. Ordinate: swimming speed of eserinized sperm expressed as percent of motility of untreated control sea water suspensions of *Arbacia* sperm. The points and ranges represent mean and standard deviations of four to six determinations. Observe biphasic response to decreasing concentrations of eserine. b. Effect of ouabain on sea urchin sperm motility. Abscissa: log molar concentration of ouabain in sea water/sperm suspensions. Ordinate: Swimming speed of treated sperm as percent of control sperm swim rate. Biphasic response again noted. c. Effect of quaternary ammonium compounds on sperm motility. (Each point represents average of two to three determinations.) Ordinate: swimming speed of spermatozoa expressed as percent of control motility. Abscissa: concentrations (log molarity in sea water/sperm suspensions) of acetylcholine chloride—△; tetraethylammonium chloride—×; neostigmine bromide—○. Note that in each case, the relatively low degree of sperm cell permeability to the highly polar compounds apparently limits their effectiveness to between only 15% slowing below and 10% speeding above the control swim rates, in comparison to the more pronounced effects of ouabain and eserine.

Ouabain evoked somewhat similar responses in the spermatozoa, although ouabain is effective down to 1 nM with a peak response between 10^{-6} and 10^{-7} M. At 2×10^{-4} M the spermatozoa could move at only about 65% of the control rate but speeded up to about 125% between 5×10^{-6} and 5×10^{-8} M. As the ouabain concentration is further decreased, its effects gradually fade away and disappear finally at 10^{-9} M (Fig. 2b).

Figure 2c shows that quaternary ammonium compounds exert relatively little effect, ranging only from 10 to 15% above and below the speeds of the untreated spermatozoa. Acetylcholine (as well as acetylthiocholine), neostigmine and tetraethylammonium could only slightly modify the swimming behavior of the *Arbacia* sperm, in spite of their being quite potent pharmacological agents, over a concentration range extending from 10 μM to 10 mM.

DISCUSSION

A coherent pattern of sperm cell behavior on exposure to this diversity of substances emerges and may be interpreted in the context of the response of a motility control device to externally applied uncouplers. Under conditions in which the agents have only limited access to a system coupling an excitatory or coordinating device and propulsive contraction waves, the system may continue to function at a near normal level. When the agents can reach and affect such a regulatory center, then such manipulation may evoke a dose-dependent response. The degree of response to the different agents may also reflect the complexity of a multifaceted control mechanism.

Sea urchin spermatozoa respond with extreme sensitivity to relatively small changes in external K^+. Woods Hole sea water contains 9 meq K^+/kg (Steinbach, 1940). Relatively small excesses, e.g., going to 15 and 20 meq, cause 30 to 50% slowing, respectively, while on the other hand a 10% dilution of the sea water by the addition of distilled water has no observable effect. Evidently the spermatozoa of some species are somewhat less adversely affected by variation in K^+. For example, *Mytilus* spermatozoa suspended in isotonic KCl reportedly (Nelson, 1965) manifest no immediate microscopically visible alterations in their swimming behavior. In this regard, it must be noted that tetraethyl ammonium in other excitable systems appears to exert its effects as a replacement for K^+ which it resembles in size and charge (Freeman, 1968); whereas in the *Arbacia* sperm cell even though excess K^+ serves as a potent inhibitor of motility, tetraethylammonium (TEA) evokes only a minimal response. Since the TEA is a highly polar ion, it along with other quaternary ammonium compounds apparently encounters difficulty in penetrating the sperm cell membrane. Acetylcholine, acetylthiocholine and neostigmine fall into the same category, namely, quaternary ammonium ions which if they do penetrate, enter in amounts insufficient to produce any appreciable effect.

Eserine (or physostigmine), however, a tertiary amine, apparently can penetrate and presumably as a competitive inhibitor of acetylcholinesterase affects the sperm motility. Diisopropylfluorophosphate also an esterase inhibitor, was found to increase *Asterias* sperm cell motility by 60% although eserine speeded them up by only 25% (Shelley and Nelson, 1969). Using a stroboscopic device, Applegate (Applegate and Nelson, 1962) clearly demonstrated that the eserine operated at a level that was translatable in terms of motility control by accelerating the frequency of Mytilus sperm flagellar wave motion at 5×10^{-5} M. Those (low) concentrations of ouabain which specifically inhibit the Mg^{2+}-dependent, Na^+, K^+-activated ATPase, may thereby provoke at least for the duration of the measurements, a shift in the ionic balance physiologically equivalent to a decrease in K_o^+.

The sperm cell thus appears to comprise a single unit excitable system in which flagellar wave production provides a visible manifestation of that system's activity. While initiation (organelle and mode) of the flagellar wave remains to be elucidated, and while the wave itself has been variously and inadequately described as "self-propagating" or "intrinsic," the present investigations offer evidence for intracellular modulation of the rate of sperm cell propulsion. (We hope, by means of high speed cinephotomicrography, eventually to determine which parameters, frequency, amplitude or both, participate in the response.) Spermatozoan flagella possess what appears to be a highly active "true specific" acetylcholinesterase (Nelson, 1964), i.e., characteristically they preferentially hydrolyze acetylcholine over the butyryl and benzoyl esters, while acetylcholine hydrolysis exhibits a sharp substrate optimum and is subject to competitive inhibition by eserine. Eserine a competitive inhibitor of acetylcholinesterase, and diisopropylfluorophosphate, a nonspecific cholinesterase inhibitor (Shelley and Nelson, 1969) exert biphasic effects on sperm motility. Preliminary experiments now show that under conditions favoring penetration of acetylcholine, the sperm cells exhibit marked dose-dependent increases and decreases in swimming rate. We therefore tentatively conclude that regulation of the flagellar wave may be mediated by an acetylcholine–acetylcholinesterase system. The sensitivity of the sea urchin spermatozoa to alterations in environmental and intracellular K^+ further points to a significant role in control of the cell's motility by critical levels of this and perhaps other cations, possibly via membrane regulated processes.

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