MECHANICAL HYPOTHESIS OF SPERM PENETRATION

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ABSTRACT Sperm have generally been supposed to penetrate the zona pellucida surrounding the mammalian egg with the aid of a protease, acrosin. Difficulties associated with this view are discussed and an alternative, mechanical hypothesis introduced. The calculated force exerted by individual sperm is too small to permit the rupture of any but the weakest of secondary chemical bonds. Mechanical progress through the zona must rely on stress relaxation in a viscoelastic medium. The known properties of the zona appear to be consistent with such a mechanism of penetration.

INTRODUCTION

The mammalian egg is a spherical cell that, in the common species, has a diameter of 80–150 μm (for reviews of mammalian fertilization see Austin, 1975; Edwards, 1980). Eggs are surrounded at fertilization by two concentric investments. The innermost is the zona pellucida made of an amorphous material and ~10 μm thick; outside the zona lies the cumulus oophorus, composed of residual follicle cells. The function of the zona appears to be the exclusion of cells, which are immotile.

In the course of fertilization, sperm pass between the cumulus cells and then penetrate the zona. This they do by first adhering to the zona surface, approximately tangentially, and then moving through the zona in a curved path, leaving a permanent penetration slit. There is still considerable doubt as to how mammalian sperm produce the slit (Edwards, 1980). The traditional view (Austin, 1975) is that sperm penetrate the zona by proteolytic digestion, the function of their motility at this stage being merely to ensure that the zona lysin and zona come into continuous contact. The alternative view is that sperm penetrate solely by mechanical means, without the assistance of an enzyme (Green, 1978). Historically, the experimental evidence has always been taken to support enzymic penetration, and the case for purely mechanical penetration has gone by default.

MORPHOLOGY OF PENETRATION

Sperm will only penetrate the zona if they have undergone the acrosome reaction. This morphological change activates and exposes the proteolytic enzyme, acrosin. Acrosin has properties similar to trypsin, and both trypsin and crude acrosin extracts remove the zona. Because of this acrosin has for many years been regarded as the principal contender for the role of zona lysin (Austin, 1975). However, to account for the fact that sperm produce a penetration slit, and not wholesale erosion of the zona, acrosin must remain on the sperm surface after the acrosome reaction. Experimental evidence for this view is equivocal, for although guinea-pig sperm do retain some acrosin on their surface after the acrosome reaction (Green and Hockaday, 1978), bull sperm, which have precisely the same problems of penetration, apparently do not (Shams-Borhan et al., 1979). Again, the zona can be rendered resistant to trypsin digestion without affecting the ability of sperm to penetrate it (Bedford and Cross, 1978). Bedford (1968) also provided evidence that in the rabbit egg, 8 h postovulation, sperm split the zona anterior to the sperm tip: presumably, the crack tip propagates as the sperm advances. Eggs closer to ovulation did not produce zona splitting, and this suggests that splitting is not an artefact due to sperm retraction. Enzymic penetration cannot account in any simple way for crack formation and propagation anterior to the sperm tip.

There is another objection to enzymic penetration that has not been properly confronted; namely, that an immobilized substrate and an immobilized enzyme (admittedly fixed to the periphery of a mobile raft) are expected to meet with the intimacy that characterizes occupation of active sites. The available evidence shows that where receptors on two surfaces are immobile, adhesion fails (Rutishauser and Sachs, 1975), which suggests that effective collision between substrate and enzyme would be difficult to achieve.

If the acrosome reaction is needed for zona penetration, it clearly has to fulfill some function, and the mechanical theory of penetration must provide a plausible candidate. The most obvious morphological feature of the reaction is exposure of the perforatorium, a rim of material that surrounds the anterior edge of the nucleus and that before exposure is concealed beneath the acrosome. The thickness of the perforatorium varies from ~20–25 nm in the rabbit to ~80 nm in guinea-pig sperm. The cross-sectional area presented by a penetrating sperm (human, bull, ram, rabbit, etc.) before the acrosome reaction would typically be of the order of 2 μm^2. Measurements for rabbit sperm taken from Bedford (1968) give a maximum thickness for the head of 400–450 nm. The width is ~5 μm. After the reaction, the cross-sectional area presented by the perfora-

torium is ~0.1 μm^2. On the mechanical theory of penetration, therefore, the perforatorium acts as a sharpened leading edge to concentrate stress induced in the zona.

FORCE GENERATED BY SPERM

The average forward velocity, U_o of sperm swimming freely in a watery medium of viscosity $\eta = 0.001$ Pa s is, in round figures, 100 μm/s (Rothschild, 1953; Rikmenspoel et al., 1969). Following contact with the zona, the velocity falls to less than a thousandth of its free swimming value. An estimate of the force, f, exerted by sperm when thus obstructed is of pivotal importance for a mechanical theory of penetration.

An extremely crude estimate can be made by supposing that the viscous resistance to swimming is due entirely to the sperm head, and that the head may be represented by an equivalent sphere of radius $r = 2.5$ μm. Stokes' law then gives $f = 6\pi r\eta U_o = 5$ pN. A more defensible approach is to suppose that resistance is due entirely to the sperm tail. Lighthill's (1976) treatment of the force exerted by a stationary flagellum of length l and radius a shows (on substitution in his Eqs. 105 and 126) that $f = 2\pi l\eta U_o / \ln[l/3a]$. Putting $l = 60$ μm, and $a = 0.2$ μm, we obtain the estimate $f = 8$ pN.

Force exerted by the sperm is applied to the zona over an area of ~0.1 μm^2 (Morphology of Penetration section). A well- known result in elasticity theory is that the maximum shear stress produced by pressure applied to a semi-infinite elastic solid is of the order of one-third the pressure. This result holds whether the pressure is applied over a circular region (Saada, 1974), or over a strip (Jaeger, 1969). If $f =$ 8 pN, the maximum shear stress induced in the zona is ~27 N/m^2.

These estimated values of force and stress are small. Because the calculated force required to break a single covalent bond is of the order of 3 nN (Bell, 1978), sperm could not penetrate the zona if it were a covalently cross-linked structure. Penetration must occur only by separation of reversible (secondary) chemical bonds.

The appropriate vehicle for assessing the effect of stress on bond lifetime is the kinetic theory of fracture. In Bell's (1978) notation bond lifetime, τ, as a function of applied force, f, is given by $\tau(f) = \tau_o \exp[(E_o - \gamma f)/kT]$, where τ_o is a vibration period, E_o is bond energy, and γ is an empirical length parameter. Bell (1978) estimated that for a typical antigen-antibody interaction, γ is of the order of 0.5 nm. The factor by which bond life is shortened by applied force is $\tau(0)/\tau(f) = \exp[\gamma f/kT]$. For a force of 8 pN this factor is 2.5; thus, even if the whole force of a sperm were directed at a single bond, bond lifetime would be reduced only to 40%. It is unreasonable to expect that the whole force of the sperm is directed at one bond at a time, and for practical purposes, therefore, sperm can apparently make no significant impact on bond lifetime.

There are, however, reasons to believe that the value of the force is an underestimate. Firstly, viscous resistance to swimming is contributed by both the head and the tail; the estimate of force obtained from Lighthill's equation should be increased by an unknown (but small) amount. Secondly, recent estimates of swimming speed are as high as 150 μm/s (Wilson and Harvey, 1983). Thirdly, the peak force generated during a cycle of flagellar movement may be greater than the mean. Finally, estimates of the force exerted depend on measured parameters for single sperm. At no time has any attempt been made, either in these studies, or in studies on demembranated mammalian sperm (Lindemann and Gibbons, 1975), to allow for activation. Activation occurs during capacitation of sperm prior to penetration. It includes a substantial increase in the amplitude, and sometimes the frequency, of flagellar movement (Yanagimachi, 1970, 1972) and must therefore increase the force exerted. In the absence of any clearly defined attempt to induce capacitation, it is unlikely that any sperm whose swimming speeds have been measured had been activated.

These considerations taken together allow the estimated force exerted by sperm to be increased, perhaps to as much as 100 pN. Even this value is probably still too small to invalidate the above conclusions about bond breaking. Given these constraints, the only mechanism by which sperm could progress through the zona would be one of stress relaxation in a viscoelastic medium.

COMPOSITION OF THE ZONA

The zona appears to be a protein gel. In the mouse, its protein content is 4.8 ng/zona, equivalent to a protein concentration of 1.4% (Bleil and Wassarman, 1980). A similar value has been found for hamster egg zona using interference microscopy (Green, unpublished observations). The intact mouse zona is dissolved by the detergent sodium dodecyl sulfate without disulphide reduction (Inoue and Wolf, 1974), indicating that it is not covalently cross linked. Pig zonae are also dissolved without reduction by a number of agents, including sodium dodecyl sulfate, urea, and distilled water (Dunbar et al., 1980).

Various lines of evidence indicate that the zona is permeable to a number of large macromolecules, ferritin (Hastings et al., 1972) and ferritin conjugates, immunoglobulins (Sellens and Jenkins, 1975), and mengovirus (Gwatkin, 1967). This is consistent with an open porous structure. Ferritin, for example, has a diameter of 12 nm and conjugates would be expected to have diameters of 20–25 nm; mengovirus has a diameter of ~28 nm. There is also morphological evidence that the zona has a spongelike microstructure (Phillips and Shalgi, 1980).

It is possible to make an estimate of the distribution of proteins in the zona using the information available from the mouse egg (Bleil and Wassarman, 1980). For a total protein content of 4.8 ng/zona, the contributions of the three major proteins are as follows: ZP1, 36% by weight, 200,000 mol wt; ZP2, 47%, 120,000 mol wt; ZP3, 17%, 83,000 mol wt. From these figures the protein concentra-

tion can be calculated as 110 μM. If protein molecules are distributed in a cubic lattice, the density of protein-protein interactions in a plane is 1,600/μm^2.

Sperm move through the zona in a curved path of ~10–20 μm radius. For a zona that is 10 μm thick, and for tangential entry, the slit length is ~15–25 μm. Lateral slit width appears to be no wider than the sperm head (Austin, 1965; Bedford, 1968), say 5 μm. This gives an area of zona separated of 75–125 μm^2. Sperm cross the zona in as little as 5 min (Edwards, 1980). The rate at which they travel, therefore, may be as high as 50–83 nm/s and consequently the area of zona separated per second as high as 0.25–0.4 μm^2. On the simple model for the density of proteins in the plane of fracture, the number of interactions separated would be of the order of 400–640 per second, dictating an average extraction time of ~2 ms.

STRESS RELAXATION AND THE LIFETIMES OF SECONDARY INTERACTIONS

If sperm penetrate the zona mechanically, they do so by placing linkages under tensile load and waiting for the interaction, which prevents further progress, to dissociate spontaneously. Such a process is one of stress relaxation and it has one of two underlying molecular mechanisms. It represents either the relaxation of chain entanglements, or the spontaneous breakage, redistribution, and reformation of chemical bonds. Too little is known of chain entanglements to assess their importance in the present context, but stress relaxation due to dissociation and reformation of chemical bonds has been studied for many years and has a substantial theoretical basis (Flory, 1960).

It is possible to make an estimate of the relaxation times for some of these interactions. For example, a typical antigen-antibody interaction has an equilibrium constant $K = 10^6\ M^{-1}$, and a measured dissociation constant $k_{-1} = 10^2\ s^{-1}$ (Bell, 1978). The lifetime of such an interaction is ~10 ms. If, therefore, the zona were constructed of globular proteins whose equilibrium constants were of the order of $10^6\ M^{-1}$, their lifetimes would be commensurate with the calculated interaction lifetime required for mechanical penetration. Weaker interactions will have shorter lifetimes. For example, hydrogen bonds have equilibrium constants of between 1 and $10^2\ M^{-1}$ (Taft et al., 1969; Arnett et al., 1970). If the forward rate constant is regarded as being diffusion limited, which is a reasonable assumption for such a simple interaction, the dissociation rate constant, k_{-1}, is 10^7–$10^9\ s^{-1}$. The lifetime of hydrogen bonds would therefore be ~1–100 ns. Other secondary bonds would be weaker and would have correspondingly shorter lifetimes. There will, of course, be further delays imposed by the re-establishment of new linkages at equilibrium, while the original linkages are still under stress. Empirically, however, it is known that these form slowly (Miller et al., 1951), and to a first approximation they can probably be ignored.

If the crude Stokes' law argument outlined earlier (Force Generated by Sperm section) is accepted as giving a reasonable value for the force exerted by sperm, then the rate of penetration can be used to provide an estimate of the viscosity of the zona. This we calculate to be not less than 2 Pa s.

A material whose properties might be expected to resemble those of the zona is gelatin. A 2% gelatin gel, aged for 48 h at 22°, can be transferred to water without dissolution; as far as these matters can be judged subjectively it behaves remarkably similarly to the zona when handled with micropipettes. The viscosity at 22°, estimated from the rate of fall of ball bearings, is ~260 Pa s (Green, unpublished observations), a value considerably higher than that estimated for the zona. However, the viscosity of protein gels is strongly dependent on concentration, chemical constitution, temperature, and age. Since there is no reason to suppose that gelatin is an accurate model of the zona proteins, the discrepancy does not seem to us a serious one.

The penetration slit formed by the sperm in the zona persists after sperm passage, and shows little, if any, elastic recoil. Assuming that it is formed mechanically, stress relaxation must be rapid and the zona weak. However, its rigidity may be relatively high. For example, in the aging rabbit egg, it prima facie becomes high enough to ensure that the penetration slit propagates as a crack anterior to the sperm tip (Bedford, 1968).

CONCLUSION

The two principal alternatives for the mechanism of sperm penetration are enzymic and mechanical. In this paper we calculate for the first time, the force which sperm could exert. It is sufficiently small that it can be shown to have a negligible effect on bond lifetime. Thus if sperm penetrate the zona mechanically, the zona must behave as a liquid. Experimental evidence of the structure of the zona is consistent with this view. The question of whether sperm form the penetration slit through the zona pellucida enzymically or mechanically cannot be decided, therefore, by assuming that the latter is a priori impossible.

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